Cartilage-derived morphogenetic proteins

Key regulators in chondrocyte differentiation?

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Biological regeneration of tissues can be considered a feasible goal in modern medicine largely because of scientific advances in this past decade. These advances clearly demonstrate that the cellular and molecular events taking place during the formation of tissues in embryogenesis are recapitulated during their repair or regeneration. A striking example of this biological principle is bone remodeling and fracture repair, showing a virtually identical cascade of events as seen in the formation of long bones in development. The potential therapeutic implications in diseases such as osteoporosis and osteoarthrosis, in a number of clinical problems such as delayed unions and avascular necrosis and for a large number of congenital osteochondrodysplasias, have intensified the research efforts in the field of skeletal development. This has resulted in major breakthroughs in the characterization of the molecular signals involved in the formation of the skeleton (Erlebacher et al. 1995). An example of this is the discovery of the family of Bone Morphogenetic Proteins (BMPs), originally defined by their ability to induce de novo in an ectopic site endochondral bone formation in vivo (Urist 1965, Wozney et al. 1988, Luyten et al. 1989, Sampath et al. 1990).

The role of bone morphogenetic proteins in skeletal development

Intense cloning approaches have led to the characterization of an increasing list of BMP family members with a structural homology to the TGF-ß superfamily (Kingsley 1994). This superfamily comprises a large group of signaling molecules that are secreted as biologically active dimers with a highly conserved carboxyl-terminal domain containing seven cysteines. This high conservation in evolution, with close relatives present in Drosophila (Wharton et al. 1991, Doctor et al. 1992), suggests that the BMPs existed long before the appearance of cartilage and bone. In addition, several BMPs produced as recombinant protein induce cartilage and bone formation when subcutaneously implanted in rats (Reddi 1992), demonstrating that their physiological roles have yet to be determined. Localization studies exhibiting their expression in many different tissues, suggested that the BMPs may play diverse roles in higher animals.

The physiological role of the BMPs can be examined by studying the phenotypes associated with deletions or mutations of the genes. The first evidence that at least some BMPs are key players in the formation of skeletal structures was provided by the defects in many small bone and cartilage structures in short ear mice caused by a nonsense mutation in the middle of the Bmp5 coding region (Kingsley et al. 1992).

The involvement of other BMPs in cartilage and bone formation is most likely, because most of them have profound effects on in vivo and in vitro models of chondrogenesis and osteogenesis (Vukicevic et al. 1989, Vukicevic et al. 1991, Luyten et al. 1992, Ripamonti et al. 1992, Chen et al. 1993, Luyten et al. 1994, Chen et al. 1995), and are present in active form at specific stages of skeletal development (Vukicevic et al. 1994). Ongoing inactivation studies using a variety of approaches including transgenic and gene "knock-out" experiments will further establish their importance in controlling the formation, growth and repair of skeletal tissues. In the meantime, applications using BMPs as agents promoting bone repair in plastic and reconstructive surgery show great potential (Ripamonti and Reddi 1995).

Discovery of the cartilage-derived morphogenetic proteins, novel members of the BMP family

The unexpected finding that the 0.15 M NaCl eluate of articular cartilage extracts after ion exchange chromatography (DEAE-Sephadex) induced the formation of several islands of chondrocytes in the in vivo subcutaneous implantation model, prompted us to further characterize the potential chondrogenic

factors in cartilaginous tissues. We focused on the articular cartilage since this unique tissue never undergoes bone formation. In addition, localization studies at that time indicated the absence of known BMPs in the chondroblasts and chondrocytes of developing skeletal structures. Partial purification of the activity using heparin-Sepharose affinity chromatography and molecular sieve chromatography, confirmed the presence of chondrogenic/osteogenic factors in the cartilage extracts. Highly purified fractions were obtained after ConA-Sepharose chromatography, SDS polyacrylamide gel electrophoresis followed by gel elution, indicating that the activity resided in the area between 35–40 kDa. Protein sequencing data of tryptic digests of the gel eluted chondrogenic activity did reveal the presence of unique peptides, and surprisingly no sequencing data corresponding to any of the known BMPS. These data, together with the loss of activity after reduction and alkylation, suggested the possibility of the existence of other BMP members produced by chondrocytes. At this point we decided to pursue this hypothesis using molecular biology approaches. Based on the high degree of conservation of the C-terminal domain of the BMPs and TGF-ß superfamily members (see above), a large number of degenerate primer sets were designed and used in RT-PCR, a strategy previously successful when used to identify TGF-ß superfamily members in Drosophila (Wharton et al. 1991). Using mRNA prepared from newborn articular cartilage, we identified 2 novel members of the BMP family, designated Cartilage-derived Morphogenetic Proteins-1 and -2 (CDMP-1, CDMP-2) (Chang et al. 1994). They can be classified as members of a novel subfamily, structurally and probably functionally related based on the identity of their carboxyl-terminal domains (about 80%). Genomic cloning resulted in the identification of a third member, CDMP-3 (Thomas et al. manuscript in preparation).

CDMP-1 and CDMP-2 are expressed and transcriptionally regulated in postnatal cartilage

At this point it was not clear if the CDMPs, despite their cloning from cartilage cDNA libraries, were involved in the formation, growth or maintenance of cartilaginous tissues. Northern blot analysis performed with specific cDNA probes designed from the pro-region of CDMP-1 and CDMP-2 revealed that both genes are expressed postnatally in newborn bovine articular and cricoid cartilage. The levels of CDMP-2 were significantly higher then those of CDMP-1. Interestingly, while the expression of CDMP-2 was down regulated as a result of in vitro culturing, as was seen for typical cartilage markers such as collagen type II, CDMP-1 expression was upregulated both in monolayers (data not shown) and explant cultures (Figure 1). In addition, in serum-free conditions, growth hormone did not have any effect on the expression of the CDMPs , while IGF-I significantly downregulated CDMP-1 (Figure 1). These data, together with the low levels of expression in other postnatal tissues, suggest that the CDMPs, especially CDMP-2, contribute to the postnatal growth and maintenance of cartilage tissues. Ongoing studies are addressing their expression and synthesis in adolescent and adult cartilage as well as in cartilage disease states such as osteoarthritis.

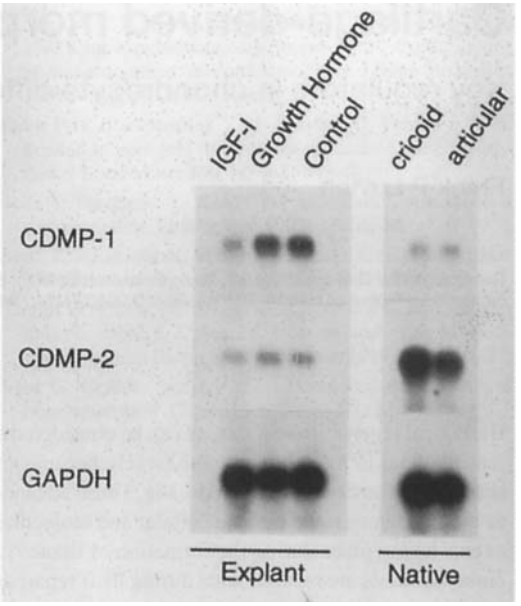

Figure 1. Regulation of expression of CDMP-1 and CDMP-2. Poly (A+) RNA was prepared from freshly prepared articular and cricoid chondrocytes (Native) or from articular cartilage explant cultures (Explant). The mRNA samples were separated on a 1.2 % agarose-formaldehyde gel, blotted and hybridized with 32P-labeled ApaI cDNA probes for CDMP-1 and CDMP-2 (Chang et al. 1994). The cartilage explants were cultured in serum-free conditions (controls) for three days, or in serum-free media containing IGF-I (20 ng/ml) or growth hormone (100 ng/ml). A human cDNA GAPDH probe was used to verify equal loading.

Role of the CDMPs in skeletal development

In situ hybridization in human embryonic development demonstrated transcripts for CDMP-1 in the pre cartilage mesenchymal condensations of the developing limb, and in the cartilaginous cores of the long bones with complete absence in the axial skeleton. Immunolocalization data using a specific peptide antiserum confirmed the synthesis of CDMP-1 in these sites. This suggests a pivotal role for this mole-

cule in the development of the appendicular skeleton. Genetic approaches confirmed this. We mapped CDMP-1 on chromosome 2 in close proximity to the brachypodism (bp) locus. Screening of the literature revealed that the bp mutation is a single gene mutation affecting several steps in early limb development resulting in a distinct shortening of the limbs. Careful analysis of the underlying cellular defect demonstrated that the primary defect in bp mice is due to the reduced ability of a specific mesenchymal cell population to provide an inductive chondrogenic stimulus (Owens and Solursh 1982). These data strongly suggest that a mutation in CDMP-1 was responsible for the bp phenotype in mice. During the course of our investigation an independent study established that effective null mutations in a novel gene Gdf-5, the mouse homologue of the human CDMP-1, were found in bp mice (Storm et al. 1994). These data unequivocally demonstrate the role of CDMP-1/Gdf-5 as a physiological chondrogenic signal.

CDMP-2 localization studies in development were less revealing. Northern analysis of poly (A+) blots of a variety of fetal human tissues showed the most prominent transcripts in cartilage, heart and liver. Mapping of CDMP-2 did not reveal any association with an obvious skeletal phenotype and will be reported elsewhere. The in situ hybridization data indicated its expression at later stages and in the more mature and hypertrophic chondrocytes of the developing limb, consistent with the higher levels of expression of CDMP-2 in postnatal cartilage tissues.

Conclusions

The presence of in vivo chondrogenic activity in cartilage extracts, and the characterization of this activity at the protein level suggested the presence of BMP-like molecules in cartilage. Using molecular biology approaches, we discovered the existence of a novel subgroup of the TGF-ß superfamily, designated the CDMPs, now containing at least 3 members. Localization studies, genetic approaches and gene regulation studies establish the involvement of CDMP-1 in the development of the appendicular skeleton as a chondrogenic inductive signal, and of CDMP-2 as a possible regulatory molecule in the growth and maintenance of cartilaginous tissues. The characterization and expression of a third member of this family, CDMP-3, will be reported elsewhere.

The physiological role of CDMP-2 and other members of the subfamily in skeletal development is as yet unknown. The genetic data support the concept that the skeleton is a mosaic structure in which the pattern is most probably the result of the combined activities of a number of BMP-like molecules. Ongoing transgenic approaches, gene "knock-outs" and gene "replacement" studies will soon provide more insight in this matter.

A major challenge—the articular chondrocyte

The TGF-ß superfamily of signaling molecules, especially the BMPs and BMP-like genes, provide the scientific community with a set of tools to further define the mechanisms involved in chondrogenesis, chondrocyte maturation and osteogenesis. Their discovery has therefore been a major contribution to provide a scientific basis for the biological regeneration of skeletal tissues including cartilage and bone. This is just the beginning of what promises to be a very exciting era in skeletal development and repair, and many questions continue to arise. Having a particular interest in the possible biological repair of joint surfaces, a major challenge today is the understanding of the process of joint morphogenesis, and articular cartilage development. What is the developmental origin of the articular surface? The discovery of the CDMPs might provide an opportunity to understand more about the inductive signals involved in chondrogenesis and chondrocyte maturation. Although the data suggest that the soluble signals involved in the development of the appendicular skeleton are distinct from the ones determining the morphogenesis of the axial skeletal, there seems to be no evidence that the BMP-like molecules involved in early joint development are distinct from the ones in long bone development. However, there are several indications that the differentiation pathway of the articular chondrocyte might be separate from the other chondrocytic lineages. For instance, agents (thyroid hormone, osteogenic protein-1, retinoic acid) known to promote maturation and hypertrophy of chondrocytes in vitro, are incapable of doing so when added to cultures of articular chondrocytes (Chen et al 1993; Chen et al 1995). In addition, we recently discovered a novel membrane associated protein, expressed in a gradient pattern and restricted to the cartilaginous anlage of the skeleton in human embryonic development (Hoang et al submitted). Strikingly, and in contrast to CDMP-1 for instance, no transcripts were seen in the 5–6 cell layers of the articular surface of the developing joint, suggesting that the developmental pathways of the joint surface may be distinct from the rest of the appendicular skeleton early on (8 1/2 weeks in human development). The development of markers which would allow the investigator to identify the "proper" articular chondrocyte, will be a vital part of the scientific basis for the development of clinical protocols in

the biological repair of the joint. These scientific breakthroughs are paving the way to dramatically reduce the invasiveness of current clinical approaches in joint and skeletal diseases.

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