In Vitro Regulation of Expression of Cartilage-Derived Morphogenetic Proteins by Growth Hormone and Insulinlike Growth Factor 1 in the Bovine Cricoid Chondrocyte

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Objectives: To delineate the endogenous growth factors that regulate cricoid cartilage growth at the molecular level. Specifically, to attempt to establish the presence of cartilage-derived morphogenetic proteins (CDMPs), cartilage-specific members of the bone morphogenetic protein family, in newborn bovine cricoid chondrocytes and to assess the expression of these endogenous growth factors with the addition of exogenous growth hormone or insulinlike growth factor 1 in an in vitro chondrocyte culture model.

Methods and Design: Basic science molecular biologic research methods, including high-density monolayer and explant chondrocyte cultures with extraction of messenger RNA and quantitation via Northern blot hybridization via radiolabeled complementary DNA probes.

Setting: Intramural basic science research laboratory.

Results: Both CDMP-1 and CDMP-2 were found in newborn bovine cricoid chondrocytes. Addition of exogenous growth hormone did not appear to influence the expression of CDMP-1 or CDMP-2. Addition of exogenous insulinlike growth factor 1 appeared to down-regulate the expression of CDMP-1 but had no effect on the expression of CDMP-2. No major differences in CDMP level of expression were noted between high-density monolayer cultures vs explant cultures. No tissue specificity differences were noted in regulation of CDMPs between cricoid and articular chondrocytes.

Conclusions: Our preliminary studies indicate the presence of endogenous morphogenetic proteins in newborn bovine cricoid chondrocytes. These novel polypeptide hormones (CDMP-1 and CDMP-2) have not been previously reported in laryngeal cartilage chondrocytes. Change in level of transcription of these morphogenetic proteins under various in vitro conditions suggests that these proteins are subject to regulation and/or play a regulatory role in cricoid chondrocyte growth and differentiation. Further experimentation is needed to confirm these findings.


Congenital subglottic stenosis can be secondary to abnormal growth of the cricoid cartilage. The treatment varies from watchful waiting in mild cases to expansion laryngotracheal reconstructive surgery with auricular and costal cartilage grafting in severe cases. The factors that influence laryngeal cartilage growth, specifically cricoid cartilage growth, have not been delineated. In addition to structural proteins of cartilage such as type II collagen, cartilage cells synthesize a number of regulatory growth factors that are stored in the cartilage matrix.

Recent work on articular and growth plate cartilage chondrocytes has identified these various polypeptide growth factors as bone morphogenetic proteins (BMPs). Members of the transforming growth factor β (TGF-β) superfamily, these morphogens are known to affect chondrocyte proliferation and differentiation in articular and growth plate chondrocytes. At present, more than 16 members of the BMP family and related osteogenic proteins have been reported.

The newest addition to the BMP family is the subpopulation known as the cartilage-derived morphogenetic proteins (CDMPs), which are structurally similar to the BMPs. To date, 3 CDMPs have been isolated, CDMP-1 and CDMP-2, and are noted to be present in bovine articular and growth plate chondrocytes and human embryonic precartilaginous mesenchymal tissue. These novel subfamily members are most closely related to BMP-5, BMP-6, and osteogenic protein 1 (BMP-7).

Based on the work in growth plate and articular chondrocytes, this series of experiments was designed to examine cri-
MATERIALS AND METHODS

PREPARATION OF NEWBORN BOVINE AND ARTICULAR CHONDROCYTE CULTURES

The larynges of 5 newborn (2- to 6-week-old) calves obtained from a slaughterhouse were received less than 12 hours after slaughter. The specimens were initially cleansed with providone-iodine solution, placed in a HEPES-antibiotic solution, washed 3 times in the HEPES-antibiotic solution after rotary stirring, and immediately dissected under sterile conditions. The cricoid cartilage was isolated, and all overlying soft tissue and perichondrium were removed. Each cricoid cartilage sample was cut into 1 × 1 × 1-mm pieces and placed into a serum-free medium consisting of Dulbecco modified Eagle medium (DMEM) with high glucose content, 4.3 g/L (Whittaker Bioproducts, Inc, Walkersville, Md), with 0.2% bovine serum albumin (1 g per 500 mL of DMEM) containing 10 mL of antibiotic solution (penicillin, 100 U/mL; streptomycin sulfate, 100 µg/mL; amphotericin B, 250 ng/mL [Life Technologies, Bethesda, Md; catalogue No. 15240-013]; gentamicin sulfate, 20 mg per 500 mL of media [Whittaker Bioproducts]; and 5 mL of 1-mol/L HEPES [Quality Biological Inc, Gaithersburg, Md]). The samples were transferred into sterile 50-mL test tubes and washed 3 times with DMEM serum-free medium. Digestion was accomplished with 0.2% collagenase (collagenase A, Boehringer Mannheim Biochemicals, 500 mg of collagenase in 250 mL of DMEM) during 16 hours in a 37°C oven at 95% air and 5% carbon dioxide. Appropriate aliquots of each digestion were taken for direct processing into RNA (native chondrocyte specimens). High-density monolayer cultures in 75-cm² flasks (flask 3023, Falcon Co, Bethesda) were then established. For each flask, 10⁶ cells were plated and grown to high-density confluence in 16 hours in serum-containing media. Following this, the cells were washed in serum-free medium 3 times and then cultured in serum-free medium. The following were prepared: 2 flasks of chondrocytes grown in serum-free medium for 72 hours without addition of any exogenous growth factor or hormone, 2 flasks of chondrocytes grown in serum-free medium with the addition of 20 ng/mL of IGF-1 per day for 3 days, and 2 flasks of chondrocytes grown in serum-free medium with the addition of 100 ng/mL of GH (recombinant GH, Genentech, San Francisco, Calif). An identical procedure was performed on the newborn bovine articular cartilage specimens to obtain articular chondrocytes to use as a control against the cricoid chondrocyte specimens.

BOVINE CRICOID AND ARTICULAR EXPLANT SYSTEM PREPARATION

The larynges of 5 newborn (2- to 6-week-old) calves obtained from a slaughterhouse were received less than 12 hours after slaughter as described above and prepared and grown in the tissue culture explant system as described by Zalzal and Luyten. LIC The cricoid cartilage samples were dissected steriley and cut into 1 × 1 × 1-mm pieces, washed 3 times in the medium described above and placed in 50 mL of the medium. The explants were washed overnight in a 37°C incubator at 95% air and 5% carbon dioxide with continuous shaking. The basal medium was changed 3 times during this period. Sixteen hours later, the explants were then plated onto tissue wells (Costar, Cambridge, Mass), keeping the medium-tissue ratio constant throughout the whole culture period at a ratio of 1 mL per well and each well containing between 100 and 150 mg of cartilage and placed in the incubator at 37°C with 95% air and 5% carbon dioxide. The explants were incubated for 6 days to deplete endogenous growth factors. On the sixth day, the

coidal cartilage chondrocyte growth at the molecular level. The specific aims of the study were to identify endogenous BMPs and CDMPs in the cricoid cartilage chondrocytes and to examine the effect of growth hormone (GH) and insulinnlike growth factor 1 (IGF-1) on laryngeal growth and whether its effect is mediated through BMPs and CDMPs.

RESULTS

Northern blot analysis performed with specific complementary DNA probes designed from the preregion of CDMP-1 and CDMP-2 revealed that both genes are expressed postnatally in newborn bovine cricoid cartilage (Figure 1). A single CDMP-1 mRNA transcript of approximately 3 kb was observed. The CDMP-2 mRNA was present in newborn bovine cricoid cartilage as a 4.6-kb band. These findings were confirmed by comparison of this blot to Northern blot analysis of CDMP-1 and CDMP-2 vs newborn bovine articular cartilage previously shown to express these genes.

Figure 2 demonstrates the regulation of CDMP-1 and CDMP-2. The CDMP-1 is expressed in low levels in freshly isolated (native) newborn bovine cricoid chondrocytes. In cultured cricoid chondrocytes (high-density monolayer and explant cultured in serum-free media), the expression level of CDMP-1 increases substantially by about 4-fold compared with CDMP-2. Conversely, CDMP-2 was found in high levels in native newborn cricoid chondrocytes. In cultured cricoid chondrocytes, the expression level of CDMP-2 decreased dramatically by about 10-fold compared with CDMP-1. These findings were confirmed in newborn bovine articular chondrocytes that were run as controls. The addition of exogenous GH (100 ng/mL per day for 3 days) did not appear to influence the expression of CDMP-1 or CDMP-2 in either high-density monolayer cultures or explant cultures. The addition of exogenous IGF-1 (20 ng/mL per day for 3 days) appeared to down-regulate the expression of CDMP-1 but had no effect on the expression of CDMP-2.

No major differences in CDMP level of expression were noted between high-density monolayer cultures vs explant cultures. No tissue specificity differences were noted in level of transcription or degree of expression of CDMPs between cricoid and articular chondrocytes.
tissue plates were divided into the following groups: serum-free media (group 1); exogenous GH, 10 ng/mL (group 2); and exogenous IGF, 120 ng/mL (group 3). The exogenous growth factors were added daily for a total of 3 days (72 hours). Following this, the explants were digested in 6 hours with 0.2% collagenase as described above in a 37°C oven at 95% air and 5% carbon dioxide with continuous shaking. Newborn bovine articular chondrocyte control samples in equal amounts were prepared in the same fashion simultaneously.

**EXTRACTION OF TOTAL RNA**

The extraction of total RNA was accomplished by the acid-guanidinium-phenol-chloroform method of RNA isolation as described by Chomczynski and Sacchi. The concentration of total RNA was determined via spectrophotometry.

**DETERMINATION OF MESSENGER RNA**

Messenger RNA (mRNA) was extracted from the total RNA by the Promega PolyA tract isolation systems (product Z5300). The system has a biotinylated oligodeoxythymidine primer to hybridize in solution the 3' polyadenylic acid (poly(A)') region present in eukaryotic mRNA species. The hybrids were then captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA was eluted from the solid phase by the addition of ribonuclease-free deionized water. This procedure yielded an essentially pure fraction of mature mRNA (poly(A)'). One miligram of total RNA typically yielded approximately 1 µg of mRNA. The concentration and purity of the eluted mRNA were determined by spectrophotometry as described in the previous section. Pure mRNA has an OD 260/280-nm ratio of 2.0 or greater.

**NORTHERN BLOT ANALYSIS OF mRNA**

Northern hybridization was used to determine the size and amount of specific mRNA molecules. Two micrograms of messenger RNA poly(A)' for each subpopulation of chondrocytes (native, serum-free high-density monolayer, GH high-density monolayer, IGF-1 high-density monolayer, serum-free explant, GH explant, IGF-1 explant) was electrophoresed on 1.2% agarose-formaldehyde gel and transferred to Nytran nylon filters. The membranes were prehybridized for 3 hours at 42°C in hybridization buffer (5X SSPE, 5X Denhart solution, 50% formamide, 1% sodium dodecyl sulfate, and 100 µg/mL of freshly denatured salmon sperm DNA). Hybridizations with phosphate 32-labeled deoxyctydine triphosphate probes, having specific activities of at least 1 × 109 cpm/µg, were performed overnight under the same conditions as the prehybridization. Probes included the complementary DNA probe for human glyceraldehyde-3-phosphate dehydrogenase (1.1 kilobase [kb]) (Clontech), an Apal fragment (470-1155 base pairs) of CDMP-1, and Apal fragment (194-677 base pairs) of CDMP-2. Glyceraldehyde-3-phosphate dehydrogenase is a housekeeping gene that is found in eukaryotic cells, and probing for glyceraldehyde-3-phosphate dehydrogenase allows for a quantitative and qualitative assessment within the Northern blotting experiment. The CDMP-1 and CDMP-2 probes were chosen to avoid the highly conserved carboxy-terminal domain, therefore minimizing the potential for cross-hybridization with other members of the gene family. Following hybridization, the filters were washed to a final stringency of 55°C (0.4 × SSC, 0.1% sodium dodecyl sulfate). The expression levels were quantified using a Phosphorimager (Molecular Dynamics).

**COMMENT**

The current treatment of severe subglottic stenosis involves the use of a costal cartilage graft that is sculpted and sutured into the stenotic airway during laryngotracheal reconstruction. The fate of these cartilage grafts has been studied by several authors. The grafts appear to survive and act as a stent or bridge. However, the grafts have not been shown to “mesh” into the laryngeal framework at the molecular level. Zalzal et al reported that although in rabbits autograft elastic cartilage were digested by collagenase at 2 and 4 months, growth of the grafts did not occur. Cartilage is repaired slowly through proliferation of cartilage or chondroblast and subsequent synthesis and deposition of type II collagen. At a molecular level, several major growth factor families that are important in tissue growth and repair have been delineated. These polypeptide molecules are responsible for inducing mesenchymally derived cells such as monocytes and fibroblasts to migrate, proliferate, and differentiate. Growth factors are synthesized and released from various cells, especially those cells responsible for wound repair, namely, platelets, macrophages, and fibroblasts. Most growth factors belong to families of related molecules, and each growth factor has a different mechanism of action and different target cell specificity. For cartilage, growth factors such as TGF-β, BMPs, basic fibroblast growth factor, platelet-derived growth factor, IGF, and GH have been shown to stimulate chondroblast proliferation in vitro.
Members of the TGF-β superfamily are known as BMPs and have been found to be of fundamental importance both in bone formation and mesenchymal-epithelial interactions in early embryogenesis. In 1965, Urist reported that bone protein extracts that were injected into nonskeletal sites in rats induced ectopic bone and cartilage formation. Urist coined the term bone morphogenetic proteins. Wozney et al, Luyten et al, and Sampath et al subsequently independently characterized BMPs at the protein level. Six related BMPs, named BMP 2 to BMP 7, have been isolated via molecular cloning. Certain BMP genes have been localized in the human genome.

Most BMPs have been shown to have osteoinductive potential in vivo, and some participate in promotion, maintenance, and termination of the cartilage and bone cell phenotypes in vitro. In addition, BMPs play a role in numerous developmental processes, such as epithelial-mesenchymal interactions. Most recently, recombinant BMPs have been used to heal craniofacial and periodontal lesions in nonhuman primates. Biochemically, BMP proteins have a hydrophobic secretory leader sequence, a large propeptide region, and a mature domain. In the mature domain resides the 7 cysteine residues common to most members of the TGF-β superfamily. After the BMPs are synthesized by the cell, they dimerize and become glycosylated. On secretion from the cell, a cleavage even occurs such that the mature active protein is a dimer of the carboxy-terminal region of the precursor peptide. The BMPs are divided into 3 subgroups based on amino acid homologies.

The CDMPs have recently been added to this group. The CDMPs are unique in that, to date, they are the only cartilage-specific morphogenetic proteins, ie, they appear to have a cartilage-specific localization pattern compared with the ubiquitous presence of other BMP family members. Thus, investigators posit the potential role for these proteins in chondrocyte growth and differentiation. In situ hybridization has demonstrated that CDMPs are preferentially expressed in the cartilaginous cores of long bones during human embryogenesis. Mainly, CDMP-1, the human homologue of mouth growth differentiation factor 5, is localized in the precartilaginous regions of developing limbs and epiphyseal cartilage, and its primary role is believed to be as an agent promoting cartilage and bone differentiation during joint morphogenesis in the appendicular skeleton. Recombinant CDMP-1 pro-

Figure 1. Northern blot analysis of cartilage-derived morphogenetic proteins 1 and 2 (CDMP-1 and CDMP-2). A single CDMP-1 messenger RNA transcript of approximately 3 kilobases and a CDMP-2 messenger RNA of approximately 4.6 kilobases is present in newborn cricoid chondrocyte poly(A) RNA. A Northern blot analysis of multiple human postnatal tissues and newborn bovine articular chondrocytes known to express CDMPs are shown for comparison, demonstrating that both genes are predominantly expressed in cartilaginous tissues. IGFI indicates insulin-like growth factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 2. Northern blot analysis demonstrating expression of cartilage-derived morphogenetic proteins 1 and 2 (CDMP-1 and CDMP-2) in vitro. In native cricoid chondrocytes, the level of CDMP-2 is significantly higher than CDMP-1. Note up-regulation of CDMP-1 and down-regulation of CDMP-2 in the in vitro explant (control) culture. In serum-free conditions, addition of exogenous growth hormone (100 ng/μL) did not alter expression of the CDMPs, whereas addition of exogenous insulinlike growth factor 1 (20 ng/μL) significantly down-regulated CDMP-1. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase.
tein has been shown to be chondrogenetic in vitro and in vivo.6 The expression pattern of CDMP-2 during human embryonic development suggests that it is involved in terminal differentiation of chondrocytes and in angiogenesis. In addition, CDMP-2 has high levels of expression in postnatal cartilage tissues.8 Our finding of CDMP-1 and CDMP-2 in the postnatal cricoid chondrocyte suggests some role of these morphogens in airway cartilage and growth, differentiation, and maintenance.

The GH, acting in conjunction with IGF-1 and IGF-2 somatomedins, has important regulatory roles in the rate of differentiation and proliferation of chondrocytes.21,22,25 It is still unclear whether GH acts directly on cartilage growth or only in symphony with IGFs. Somatomedins are mainly produced in the liver and kidney but are also thought to be secreted by chondrocytes. Isaksson et al21,22 suggested that GH may induce the expression of genes for local growth factors in chondrocytes.

Most investigators30 have been unable to show any effect of GH alone on DNA or matrix synthesis of cartilage in vitro. However, Isaksson et al31 reported that GH injected locally into epiphyseal growth plates of hypophysectomized rats led to a significant increase of growth plate width, and Madsen et al32 demonstrated GH stimulated thymidine incorporation and proteoglycan synthesis in high-density chondrocyte cultures. Vetter et al30 reported that IGF-2 is a more potent stimulant of clonal growth of chondrocytes during fetal life, and IGF-1 is more effective in stimulating clonal growth of chondrocytes during postnatal life. Smith et al33 reported that GH and IGF-1 act together to stimulate adult chondrocyte extracellular matrix synthesis and that IGF-1 stimulated chondrocyte DNA and proteoglycan synthesis. The GH alone had no effect on either process. Neither IGF-1 nor GH altered the hydrodynamic size of proteoglycans or synthesis of collagen.34 Luyten et al34 showed that IGF-1 at 20 ng/mL is sufficient to maintain steady state metabolism for at least 6 weeks in the absence of fetal calf serum. They also demonstrated that IGF-1 has the ability to balance biosynthesis and catabolism to maintain a steady state concentration of the matrix in bovine articular cartilage explants. Our results demonstrate no alteration of expression of CDMPs with addition of exogenous GH alone, suggesting that GH may work through a mechanism independent of CDMPs. Furthermore, our data suggest that addition of exogenous IGF-1 decreases CDMP-1 in high-density monolayer and explant cultures and thereby has some regulatory effect on the expression of this endogenous morphogen. These results should be verified over various culture time intervals with various concentrations of GH and IGF-1. The exact mechanism of CDMP regulation is yet to be delineated.

The results of this preliminary series of experiments document the presence of endogenous CDMPs in cricoid cartilage chondrocytes. We suspect these endogenous polypeptides stimulate cell proliferation and metabolism. The alteration in expression of these morphogenetic proteins seen with various culture and media conditions over various time intervals in our experiment suggests these morphogenetic proteins are subject to regulation. With continued confirmatory data to our initial results, we can attempt to biochemically enhance growth in a congenitally stenotic airway by injecting the appropriate growth factor into the cricoid cartilage. Moreover, use of these morphogens may enhance incorporation of cartilage grafts into the airway during surgical reconstruction and create a viable, fully incorporated graft that is indistinguishable from native laryngeal cartilage. A final long-term goal would be to grow in vitro cartilage grafts from autologous laryngeal cartilage cells with the aid of these morphogens and to reimplant them to correct an airway stenosis requiring open surgical repair. Biologic repair of airway cartilage based on this molecular research may revolutionize medical and surgical approaches to subglottic stenosis.

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REFERENCES


