INTRODUCTION: During endochondral ossification chondrocytes undergo maturation marked by increases in type X collagen and alkaline phosphatase activity. Both bone morphogenetic proteins (BMPs) and Retinoids have been known to influence chondrocyte maturation and endochondral bone formation during skeletogenesis, and both signal through different receptors and transcription factors. The BMPs and related members of the transforming growth factor (TGF)-β superfamily signal through serine/threonine kinase receptors which phosphorylate Smad transcription factors. Retinoid signaling is mediated through the actions of nuclear retinoid receptors. These receptors act as ligand-dependent transcription factors, bind to specific responsive sequences, and modulate gene expression. Retinoic acid (RA) can act as a morphogen to influence early limb development, potentially through stimulation of BMP-2. The evidence that retinoic acid acts through BMPs to initiate skeletal tissue formation led us to examine whether there are cooperative actions of the BMPs and RA during chondrocyte maturation. Interactions between growth factors, particularly those which stimulate this process, have not been well characterized.

METHODS: Chondrocyte isolation and culture: Chondrocytes were isolated from 13 day old chick embryonic lower sterna. After primary cultured for 5-7 days, floating cells were plated in secondary culture. Chondrocytes were in culture in the presence of ascorbate (50µg/ml) each day. Treatments for BMP-2, RA and RA antagonist AGN 193109 were added to the cultures in some experiments at the first day of plating in serum-containing medium. To infect the chondrocytes with the RCAS virus containing BMP receptor DNA constructs, cells were cultured in media containing 25% filtered viral stock and 75% sternal chondrocyte media. After 2 days, the medium was changed to 100% sternal chondrocyte media.

Immunocytochemistry: Chondrocytes were grown in 8-well, plastic chamber slides for immunocytochemical analysis. Anti-gag (3C2 antibody) was probed for expression of the viral gag gene in chondrocytes.

RNA Isolation and Analysis: Messenger RNA was isolated from cultures using the RNeasy Kit. Northern analysis was performed on denaturing formaldehyde/agarose gels with 5 µg of RNA loaded per lane.

Alkaline Phosphatase Activity: Culture medium was aspirated from chondrocytes cultured in 24 well plates. One milliliter of reaction buffer, containing 0.25M 2-methyl-2-amino propanol, 1mM magnesium chloride, and 2.5 mg/ml of p-nitrophenyl phosphate was added to the wells. The reaction was stopped after 15 minutes with the addition of 0.5 ml of 0.3 M Na3PO4, and absorbance measured at 410 nm.

RESULTS: Retinoic acid signaling stimulates chondrocyte maturation. All trans-retinoic acid stimulated the expression of type X collagen in secondary cultures of lower sternal chondrocytes. The effect was dose dependent in maximal at 100 ng/ml. The effect was maximal in day 8 and day 12 cultures, where levels of type X collagen expression were markedly increased over basal level. Similar effects were observed in cultures treated with either cis or trans retinoic acid. Retinoic acid also stimulated alkaline phosphatase activity. Although type X collagen expression increased in the untreated cultures up to 20 days, the levels achieved in untreated cultures did not approach the levels observed in retinoic acid treated cultures.

Retinoic acid and BMP-2 synergistically enhance maturation. The addition of BMP-2 (1-50 ng/ml) also resulted in a dose dependent stimulation in expression of Type X collagen with effect quantitatively similar to those observed with retinoic acid. The treatment of lower sternal chondrocytes with both BMP-2 and retinoic acid together resulted in a synergistic increase in type X collagen with levels markedly higher than those observed with either BMP-2 or retinoic acid alone in 8 day cultures. Similarly, both BMP-2 and retinoic acid alone increased alkaline phosphatase activity in 8 day cultures, but a synergistic effect was observed in cultures treated with both factors together.

Retinoic acid effects are dependent upon BMP signaling. Lower sternal chondrocytes were infected with RCAS viruses expressing a dominant negative BMP receptors, BMPRIA and IB. In the presence of the dominant negative BMPR IB, which has previously been shown to block receptor signaling, the effect of retinoic acid on both type X collagen and alkaline phosphatase were essentially completely blocked (Figure). In contrast in cultures treated with the dominant negative BMPR IA, which does not completely block the onset of maturation in the presence of BMP-2, the effect of retinoic acid on alkaline phosphatase was not altered, and its effect on type X collagen stimulation was only partially inhibited.

DISCUSSION: The current study demonstrates the interdependence of both retinoic acid and BMP-2 signals in regulation of the expression of chondrocyte maturational characteristics, including type X collagen and alkaline phosphatase activity. While both of these agents alone stimulate chondrocyte differentiation, in combination they have a synergistic effect on type X collagen and alkaline phosphatase activity. Agents which block either BMP or retinoic acid signals prevent chondrocyte differentiation, even in the presence of a strong differentiating agent involved in a separate signaling pathway. The findings suggest the presence of BMPs and retinoic acid in the cultures under basal conditions. In the absence of added factors, the chondrocyte cultures slowly express differentiated characteristics. Retinoic acid is present in serum, and synthesis of BMPs by chondrocytes both in-vivo and in-vitro, have been described. It is likely that these factors are important in the differentiation of chondrocytes, both under basal conditions as well as during stimulation of maturation by the addition of a single factor. The manner in which the retinoic acid in BMP signaling factors interact is unclear. Both of the signaling pathways are complex, and interactions are possible in a number of steps. These include possible signaling process, receptor expression or activation, or the interaction in the transcriprional apparatus. Elucidation of these interactions will require further study, but will likely define important events in chondrocyte differentiation.