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1992

http://hdl.handle.net/10539/20438

Luyten,F.P., Cunningham,N.S.,Vukicevic,S.,et al. 1992.Advances in osteogenin and related bone morphogenetic proteins in bone induction and repair. Acta orthopaedica belgica;58 Suppl 1:263-267

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ADVANCES IN OSTEOGENIN AND RELATED BONE MORPHOGENETIC PROTEINS IN BONE INDUCTION AND REPAIR

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Bone matrix is a repository of growth and differentiation factors as demonstrated by the induction of local cartilage and bone formation in rats. The bone inductive activity, termed osteogenin, can be dissociatively extracted, and it was isolated by heparin affinity, hydroxyapatite and molecular sieve chromatography. Osteogenin has been purified to homogeneity from bovine bone matrix and the sequences of several tryptic peptides have been determined. The sequences were similar to portions of the amino acid sequence deduced from the cDNA clone of bone morphogenetic protein-3 (BMP-3). The carboxyl-terminal quarter of osteogenin has sequence identity to the corresponding regions of two related proteins BMP-2A and BMP-2B. The bone inductive proteins are members of the TGF-β superfamily, by virtue of the location of the highly conserved cysteines in their carboxyl-terminal region. Osteogenin and related BMPs initiate cartilage and bone formation *in vivo*. The study of the mechanism of action of these proteins will add considerable new information on the molecular signals controlling endochondral bone formation. *In vitro* data indicate that osteogenin stimulates the expression of the osteogenic and chondrogenic phenotypes. Our results demonstrate their profound influence on proteoglycan synthesis and degradation in bovine cartilage explant cultures. High affinity specific binding sites have been identified in both MC3T3 cells and articular chondrocytes. *In vivo* experiments demonstrate the efficacy of primate osteogenin in restoring large calvarial defects in adult baboons, establishing a primary role for osteogenin in therapeutic initiation and promotion of osteogenesis.

Keywords : osteogenin ; morphogenetic proteins ; bone induction ; bone repair.

Mots-clés : ostéogénine ; protéines morphogénétiques ; induction osseuse ; réparation osseuse.

The remarkable potential for repair and regeneration of bone is well known. This potential can be partly explained by the bone inductive properties of demineralized bone-matrix implantation at heterotopic sites. The likelihood that bone contains one or more osteogenic substances has been suggested by Pierre Lacroix in Belgium when he demonstrated that alcohol extracts of epiphyseal cartilage and bone induced the formation of an ossicle with bone marrow when implanted under the kidney capsule (6). The finding of Urist that demineralized bone matrix induced endochondral bone formation at a heterotopic site in vivo (20), triggered the interest of many basic scientists. The continuous research efforts of cell biologists resulted in the characterization of the cascade of events involved in the endochondral bone induction by demineralized bone matrix and the development of a reliable in vivo endochondral bone formation bioassay (12, 13).

This multistep cascade consists of chemotaxis, attachment of mesenchymal stem cells to the matrix, proliferation of progenitor cells, and differentiation of cartilage, followed by cartilage resorption and bone formation, and finally culminating in the formation of an ossicle filled with hematopoietic marrow. The events are taking place in a well-defined time course : at day 1,

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fibronectin is deposited on the matrix facilitating cell attachment; at day 2 and 3, a pronounced proliferation of mesenchymal cells takes place; this cell-matrix interaction results in the differentiation of chondroblasts at day 5, with culmination of a cartilaginous matrix as shown by an increased [35S]sulfate incorporation into proteoglycans, and the appearance of hypertrophic chondrocytes at day 7; angiogenesis with cartilage resorption starts on day 9, and new bone formation in association with [45Ca] incorporation at day 11; extensive bone remodeling with hematopoietic marrow formation as evidenced by [59Fe] incorporation appears on day 21. This developmental program is a single cycle of cartilage and bone formation and recapitulates the embryonic development of limbs, and could be a useful model for fracture healing. Moreover, this system permits molecular studies at the different stages of endochondral bone formation and a better understanding of the role of endogenous and exogenous factors involved. Our research goal has been the isolation and characterization of factors involved in this cascade of events, and subsequently the study of the mechanisms of action of the proteins initiating this differentiation process. We termed this cartilage and bone inductive activity "osteogenin", in honor of Pierre Lacroix who first described the hypothetical existence of these proteins in bone (6).

A breakthrough in the isolation of osteogenin has been the dissociative extraction of demineralized bone matrix by 4M guanidinium hydrochloride, or 8M urea (16). Neither the soluble extract, nor the residual bone powder alone had detectable activity when implanted subcutaneously in the rat. However, if the extract was reconstituted with the inactive residue, the biological activity of bone and cartilage formation was recovered. This reconstitution assay has permitted the further purification of osteogenic components. Additional experiments revealed that irrespective of the species, the lower molecular weight fractions of a 4M guanidinium hydrochloride extract following molecular sieve chromatography induced new cartilage and bone formation in rats (17). The stage was set for the large-scale purification of osteogenin.

The purification flow chart of osteogenic activity is shown in fig. 1. Extraction of demineralized bone powder (particle size 75-400 μm) with 6M urea, followed by hydroxyapatite chromatography, heparin affinity, molecular sieve (Sephacryl S-200) chromatography, and finally gel elution are the major steps in the purification schedule (7, 18). Preparative SDS polyacrylamide gel electrophoresis followed by gel slicing, electroelution and reconstitution for the in vivo bioassay, localized the osteogenic activity to a region between 28-40 kDa, with the peak of the activity between 30-35 kDa (7). N-terminal sequencing data could not be obtained. Sequences of tryptic peptides of the highly purified material showed similarities to the amino acid sequence deduced from the cDNA clones of bone morphogenetic protein-3 (BMP-3) (26). The carboxyterminal quarter of osteogenin has sequence identity to the corresponding region of two related proteins BMP-2A and BMP-2B (now BMP-4) (26).

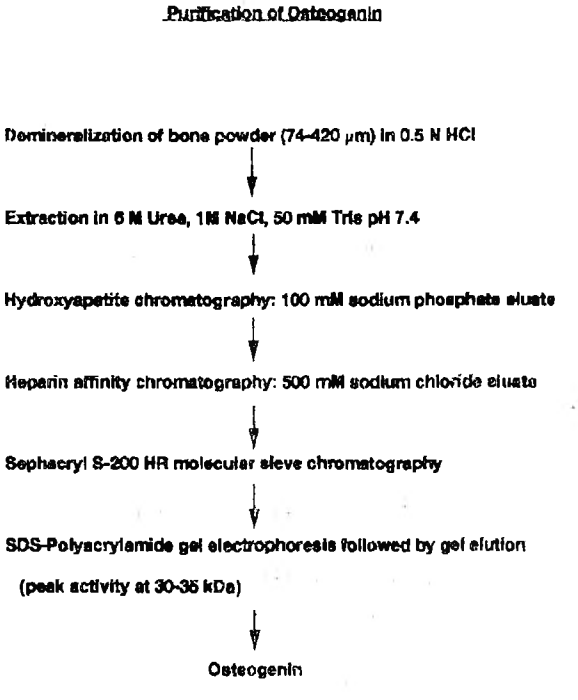


Fig. 1. — Purification process of osteogenin (7).

Abbreviations: HCl, hydrochloric acid; NaCl, sodium chloride.

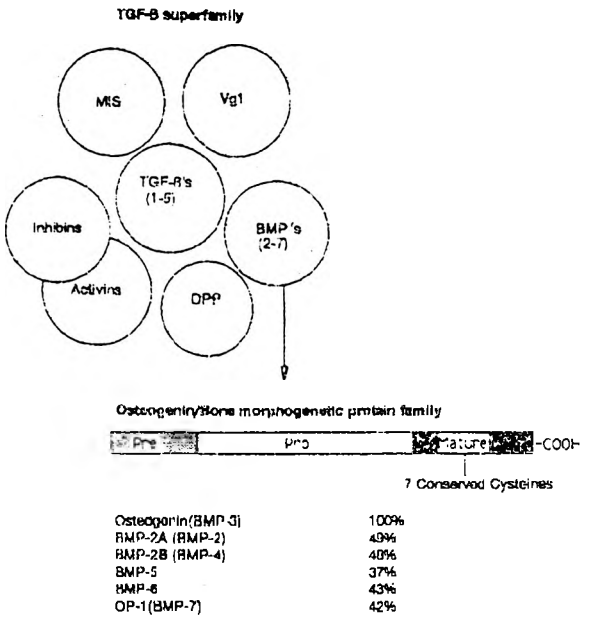


Fig. 2. — Gene product and sequence identity of osteogenin/bone morphogenetic proteins, members of the transforming growth factor-β (TGF-β) superfamily.

Abbreviations : MIS, Mullerian inhibiting substance ; DPP, decapentaplegic product of *Drosophila* ; Vg1, vegetal pole product of oocytes in *Xenopus* ; OP-1, osteogenic protein-1 (19).

Protein sequencing and cDNA cloning data from several groups indicate the existence of a family of bone inductive proteins, which belong to the TGF-β superfamily, by virtue of the location of 7 highly conserved cysteines in their carboxy-terminal region (fig. 2). The family of the osteoinductive proteins now contains 7 members, BMP-1 through BMP-7 (26, 3). BMP-1 does not appear to be a member of the TGF-β superfamily. The other members are homodimers, and reduction to the monomeric form results in the loss of biological activity. BMP-3 is the most abundant one in the highly purified preparations, but other members, especially BMP-2 and BMP-7 (also called OP-2 and OP-1 respectively (19)), are also present in the bioactive highly purified bone derived fractions. BMP-2, BMP-3, and BMP-4 have been reported to induce cartilage and bone as single recombinant proteins in conjunction with residue (5, 25, 26). The other members, BMP-5 and BMP-7, are also bioactive as recombinant preparations (J. Wozney and T. K. Sampath, personal communication). The cartilage- and bone-inducing activity in a similar in vivo bioassay by the osteoinductive factor (OIF) reported by another group (1), was later discovered to be due to the presence of BMP-2 and BMP-3 in their bioactive fractions. The data available today show that none of the individual recombinant proteins have the same in vivo bioactivity in a dose response experiment when compared to highly purified preparations, indicating that different members of this family may probably potentiate or act in synergism during bone induction in vivo. The availability of all the recombinant proteins will allow us to address this more accurately and to define their precise roles. The study of the mechanism of action of the osteogenin/bone morphogenetic proteins will add considerable new information on the molecular signals controlling cartilage and bone formation, as well as cartilage and bone regeneration. Recently, we have been conducting experimental studies addressing several aspects regarding the role of osteogenin and related bone morphogenetic proteins in development, interaction with extracellular matrix, cellular binding sites and in vitro activities.

Autoradiographic localization of osteogenin binding sites during rat embryonic development showed maximal binding during days 11-15 in the perichondrium during limb development and vertebral morphogenesis (24). By day 18, periosteum exhibited the highest concentration of autoradiographic grains. The specific affinity of osteogenin and BMP-2B for type IV collagen of basement membrane matrix (10) underscores the potential critical role of extracellular matrix in sequestering differentiation factors and regulating their bioavailibility. Moreover, it also sheds light on the possible role that basement membrane components of blood vessel walls (as laminin and type IV collagen) could have in bone formation and regeneration (23).

In vitro studies allowed us to explore the effects of osteogenin on cartilage and bone cells. Osteogenin stimulated alkaline phosphatase activity and collagen synthesis in rat periosteal cells and calvarial osteoblasts (21). There was also an increase in the formation of alkaline phosphatase positive

colonies in rat bone marrow stromal cell cultures (21). The promotion of the osteogenic phenotype was confirmed in experiments with MC3T3-E1 osteoblastic cells : osteogenin inhibited growth and stimulated the alkaline phosphatase activity within 72 h, while usually about 12 days are needed for their spontaneous differentiation in vitro (22). In the same cells high affinity receptors for BMP-2B have been identified and partially characterized (11). A profound stimulation of proteoglycan synthesis in fetal rat chondroblasts and rabbit articular chondrocytes (21), together with increased cartilage matrix synthesis in chicken limb-bud cell cultures (2, 4), demonstrated the role of osteogenin in the promotion of the chondrogenic phenotype. Osteogenin and BMP-2B (BMP-4) were equipotent in the maintenance of proteoglycan metabolism in articular cartilage explant cultures by increasing proteoglycan synthesis and decreasing proteoglycan catabolism (8). Taken together, these in vitro data suggest that osteogenin and related bone morphogenetic proteins do not only play a role in the initiation of cartilage and bone formation, but clearly promote the expression and maintenance of the chondrogenic and osteogenic phenotype.

What is the in vivo role of osteogenin ? Work on in vivo activity of osteogenin on bone induction and regeneration in primates has been performed recently. Based on the in vivo bioassay, we expected osteogenin to have a therapeutic potential in the restoration of skeletal defects. Before clinical studies can be undertaken, it is essential to demonstrate its biological activity in primates, and to define conditions for optimal bone induction. In view of this, osteogenin was isolated from baboon bone matrix and purified as described above. The protein fractions were assayed for cartilage- and bone-inducing activity by subcutaneous implantation in rats. The partially purified material (Sephacryl S-200 fractions), showing activity in rats, was subsequently implanted with insoluble collagenous matrix and carrier into the rectus abdominis of 16 baboons, as well as into 24 nonhealing critical size (25-mm diameter) calvarial defects of 8 adult male baboons (14, 15). Implantation resulted in rapid formation of bone and high alkaline phosphatase activity. As early as 30 days after implantation, new bone formation was observed in all the calvarial defects, and at 90 days, baboon osteogenin induced complete regeneration of the critical size cranial defects in adult male baboons (15).

In conclusion, significant progress has been made in the characterization of the cartilage and bone inducing proteins. A family of unique proteins, osteogenin and related bone morphogenetic proteins, has been uncovered and there is ample evidence they are directly responsible, in conjunction with an insoluble residue, for novo cartilage and bone formation in vivo. Extensive research is underway to develop an appropriate and optimal delivery system (9), which is partly dependent on the clinical indication. The availability of highly purified and recombinant osteogenin and related bone morphogenetic proteins, allows the generation of additional tools such as antibodies and cDNA probes, which enables one to perform the experiments required to better understand their role in embryogenesis and skeletogenesis. As tissue regeneration probably recapitulates the developmental cascade of tissue formation, it is very likely that the osteogenins will play a crucial role in bone regeneration and repair. The above mentioned baboon studies seem to establish their primary role in the initiation and promotion of osteogenesis in primates and imply a potential therapeutic application in bone grafts. With the current discoveries of growth and differentiation factors and the powerful research tools, there will be major developments in the near future in biological regeneration of tissues, influencing directly the way clinicians will handle orthopedic problems. Close interaction between basic scientists, applied researchers and orthopedic surgeons will ensure rapid developments and bring them faster to the patients.

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