Retinoic Acid Stimulates Chondrocyte Differentiation and Enhances Bone Morphogenetic Protein Effects through Induction of Smad1 and Smad5

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Whereas bone morphogenetic protein (BMP)-signaling events induce maturational characteristics in vitro, recent evidence suggests that the effects of other regulators might be mediated through BMP-signaling events. The present study examines the mechanism through which retinoic acid (RA) stimulates differentiation in chicken embryonic caudal sternal chondrocyte cultures. Both RA and BMP-2 induced expression of the chondrocyte maturational marker, colX, in chondrocyte cultures by 8 d. Though the RA effect was small, it synergistically enhanced the effect of BMP-2 on colX and phosphatase activity. Inhibition of either RA or BMP signaling, with selective inhibitors, interfered with the inductive effects of these agents but also inhibited the complementary pathway, demonstrating a codependence of RA and BMP signaling during chondrocyte maturation. BMP-2 did not enhance the effects of RA on an RA-responsive reporter construct, but RA enhanced basal activity and synergistically enhanced BMP-2 stimulation of the BMP-responsive chicken type X collagen reporter. A similar synergistic interaction between RA and BMP-2 was observed on colX expression. RA did not increase the expression of the type IA BMP receptor but did markedly up-regulate the expression of Smad1 and Smad5 proteins, important participants in the BMP pathway. Inhibition of RA signaling, with the selective inhibitor AGN 193109, blocked RA-mediated induction of the Smad proteins and chondrocyte differentiation. These findings demonstrate that RA induces the expression of BMP-signaling molecules and enhances BMP effects in chondrocytes. (Endocrinology 144: 2514–2523, 2003)

In vitro studies support the concept of spontaneous differentiation by chondrocytes and suggest that bone morphogenetic proteins (BMPs) have a role in this process (9, 11). BMPs are potent inducers of chondrocyte terminal differentiation. BMP-2 and BMP 6 have been shown to induce the expression of colX and alkaline phosphatase activity in chick sternal chondrocyte cultures (12, 13). More recently, our laboratory has provided evidence demonstrating that the suppressive effect of PTHrP on chondrocyte maturation is mediated by an inhibition of BMP signaling (9). Whereas PTHrP is a potent inhibitor of colX, alkaline phosphatase activity, and BMP-6 expression, the exogenous addition of BMP-6 to PTHrP-containing cultures leads to an accelerated rate of chondrocyte differentiation (9). A role for BMP signaling in the induction of a differentiated phenotype is further supported by studies demonstrating the induction of maturation by constitutively active BMP receptors (BMPRs) and inhibition of differentiation by dominant negative BMPRs (DNBMPRs) (14).

Whereas BMPs induce maturational characteristics in vitro, other regulatory molecules have also been shown to have a similar effect. 1α,25 Dihydroxyvitamin D3 enhances alkaline phosphatase activity in rat sternal chondrocyte cultures (15), whereas T4 induces cellular hypertrophy, colX expression, and alkaline phosphatase activity in rat neonatal limb chondrocytes (16, 17). Similarly, retinoic acid (RA) stimulates the expression of colX and alkaline phosphatase in embryonic vertebral and sternal chondrocyte cultures and promotes chondrocyte differentiation in vivo in the developing chick limb bud (18–20). However, recent evidence

Abbreviations: BMP, Bone morphogenetic protein; BMPR, BMP receptor; DNBMPRs, dominant negative BMPR; RA, retinoic acid; RAR, RA receptor; RCAS, replication-competent avian leukemia virus with splice acceptor.

The skeleton forms from a cartilaginous template that develops early in embryogenesis (1). Chondrocytes subsequently undergo a complex process of differentiation characterized by marked changes in physical and biochemical features that culminate in calcification of the cartilaginous matrix and subsequent replacement by bone (2–4). This process of endochondral ossification persists during adolescence and is recapitulated during fracture healing and other reparative processes in the adult (5).

The rate of chondrocyte differentiation during endochondral ossification is regulated by a signaling pathway involving PTHrP and Indian hedgehog (6). PTHrP is a potent suppressor of chondrocyte maturation and prevents cell hypertrophy and the associated expression of type X collagen (colX) and alkaline phosphatase, both of which are necessary for the terminal differentiation of growth plate chondrocytes (7–9). In PTHrP knockout mice or mice defective in PTHrP signaling, chondrocytes undergo accelerated maturation, leading to disorganization of the growth plate and severe skeletal malformations (7, 8). In contrast, overexpression of PTHrP inhibits terminal differentiation, also resulting in widespread skeletal malformations (10). These findings suggest that there is a default signal in chondrocytes that commits them to undergo differentiation, thus explaining the spontaneous maturation that occurs in the absence of PTHrP.
suggests that the effects of these other regulators might be mediated through BMP-signaling events. The effect of T4 in rat chondrocyte differentiation is associated with an increase in BMP-2 expression and can be blocked by the addition of noggin, suggesting a critical role for the BMPs in T4-mediated differentiation (17). Similarly, in rat osteoblasts, glucocorticoid-induced differentiation is secondary to up-regulation in BMP-6 expression (21). Thus, it is unclear whether there is a BMP-independent mechanism for chondrocyte differentiation.

The cellular pathway through which the TGF-β receptor family signals from the membrane to the nucleus has been recently elucidated. The critical regulators are called Smads, which are phosphorylated and released from activated receptors. In the case of BMP-signaling, Smad1 and Smad5 have been shown to be proteins that specify BMP-signaling events. Activated Smad1 and Smad5 associate in the cytoplasm with a common mediator, Smad4, and this Smad complex subsequently translocates to the nucleus, where gene transcription is influenced. At present, there is limited information regarding the expression or regulation of the Smad-signaling molecules during chondrocyte development. Whereas TGF-β-specific Smad2 is regulated by signaling events, as are the inhibitory Smad molecules (Smad6 and Smad7), no factors that directly regulate Smad1 or Smad5 expression have been identified thus far.

In the present study, we examine the mechanism through which RA stimulates differentiation in chicken embryonic caudal sternal chondrocyte cultures. We show that in combination, BMP-2 and RA synergistically enhance collagen αX and the expression of alkaline phosphatase activity. Furthermore, we demonstrate that the effects of RA are associated and dependent on an increased responsiveness to BMP-mediated signals. Although RA does not alter the expression of BMPR IA, it does lead to a marked increase in protein expression of the BMP-signaling molecules, Smad1 and Smad5.

Materials and Methods

Reagents

BMP-2 was a gift from Genetics Institute (Cambridge, MA) and was obtained in solution (1 mg/ml) and maintained at 4 C; 13-cis-RA (Sigma, St. Louis, MO) was reconstituted in 95% ethanol at a stock concentration of 1 mm and maintained at −20 C. The RA antagonist, AGN 193109, was a gift from Allergan, Inc. Pharmaceutical (Irvine, CA) and was reconstituted in 10% DMSO at 1 mm and maintained at −20 C. Antibodies for Smad1 and Smad5 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); β-actin antibody was obtained from Sigma.

Chondrocyte cell culture

Embryonic cephalic sternal chondrocytes (d 13) were prepared and cultured as previously described (22). After isolation and primary culture for 5–7 d, floating cells were plated in secondary cultures at 1.5 × 106 cells/10-cm culture dish, or at 3 × 105 cells/well/6-well plate, or at 2 × 104 cells/well/24-well plate. The cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) containing 10% NuSerum IV (Collaborative Research, Bedford, MA), 4 U/ml hyaluronidase (Sigma), and 2 mm l-glutamate (Sigma). Chondrocytes were cultured in the presence of ascorbate (50 μg/ml). BMP-2 (50 ng/ml) or all trans-cis-RA (1–100 nm) were added to the cultures, in indicated experiments, after 24 h in serum-containing medium. Chondrocytes were infected with replication-competent avian leukemia virus with splice acceptor (RCAS) viruses at the time of secondary plating in culture medium containing 25% filtered viral supernatant and 75% sternal chondrocyte media (high-glucose DMEM + 10% Nusenum IV, plus hyaluronidase). The medium was changed to 100% sternal chondrocyte medium after 2 d. AGN 193109 was added to the cultures at a concentration of 1 μm as previously described (23). Medium was changed, in all experiments, with identical fresh medium (+2 growth factors) every 2 d.

Viral vectors

Type I DN BMPR constructions in RCAS were provided by Niswander and co-workers (1). The dominant negative receptors were constructed by mutating a lysine to arginine within the ATP-binding site, which dramatically reduces kinase activity of the receptors (1). The DN BMPR IA was human, whereas the DN BMPR IB was derived from chick CDNA. Flag-tagged wild-type Smad1 was obtained from Dr. Rick Derynck (University of California, San Francisco, CA) and was cloned into RCAS as previously described (24). Viral stocks were produced in chicken embryonic fibroblasts after transfection with RCAS viruses using Lipofectamine (Life Technologies, Inc.). The chicken embryonic fibroblasts were prepared from the soft tissues of d-10 chicken embryos as previously described (25). Serum-free medium was then placed on the fibroblast cultures for 24 h, and retroviral conditioned medium was harvested. The conditioned media was filtered through a 0.45-micron filter (Corning, Inc., Corning, NY) to remove cellular contaminants. The viral stocks were frozen in single-use aliquots at −80 C. We have previously reported approximately 75% infection rates with these methods (9).

RNA isolation and Northern analysis

Total RNA was isolated from 10-cm cultures using the RNAeasy kit according to the manufacturer’s instructions (QIAGEN, Valencia, CA). The RNA concentration was quantified by measuring absorbance at 260 nm and run on denaturing formaldehyde/agarose gels with 5 μg RNA loaded per sample well. Ethidium bromide (40 mg/ml final concentration) was added to the sample loading buffer to permit visualization of the ribosomal bands. After electrophoresis, the gels were photographed with Polaroid (Boston, MA) film, and RNA was transferred to Gene Screen nylon membranes (DuPont, Wilmington, DE) by capillary transfer. The blotted RNA was baked for 1.5–2 h and hybridized with DNA probes.

Chicken cDNA sequences encoding Smad1 and Smad5 were obtained from Dr. John Lough (Medical College of Wisconsin, Milwaukee, WI). BMPR IA was obtained from Dr. Lee Niswander (Memorial Sloan-Kettering Cancer Center, New York, NY) (1). 32P-labeled cDNA probes were synthesized using a random priming kit (Life Technologies, Inc.). A synthetic type X oligonucleotide was end-labeled with T4 kinase, using a kit (Life Technologies, Inc.,) as previously described (9). The type X collagen oligonucleotide probe was hybridized at 73 C for 1 h in QuickHyb Hybridization Solution (Stratagene, La Jolla, CA), whereas the other probes were hybridized in the same solution at 68 C for 1 h. Repeated washes were performed and were identical for each of the probes, with the final wash performed in a solution composed of 0.1 × saline sodium citrate and 0.1% sodium dodecyl sulfate at 60 C for 30 min. Probed membranes were exposed to XAR film (Eastman Kodak Co., Rochester, NY). The ratios of type X collagen to the 28S ribosomal RNA was measured by NIH Image version 1.61.

Real-time RT-PCR assay

Total RNA was extracted from cultures using the RNAeasy kit (QIAGEN). One microgram of total RNA was reverse-transcribed using Advantage RT-for-PCR kit from CLONTECH Laboratories, Inc. (Palo Alto, CA). Real-time PCR was performed using the RotorGene real-time DNA amplification system (Corbett Research, Sydney, Australia) and the fluorescent dye SYBR Green I to monitor DNA synthesis (SYBR Green PCR Master Mix; PE Applied Biosystems, Foster City, CA). The sequences of the forward and reverse primers are listed in Table 1 and were normalized to chicken glyceraldehyde 3-phosphate dehydrogenase (5′-tgatggtatatcaaggaggtg-3′; 5′-tgatgacaacctgtcatac-3′). The PCR protocol included a 95°C denaturation step for 10 min, followed by 45 cycles of 95°C denaturation (20 sec), annealing (20 sec), and 60°C extension (30 sec). The annealing temperatures were 47 C for all primers.
Detection of the fluorescent product was carried out at the end of the 68-C extension, and detection. PCR products were subjected to a melting curve analysis, and the data were analyzed and quantified with the RotorGene analysis software. Dynamic tube normalization and noise slope correction were used to remove background fluorescence.

Alkaline phosphatase activity

Alkaline phosphatase activity was measured using a previously described protocol (9). Medium was aspirated from chondrocytes cultured in 24-well plates. The plates were rinsed with 150 mM NaCl; and 1 ml reaction buffer, containing 0.25 M 2-methyl-2-amino propane, 1 mM magnesium chloride, and 2.5 mg/ml p-nitrophenyl phosphate (Sigma) at pH 10.3, was added to the wells at 37 C. The reaction was stopped, after 30 min, with the addition of 0.5 ml 0.3 M Na2PO4 (pH 12.3). The alkaline phosphatase activity was determined by measuring absorbance of light at 410 nm and comparing the experimental samples with standard solutions of p-nitropheno1 and an appropriate blank. Alkaline phosphatase was normalized for protein concentration using BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL), as measured by spectrophotometry (562 nm), and compared with standard protein concentrations. Data presented is the mean of four to six samples, and error bars represent ± the mean. Statistical analysis was performed using one-way ANOVA.

DNA reporter constructs

The BMP-responsive chicken type X collagen luciferase reporter construct was a gift from Dr. Phoebe Leboy (University of Pennsylvania, Philadelphia, PA) (26). The promoter used was the b2/640-luciferase promoter, which contains a 643-bp fragment derived from bases −2649 to −2007 ligated to a 640-bp proximal promoter that contains the transcriptional start site and 5'58 base pairs of 5' flanking sequence (26). This construct was ligated into the polylinker region of the promoterless Renilla luciferase reporter plasmid, PRL-null (Promega Corp., Madison, WI). A 20- to 30-fold induction in activity has previously been demonstrated with this reporter in response to BMP treatment (26). For control experiments, the B5353/640-luciferase promoter was used. This construct contains sequences from −3193 to −1583 ligated to the proximal 640-bp proximal promoter from which a 533-bp fragment (−2866 to −2333) has been removed. Removal of this region, which includes 316 bases contained in the b2/640-luciferase construct, results in loss of BMP responsiveness (26). The RA receptor (RAR) responsive reporter construct, pRARβ-CAT, was a gift from Dr. Ronald Evans and responds to all three subtypes of RAR but not to thyroid hormone, estrogen, glucocorticoid, or vitamin D (27).

Transient transfection and luciferase assay

Lower sternal chondrocytes, cultured at 30–40% confluence in 6-well plates, were transfected with a total of 1 μg plasmid DNA, 24 h after plating, using the transfection reagent Superfect (Qiagen). The renilla luciferase reporter was transfected at a concentration of 1 μg/culture, whereas pGL3 firefly-luciferase reporter plasmid containing the SV40 early promoter was cotransfected (20 ng/culture) to control for transfection efficiency (Promega Corp.). The plasmid constructs were added to the cultures in 100 μl serum-free DMEM; and after 10 min, medium containing 10% serum and antibiotics was added. At 3 h, the medium was changed to standard culture medium. Sixteen hours later, the cultures were rinsed; and serum-free DMEM, containing hyaluronidase (4 U/ml), penicillin/streptomycin, 10 pm triiodothyronine (Sigma), 60 ng/ml insulin, and 1 mM cysteine (Sigma), was added. Four hours later, 50 ng/ml BMP-2 or 100 nM trans-RA were added to selected treatment groups. Forty-eight hours later, a chondrocyte protein extract was obtained and assayed for luciferase activity using the Promega Corp. dual-luciferase assay system, as previously described (28). Firefly luciferase values were used to normalize each sample for transfection efficiency. Data presented is the mean of triplicate samples, and error bars represent ± the mean. Statistical analysis was performed using one-way ANOVA.

Chloramphenicol acetyltransferase assay

Chondrocyte transfections were also performed as described above, with 1 μg pRARβ-CAT reporter construct. A pSv40-galactosidase reporter plasmid (Promega Corp.), containing the SV40 early promoter, was cotransfected (20 ng/culture) to control for transfection efficiency. Twenty-four hours later, a chondrocyte protein extract was obtained and assayed for CAT activity using the CAT Enzyme Assay System (Promega Corp.) according to the manufacturer’s instructions. β-Galactosidase activity was determined using the β-galactosidase Enzyme Assay System (Promega Corp.). The β-galactosidase level was determined spectrophotometrically at 420 nm, in comparison with β-galactosidase concentration standards, with values used to normalize each sample for transfection efficiency. Data presented are the mean of triplicate samples, and error bars represent ± the mean. Statistical analysis was performed using one-way ANOVA.

Western blot

After rinsing the cell layer with PBS, whole-cell proteins were extracted from lower sternal chondrocyte cultures using Golden lysin buffer containing protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Indianapolis, IN). The lysate was centrifuged at 12,000 × g, and insoluble material was removed. Protein concentration of the soluble material was determined using the Coomassie Plus Protein Assay kit (Pierce Chemical Co.). One-hundred-microgram aliquots of protein extract were separated by sodium dodecyl sulfate-10% PAGE and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). The blots were incubated overnight at 20 C with either a goat-antimouse Smad1 polyclonal (Santa Cruz Biotechnology, Inc.) or a goat-antimouse Smad5 polyclonal (Santa Cruz Biotechnology, Inc.), each at a 1:3,000 dilution. After washes, blots were further incubated, for 1 h at 20 C, in the presence of alkaline phosphatase-conjugated secondary antibodies against goat (Sigma) at a dilution of 1:5,000. The immune complexes were detected via color change directly on the blots.

Analysis

Results from the experiments were analyzed using a one-way ANOVA (StatView 4.5; Abacus, Berkeley, CA). The P values are described in the figure legends.

Results

The effects of both cis- and all-trans-RA, as well as BMP-2, on colX mRNA expression were evaluated in secondary cultures of lower sternal chondrocytes (Fig. 1). Control cultures of lower sternal chondrocytes have undetectable colX ex-
pression, by Northern blot, through 12 d, consistent with previous reports documenting the slow rate of differentiation in these cultures (9, 11, 29). Though both forms of RA induce expression of colX, this effect is observed slightly earlier in cultures treated with all-trans-RA, where a low level of induction in colX is observed after 8 d of treatment (Fig. 1A). By 12 d, high levels of colX were observed in cultures treated with both cis- and all-trans-RA. In contrast to the effect of RA, BMP-2 is a more potent inducer of chondrocyte differentiation and causes a marked up-regulation of colX expression in 8-d cultures (Fig. 1B). Similar effects were observed with doses ranging from 10–100 ng/ml BMP-2. Thus, relatively low concentrations of BMP-2 have maximal effects in long-term cultures of lower sternal chondrocytes. All-trans-RA (100 nM) was used in subsequent experiments with a maximally effective concentration of BMP-2 (50 ng/ml).

We evaluated the effect of BMP-2, alone and in combination with RA, in caudal sternal chondrocyte cultures (Fig. 2). BMP-2 (50 ng/ml) caused a large induction in colX expression in 8-d cultures, whereas RA (100 nM) induced only a low level of expression, consistent with our prior results. However, RA and BMP-2 in combination were synergistic and resulted in a marked up-regulation of colX expression (Fig. 2A). The mean increase in three separate experiments, compared with control values, was approximately 5-fold in RA-treated cultures, 55-fold in BMP-2-treated cultures, and 170-fold in cultures treated with a combination of RA and BMP-2 (Fig. 2B).

The effects of RA and BMP-2 on alkaline phosphatase were evaluated to determine whether synergistic effects were also present for other markers of chondrocyte maturation. Similar to the effects observed with colX, RA caused a relatively small increase in alkaline phosphatase activity (1.9-fold), whereas BMP-2 resulted in a larger increase (2.5-fold) (Fig. 2C).
combination, the induction was greater than that observed with either growth factor alone (5.4-fold), further suggesting an interrelationship between BMP-2 and RA on chondrocyte differentiation.

We next performed a series of experiments to determine whether the maturational effects of BMP and RA are interdependent. It has been previously shown that BMP-mediated induction of colX expression can be blocked by a DNBMPR IB, whereas DNBMPR IA has a smaller effect (14). Alone, RA had no observed effect on colX expression in these 8-d cultures but markedly enhanced the effect of BMP-2 (Fig. 3A). DNBMPR IB inhibited the BMP-2-mediated induction of colX to a greater extent than DNBMPR IA, and the inhibition was observed in both the presence and absence of RA. These findings confirm that RA effects on colX are dependent on BMP signaling. The incomplete inhibition may be explained, in part, by our observation that maximal infection rates in our cultures are between 75 and 80% (30). To further assess the interaction between RA and BMP signaling in chondrocytes, we examined the effect of AGN 193109, a selective RA inhibitor (23). AGN 193109, at a concentration of 1 μM, inhibited the induction of colX in RA-treated cultures at both 8 and 12 d, confirming the inhibitory activity of this compound (Fig. 3B). AGN 193109 reduced the induction of colX by BMP-2 and blunted the synergistic effect of RA with BMP-2 (Fig. 3C). The effect of BMP-2 alone was inhibited 49.1 ± 4.5% by AGN 193109 (n = 3 independent experiments), whereas the combination of BMP-2 and RA is inhibited by 45.5 ± 10.9% (n = 3 independent experiments). These latter findings suggest that basal RA signaling is permissive for BMP-mediated effects.

We next examined whether the interactive induction of chondrocyte maturation by BMP-2 and RA was attributable to: 1) RA-mediated enhancement of BMP signaling; and/or 2) BMP-2-mediated enhancement of RA signaling. Sternal chondrocytes were transfected with a BMP or RA reporter construct, in the presence and absence of BMP-2 or RA individually or in combination (Fig. 4A). Whereas RA resulted in an 18-fold stimulation of the RA reporter construct, BMP-2 alone had no effect and did not enhance RA-induced CAT activity. As anticipated, AGN 193109 inhibited the RA-mediated increase in CAT activity. The inhibition was partial (72%) and was similar in the presence and absence of BMP-2. Thus, BMP-2 did not enhance the responsiveness of chondrocytes to RA-mediated signaling events.

The BMP-responsive chicken type X collagen promoter was used to examine the effects of RA on BMP signaling. As anticipated, BMP-2 caused a 19-fold stimulation of luciferase activity. Though RA caused a small induction (3.5-fold), it markedly enhanced the effect of BMP-2-induced luciferase activity (48-fold; Fig. 4B). In contrast, when the BMP-responsive portion of the type X collagen promoter was deleted (26), the effects of both RA and BMP-2 were absent (Fig. 4C).

The enhancement of BMP signaling by RA may be attributable to an alteration in the expression of BMP-signaling molecules that favors enhanced BMP effects. We focused these experiments on the BMP type I receptor and its associated activating Smad-signaling molecules. Prior work has demonstrated that chondrocytes primarily express the BMP IA receptor. However, neither RA nor AGN 193109 altered the expression of BMPR IA (Fig. 5A). It has previously been shown that the effects of both TGF-β and BMPs are markedly enhanced by increased expression of Smad proteins that are downstream targets of these growth factors (31, 32). For this reason, we investigated the effects of RA on the expression of Smad1 and Smad5, two Smads involved in the transduction of BMP signals and activation of BMP responsive genes. Whereas Smad1 expression is not readily detectable by Northern blot in basal or RA-treated cultures (data not shown), RA stimulated Smad5 expression dose dependently.

![Fig. 3. DNBMPR receptors and AGN 193109 inhibit the effects of BMP-2 and RA on type X collagen. The cultures were treated with control medium or medium containing BMP-2 (50 ng/ml) or all-trans-RA (100 nM). A, Secondary cultures of lower sternal chondrocytes were infected with an RCAS virus expressing the DNBMPR IA (DNIA) or the DNBMPR IB (DNIB), on the day after secondary plating in monolayer culture, in the presence or absence of BMP-2 or RA, either alone or in combination, for 8 d. B, Cultures of lower sternal chondrocytes were treated with AGN 193109, on the day after secondary plating in monolayer culture, in the presence or absence of BMP-2 or RA, either alone or in combination, for 8 d. C, Cultures of lower sternal chondrocytes were treated with AGN 193109 on the day after secondary plating in monolayer culture, in the presence or absence of BMP-2 or RA, either alone or in combination, for 8 d. Total RNA was separated by gel electrophoresis, and Northern blot analysis was performed as described in Materials and Methods. RNA loading was standardized by evaluation of the 28S ribosomal RNA.](image-url)
with effects observed at both 8 and 12 d (Fig. 5B). However, RA markedly increased Smad1 and Smad5 protein levels in the chondrocyte cultures (Fig. 5C). The effect was observed only in 12-d cultures, corresponding with the ability of RA to induce colX, as shown in Fig. 1. Furthermore, cotreatment of the cultures with AGN 193109 prevented the induction Smad 5 mRNA (Fig. 5D) and of Smad1 and Smad5 protein (Fig. 5E), demonstrating the dependence of RA signaling on this effect. The findings suggest that effects of RA on maturation are attributable, at least in part, to an induction of Smad 1 and Smad5.

To verify the role of Smad1 and its association with RA signaling in inducing chondrocyte maturation, we overexpressed Smad1 in 8-d cultures, using an RCAS expression vector in the presence and absence of RA (Fig. 6). Though both RA treatment and Smad1 overexpression resulted in a slight increase in colX expression at 8-d, the combined overexpression of Smad1 and treatment with RA resulted in a marked increase in colX expression. This experiment further confirms a role for the Smad proteins in inducing chondrocyte maturation and demonstrates, in this gain-of-function experiment, the interactive effect of Smad1 with RA signaling.

Because of the complexity of the BMP-signaling event and the number of both positive and negative regulators, we also examined the effect of 8-d exposure of RA on the expression of a number of other genes involved in regulating BMP signaling, by real-time RT-PCR (Fig. 7). These genes include BMPs, the inhibitory Smads, and the BMP-binding antagonists, chordin and noggin. Some changes in gene expression would tend to enhance signaling, whereas others would reduce BMP-signaling events. Thus, RA induced BMP-2 (~4.4-fold) and reduced the antagonist, noggin, 60%, events that favor an increase in BMP signaling. In contrast, chordin was increased 2.8-fold, whereas the inhibitory Smad, Smad6, was increased 6.7-fold. The induction of Smad6 is consistent with our recent findings showing that Smad6 is induced during chondrocyte differentiation (33). Because both Smad6 and chordin down-regulate BMP signaling, their effects would be opposite of the observed effect of RA as a stimulator of BMP signaling. In contrast, Smad7 levels were unchanged, and minimal effects were observed on BMPs 4, 6, and 7. These findings demonstrate that RA alters the expression of multiple components of the BMP-signaling pathway and that factors other than Smad1 and Smad5 also contribute, in a complex manner, to the overall effects of RA on BMP signaling and chondrocyte maturation.

Discussion

Our study demonstrates that RA and BMP signaling synergistically stimulate the expression of a mature phenotype in caudal sternal chondrocytes. In the absence of stimulation, the cultures undergo a very slow rate of spontaneous differentiation, suggesting that these cells are relatively immature, compared with chondrocytes obtained from the embryonic cephalic sternum or adolescent growth plate (9, 11, 29). Though the addition of BMP-2 did not enhance the effects of RA on an RA-responsive reporter construct, addition of RA to cultures transfected with the BMP-responsive
chick type X collagen reporter markedly enhanced reporter activity. This effect paralleled the synergistic effect of RA and BMP-2 on collagen expression and suggests that RA increases the responsiveness of chondrocytes to BMP-signaling. Because of the complexity of BMP signaling, RA could positively influence BMP-signaling events at a number of steps, including increased secretion of BMPs and enhanced expression of BMPRs or downstream signaling molecules. Similarly, the down-regulation of secreted BMP antagonists such as noggin or chordin, or decreased expression of the intracellular signaling antagonists, Smad6 or Smad7, also could enhance BMP-signaling events. Though RA affects a number of these events, the current manuscript focused on one potential mechanism and found that the RA effect is attributable, in part, to an increase in Smad1 and Smad5 protein expression.

Interactive effects between RA and BMPs have previously been defined in a number of cell types (34, 35). RA activation promotes induction of BMP-mediated apoptosis in P19 embryonal carcinoma cells (36). BMP signaling leads to a decrease in RARβ expression in 3T3 cells (37). RA has also been shown to mediate effects through stimulation of TGF-β expression, as well as the expression of the TGF-β receptor (38–40). Recently, RA and BMP were shown to synergistically induce osteoblast differentiation from preadipocytes through an undefined mechanism (41). Though both RA and BMP have been defined as critical regulators of limb development and chondrocyte differentiation (1, 20), the current study is the first to demonstrate complementary and synergistic interactions between RA and BMPs in chondrocytes, as well as demonstrate that RA induces Smad expression.

RA caused a potent induction in the expression of Smad1 and Smad5, whereas the selective RA inhibitor, AGN 193109, inhibited this effect. Smad1, 5, and 8 are signaling molecules specific for the BMP pathway (32, 42, 43). These Smads bind to the inactive type I BMPR and are phosphorylated and released after ligand-mediated receptor activation (32). Once in the cytosol, the receptor-activated Smads associate with
basal and stimulated TGF-β1, overexpression of wild-type Smads results in increased 
block signaling, BMP-mediated effects are absent. In con-
mediated effects on Smad function (42, 43). In the presence 
Smad2 and 3, whereas Smad6 and 7 are inhibitors of Smad 
gene(s) (32, 44–46). Smad4 also binds to the TGF-β-specific 
Smad2 and 3, whereas Smad6 and 7 are inhibitors of Smad 
signaling (32, 44, 47).

Prior work has demonstrated the dependence of BMP-
mediated effects on Smad function (42, 43). In the presence 
of dominant negative Smad molecules with mutations that 
block signaling, BMP-mediated effects are absent. In con-
trast, overexpression of wild-type Smads results in increased 
basal and stimulated TGF-β and BMP signaling (48). In the 
current experiments, overexpression of wild-type Smad1 in-
duced colX expression and was synergistic with RA, verify-
ning the interactive effects between Smads and RA. Thus, 
similar BMP signals result in either small or large effects, 
depending on the availability of Smads that mediate down-
stream signals.

The receptor-regulated Smads are regulated by growth 
factors and cytokines. In cultured rat articular chondrocytes, 
TGF-β1 increased the mRNA level of Smad2 (49). In contrast,

TGF-β inhibits Smad3 expression and induces Smad7 expression 
in primary cultures of skin fibroblasts (50). Our exper-
iments show that RA induces Smad1 and 5 expression and 
that the RA inhibitor AGN 193109 blocks this effect. More 
importantly, the biological significance of these findings was 
established by: 1) enhancement of BMP phenotypic effects by 
RA signaling; and 2) the induction of the BMP-responsive 
type X collagen promoter by RA.

Furthermore, because Smad6 and 7 are inhibitory, BMP 
signaling is dependent on the relative concentrations of the 
receptor-activated Smads and the inhibitory Smads (47, 51, 
52). The inhibitory Smads are functionally important; Smad6 
is highly expressed in the developing heart, and mice lacking 
this protein have severe cardiac abnormalities (53, 54). Simi-
larly, Smad6 is an important inhibitor of maturation in BMP-
treated embryonic sternal chondrocyte cultures (33). There-
fore, inhibitory Smads are critical downstream signaling 
molecules that control cellular responsiveness to TGF-β and 
BMP, and growth factors and signaling molecules alter BMP 
signaling by regulating inhibitory Smad expression (55–59). 
We show that RA induces the expression of the Smad6 by 
approximately 6.7-fold but does not alter the expression of 
Smad7. These findings are consistent with prior findings 
showing Smad6 induction by BMP-2 in cephalic sternal chon-
drocytes (33). However, because the overall effect of RA in 
these experiments is to enhance BMP signaling, the induction 
of Smad6 by RA is a modular event that likely impairs 
BMP signaling and thus does not account for the synergistic 
interaction between RA and BMP signaling.

In addition to altering Smad expression, RA could also 
have enhanced BMP effects by increasing BMP expression or 
through a reduction in the expression of the extracellular 
BMP antagonists, noggin or chordin. RA has previously been 
shown to enhance the expression of one or more Bmps in 
chick growth plate chondrocytes and in other cell types (35, 
60–62). The present data demonstrate that RA induces 
BMP-2 approximately 4.4-fold and reduces noggin 60%, both 
of which would tend to increase the amount of BMP available 
to bind to the BMPR. However, because a maximally effec-
tive concentration of BMP-2 was used in the synergy exper-
iments, it is not likely that increased availability of BMP-2 
was a major contributor to the synergistic enhancement of 
colX expression. However, the induction of BMP-2 and re-
duction in extracellular antagonists could play a more im-
portant role in the independent effects of RA on chondrocyte 
maturity.

Finally, Smads interact with other transcription factors, at 
the promoter region of responsive genes, to enhance tran-
scription (32). A model has been established with several 
transcription factors, including cJun/cFos, FAST, ATF-2, 
OAZ, and the vitamin D receptor (31, 32, 52, 63). In these 
models, Smads enhance the activity of these other transcrip-
tion factors by binding to consensus sequences at adjacent 
sites that facilitate interaction with these other transcription 
factors and permit recruitment of transcriptional coactivators 
(64). TGF-β/BMP effects manifest only in the presence of 
these associated signals (31, 32). Additionally, direct inter-
actions independent of DNA binding have been observed 
between the vitamin D receptor and Smad 3. This association 
is necessary for the cooperative induction of the osteocalcin
promoter (31). Though the RAR and consensus binding sites have close similarities to vitamin D (27, 65), the interactive effects observed between RA and the BMPs do not seem to be secondary to cooperative interaction of their downstream signaling molecules. Unlike the effects observed with other promoters, BMP stimulates colX expression independent of RA. More importantly, the type X collagen promoter does not contain a consensus sequence for RA binding. The current results support induction of Smad1 and 5 expression by RA as an important mechanism involved in the enhancement of BMP-mediated effects on chondrocyte differentiation.

In summary, RA stimulates chondrocyte differentiation through BMP-mediated mechanisms. The induction of Smad 1 and 5 by RA is associated with RA effects, as well as BMP effects, on these cells. Though we focused on the effects on Smad1 and Smad5 expression, it is likely that the RA effects are contributed to by important regulatory events on other parts of the BMP-signaling pathway, including the inhibitory Smads, BMP expression, and the expression of the BMP antagonists. Further definition of the BMP-signaling pathway and regulation in chondrocytes will provide a comprehensive understanding of the mechanisms involved in the differentiation of these cells.

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