International Orthopaedics (SICOT) (1998) 22:335–342

International Orthopaedics

Current concepts

Osteoinduction by demineralised bone

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Accepted: 9 January 1998

Summary. *Bone contains several growth factors, including bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF-β), insulin-like growth factors I and II (IGF-I and IGF-II), platelet derived growth factor (PDGF) and basic and acidic fibroblast growth factor (bFGF and aFGF). The BMPs are the only factors known to provoke bone formation heterotopically by making undifferentiated mesenchymal cells differentiate into osteoblasts (osteoinduction). Much of our knowledge of osteoinduction derives from studies in rodents of heterotopically implanted demineralised bone which contains various growth factors, including BMPs. This model has been used to examine the effect on osteoinduction of different factors, including the type of host soft tissue, age and species of donor and recipient, demineralisation procedure, storage and sterilisation procedures, experimental diabetes, dietary factors, hormones, growth factors, caffeine, biphosphonates, indomethacin and biomaterials. Demineralised bone enhances bone formation experimentally in various animal models, including cranio-maxillofacial reconstructions, healing of diaphyseal defects, and spinal fusion; demineralised bone has also been used in a limited way clinically. However, sufficient osteoinduction in humans may require a higher concentration of BMPs and other growth factors than those found in demineralised bone.*

Résumé. *L'os contient plusieurs facteurs de croissance notamment: la protéine morphogénétique osseuse (BMPs), le facteur de croissance bêta (TGF-B), les facteurs de croissance insuline-like I et II (IGF-I) et IGF-II, le facteur de croissance plaquettaire (PDGF) et les facteurs de croissance fibroblastique basique et acide (bFGF et aFGF). Les BMPs sont les seuls facteurs connus à l'origine d'ossifications hétérotopiques par transformation en ostéoblastes des cellules mésenchymateuses indifférencées (ostéoinduction). La majorité de nos connaissances de l'ostéoinduction provient de l'étude chez les rongeurs des ossifications hétérotopiques qui contiennent plusieurs facteurs de croissance notamment en BMPs. Ce modèle a été utilisé pour étudier l'effet de différents facteurs sur l'ostéoinduction comme le type de tissu mou receveur, l'âge et l'espèce du donneur et du receveur, les procédés de déminéralisation les procédés de stérilisation et de stockage, ou encore le rôle des facteurs alimentaires, des hormones… L'os déminéralisé accroit la formation expérimentale d'os dans des modèles animaux variés et il a été aussi utilisé cliniquement de façon limitée. Cependant, l'ostéoinduction chez l'homme demande une plus grande concentration de BMPs que celle présente dans l'os déminéralisé.*

Introduction

Osteoinduction is the process of mesenchymal cells being stimulated by growth factors, such as those from demineralised bone, to differentiate into osteoblasts, leading to bone formation. Much of the knowledge about osteoinduction is based on studies on demineralised bone; although recombinant growth fac-

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No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

tors are now readily available, demineralised bone continues to be important, both experimentally and clinically. Senn was the first to use demineralised bone [65]; he used ox bone demineralised in hydrochloric acid, believing it to have antiseptic properties. After experiments in dogs, he used this material in patients with skeletal defects caused by osteomyelitis. Xenogenic demineralised bone results in little or no osteoinduction, and the enhanced bone formation observed was probably due to osteoconductive properties. In 1965, Urist reported that allogenic bone which had been demineralised in 0.6 N hydrochloric acid caused heterotopic bone formation in more than 90% of cases when implanted in the abdominal muscles of rodents [83]. Urist named the hypothetical osteoinductive factor *bone morphogenetic protein* (BMP), and in 1979 he partly isolated this as an extract of osteoinductive glycoproteins [88]. Later, several closely related BMPs were identified [94], and at present there are at least 15. Bone contains several other growth factors which may moderate osteoinduction, including transformating growth factor beta (TGF-β), insulin-like growth factors I and II (IGF-I and IGF-II), platelet derived growth factor (PDGF), and basic and acidic fibroblast growth factor (bFGF and aFGF).

Heterotopic osteoinduction by demineralised bone

Demineralised bone or dentine, partly purified osteogenic proteins, or recombinant BMPs induce heterotopic bone formation when implanted in soft tissue such as the abdominal muscle of rodents. The major phases of heterotopic osteoinduction are the chemotaxis of mesenchymal cells which are pluripotent progenitor cells, mitosis of osteoprogenitor cells (day 3), differentiation of cartilage (day 5), vascular invasion (day 7), hypertrophy of chondrocytes and calcification of cartilage matrix (day 9), bone differentiation (day 10), and formation of an ossicle filled with bone marrow elements (by day 16 to 21) (Fig. 1) [57, 83]. The volume of induced new bone is proportional to the volume of implanted demineralised bone [71]. It is postulated that the separate events of the induction cascade are mediated by a series of growth factor released from the demineralised matrix, which acts as a sustained delivery system.

Demineralised bone-induced heterotopic osteogenesis in rodents has been used as an experimental model to investigate osteoinduction itself [33, 57, 83], as well as to study the effects of several influences on osteoinduction: (1) type of host soft tissue [20]; (2) age [2, 77] and species [5, 63, 85] of the donor

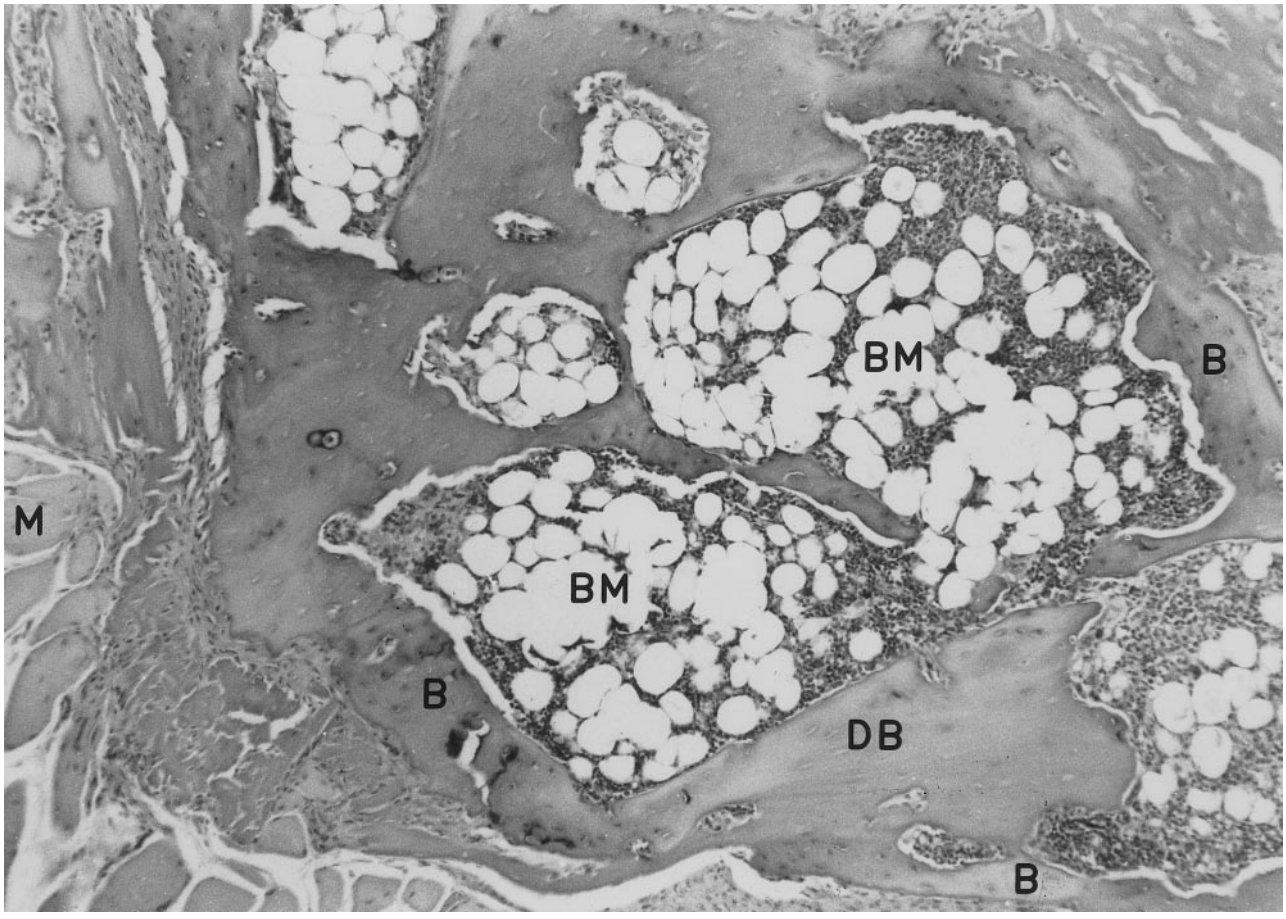

Fig. 1. Photomicrograph, 4 weeks after implantation of allogenic demineralised bone (*DB*) in rat abdominal muscle, showing ossicles of bone (*B*) and bone marrow (*BM*) surrounded by host abdominal muscle (*M*) (hematoxylin and eosin ×100)

Table 1. Effect of dietary factors/trace elements, hormones, growth factors and drugs on demineralised bone-induced heterotopic osteogenesis

Factor	Animal	Effect on osteoinduction	References
Hypophysectomy	Rats	Inhibition of mesenchymal cell proliferation, decreased and delayed chondrogenesis, delayed and reduced vascular invasion, and impaired bone formation.	[58]
Castration	Rats	Decreased calcium levels in the induced bone; normalised by dihydrotestosterone treatment.	[40]
Estradiol and progesterone	Rats	Progesterone in combination with estradiol stimulated bone formation and mineralisation.	[18]
Experimental diabetes	Rats	Reduced calcification of cartilage and osteogenesis; corrected by insulin.	[92]
Lathyritic drug (beta-aminopropionitrile)	Rats	Elevated alkaline phosphatase activity and reduced calcium content in implants at 2 weeks. At 4 weeks, these parameters were reversed.	[25]
PDGF	Rats	Increased mRNA for collagen II, alkaline phosphatase activity and calcium content of the implant.	[37]
FGF	Rats	The amount of mineralised tissue in the FGF-treated implants increased 25%.	[4]
BMP-2	Squirrel monkeys	Higher incidence of osteogenesis.	[6]
Magnesium-deficient diet	Rat	Decreased osteogenesis; large fibrous covering formed about the implant, and cartilage was present in multiple locations under thefibrous coat.	[15]
Zinc-deficient diet	Rats	Decreased osteogenesis. Poor vascularisation appeared to be an important factor.	[14]
Copper- and manganese-deficient diet	Rats	Delayed chondrogenesis.	[76]
Manganese-depleted diet	Rats	Failure of chondrogenesis or osteogenesis.	[76]
Aluminium-treated bone matrix	Rats	Decreased osteogenesis and bone remodelling.	[96]
Fluoride	Rats	Inhibited mineralisation. At higher concentration of fluoride, increased osteogenesis.	[81]
Vitamin A excess	Rats	Decrease of mesenchymal cell proliferation, chondrogenesis, osteogenesis and bone mineralisation.	[24]
Vitamin D deficiency	Rats	Impaired mineralisation and reduced bone formation and resorption; corrected by vitamin D metabolites.	[8, 89]
Caffeine	Rats	Inhibition of proliferation of undifferentiated mesenchymal cells as well as later stages of bone formation.	[11]
Biophosphonates	Rats	The uptake of ^{45}Ca in implants decreased with increasing doses of 1-hydroxyethylidene-1, 1-bisphosphonate. Almost no ^{45}Ca activity was found with the highest dose.	[12]
Indomethacin (systemically)	Rats	Indomethacin had to be present before or at the time of implantation of demineralised bone to inhibit osteoinduction.	[80]
Indomethacin (locally)	Rats	Local delivery of indomethacin by a polyorthoester significantly inhibited osteoinduction.	[73]
Rifamycin, fucidin or gentamicin (locally)	Rabbits	Bone induction was slightly inhibited by local delivery of rifamycin by plaster-of-Paris pellets, but was not affected by fucidin or gentamicin.	[91]
Gentamicin (locally)	Rats	No effect on bone induction by local delivery of gentamicin by a polyorthoester.	[72]

and recipient; (3) properties of the demineralised bone, depending on the demineralisation procedure [23], particle size [78], storage and sterilisation procedures [44, 93]; (4) hormones, growth factors, dietary factors and drugs (Table 1), and (5) biomaterials (Table 2).

The results observed with heterotopic osteoinduction produced by demineralised bone in primates are conflicting [5, 35, 59]. There may be a decreased ability of primate host tissue to respond to some osteoinductive stimuli, such as that produced by demineralised bone [2].

Preparation and storage of demineralised bone

Several methods for preparing demineralised bone have been proposed [9, 57, 83, 87]. The more extensive processing of the bone in the antigen-extracted autodigested alloimplant (AAA) method [87] may lead to some loss of inductive potential compared to demineralisation and lyophilisation only [23]. We have used a modification [9] of the original method of Urist [83] in Wistar rats. The femur, tibia and fibula are dissected free of soft tissue immediately after death under sterile conditions. The diaphyses are crushed and the marrow is removed. The cortical bone is cut into chips, demineralised in 0.2 N HCl for 48 h at 4° C, flushed in saline, suspended in liquid nitrogen and lyophilised for 22 h.

Demineralised bone has been used in large pieces, chips or powder. Reducing the particle size of the demineralised bone may improve the handling of the material when used for filling bone defects and may allow percutaneous injection of the material [7]. The osteoinductive potential may increase with decreasing particle size [61, 78] due to the increased area of contact between the osteoinducing factors and responding mes-

Table 2. Effect of biomaterials on demineralized bone-induced heterotopic osteogenesis

Biomaterial	Animal	Host tissue response and effect on osteoinduction	References
Fibrin sealand (FS)	Rats	New bone developed without any qualitative difference between specimens with or without FS.	[64]
Fibrin sealant	Rats	FS inhibited bone induction and produced a chronic inflammation with plasma cells and multinuclear giant cells.	[52]
Fibrin-collagen paste	Rats	Fibrin-collagen paste inhibited osteoinduction and produced a chronic inflammation with macrophages and multinuclear giant cells. Part of the material was not absorbed.	[72]
Porous hydroxyapatite biomatrix (PHA)	Rats	PHA was surrounded by macrophages and multinuclear giant cells. No bone induction in 74% of the composite implants of PHA and demineralised bone. When present, bone formed only within the demineralised bone and not close to the PHA.	[60]
Dense hydroxyapatite granules (DHA)	Rats	DHA was surrounded by fibrous connective tissue containing multinuclear giant cells. DHA inhibited osteoinduction; the sparse new bone formed was never close to the DHA.	[49]
Plaster-of-Paris	Rabbits	Plaster-of-Paris pellets with incorporated fucidin or gentamicin did not affect bone induction.	[91]
Bone wax of 88% beeswax	Rats	The bone wax inhibited osteoinduction; it was not absorbed and induced a chronic foreign body reaction.	[72]
Porous polysulfone (PPSF)	Rats	The tissue response to PPSF did not prevent the osteoinductive process. Chondrogenesis was seen at 10 days, followed by ossification at 21 days.	[90]
Telon tubes with either Gore-Tex (PTFE) or Dacron felt	Rats	Inflammatory reaction was seen and osteoinduction was inhibited in the vicinity of the foreign material.	[82]
Polyorthoester	Rats	The polyorthoester did not inhibit osteoinduction and it caused only a slight inflammation that subsided within 3 weeks. The polyorthoester was mostly resorbed by 4 weeks postoperatively.	[51, 72]

enchymal cells. This may also be achieved by making multiple small perforations in the demineralised bone [28]. Pulverisation of demineralised bone to a particle size less than approximately 0.1 mm may cause reduction of its osteoinductive potential, probably by denaturation of contained growth factors [61, 78].

For practical reasons, it is desirable to store the material for some time before implantation. Bang and Johannessen found that storage of demineralised dentine for 24 weeks at 20° C caused marked decrease of osteoinduction compared to storage at 4° C or –20° C [10]. However, Hosny and et al. found no difference in osteoinduction by using demineralised bone that was either fresh, lyophilised or stored for 6 months at –70° C, –4° C or 25° C [34]. We found that storage of demineralised bone for 9 months at –70° C or 4° C did not influence the osteoinduction, whereas storage for 14 months at both temperatures resulted in decreased osteoinduction [50].

Sterilisation of demineralised bone

Whereas unsterile demineralised bone may be used in rodents without any problems, the clinical success of the material depends on its sterility. Deikers et al. found organisms of low pathogenicity in 50% and of high pathogenicity in 3% of bone allografts retrieved from cadaver donors under sterile conditions [22]. Demineralisation of bone in HCl does not ensure sterility [21]. Various methods of sterilising bone have been described, including boiling, autoclaving, treatment with antiseptic and antibiotic solutions, irradiation and ethylene oxide gas. It is essential that the inductive properties of demineralised bone are maintained during sterilisation. Wientroub and Reddi exposed demineralised bone to graduated doses of radiation (1 to 15 mrad). The osteoinductive response was reduced in doses higher than 5 mrad [93]. Inactivation of HIV requires a dose of at least 25 kGy [19]; priones probably need an even higher dose [30].

Ethylene oxide sterilisation of demineralised bone has been used for a long time experimentally [9, 48, 62, 77] and clinically [84], apparently without deleterious effects on osteoinduction. However, these studies have not included a control group of unsterilised demineralised bone. Two studies have indicated that ethylene oxide sterilisation may reduce the osteoinductive potency of demineralised bone [3, 44]. Aspenberg et al. found that 5, 30 and 240 min of sterilisation with ethylene oxide was followed by a Ca content ratio in recovered grafts of 0.6, 0.3 and 0.1, respectively, compared to the control [3].

We found no quantitative or qualitative difference in bone formation by demineralised bone implanted without prior sterilisation or sterilised in ethylene oxide gas at room temperature for 1, 3 or 6 h [74]. Recently, Zhang et al. confirmed our results by finding no significant difference in osteoinduction with implants sterilised in ethylene oxide at 40° C for 8 h compared to controls, and they concluded that this method may be recommended for clinical use [95]. Exposure to ethylene oxide at 55° C resulted in almost complete loss of osteoinductivity, probably due to heat induced inactivation of contained osteoinductive proteins. The difference in the observed effect of

ethylene oxide sterilisation on the osteoinductive potential of demineralised bone may be due to differences in sterilisation equipment and technique, processing of demineralised bone or methods of evaluating osteoinduction.

The susceptibility to ethylene gas varies among different species of bacteria. Exposure for 2 to 4 h usually secures sterilisation of bone [9, 68]. Heavily contaminated bone grafts may require exposure for 4 to 6 h [3, 44].

Effect of age of the donor and the recipient of demineralised bone

The osteoinductive response to heterotopically implanted demineralised bone in rodents decreases with increasing age of the recipient [36, 38, 77], probably due to an age-related decline in the number and activity of osteoprogenitor cells [47, 54]. The results regarding the influence of the donor's age on osteoinduction have been less conclusive.

Syftestad and Urist found no significant difference in ^{45}Ca uptake in recovered heterotopic implants of demineralised bone from Fisher rats who were 3 months and 13 months of age, whereas the uptake was reduced in implants from older donors of 28 months [77]. Reddi found that heterotopically implanted demineralised bone from 4-month-old Long-Evans rats induced more bone than that of donors aged 24 months [56]. As only two age-groups of donors were included, one should be cautious in interpreting the results as a general decline in osteoinductive property of matrix throughout the life of the rat.

Jergesen et al. found by semiquantitative histological rating that more bone was formed in heterotopically implanted demineralised bone from 8-month-old rats than in those aged 1 month [39]. We found, by estimating ^{85}Sr uptake, that the osteoinductive response by demineralised bone from newborn, 8-week-old (adolescent) or 8-month-old (prime breeding age) increased with the increasing age of the donor Wistar rats [53].

The results of these studies [39, 53, 56, 77] indicate that the osteogenic potential of the bone matrix increases from newborn to adulthood but decreases in aged rats, which may be due to changes in concentration of essential growth factors. BMP and other growth factors are stored in the bone matrix. Maturational changes in the bone resulting in more matrix than cells may lead to an increase in concentration of BMP and the other growth factors. The decreased osteogenetic potential of the matrix of aged rats may be related to osteoporotic changes in the bone.

Clinical use of demineralised bone

Demineralised bone has been shown to enhance bone healing in animals, mostly rodents, in experimental models mimicking clinical situations, including cranio-maxillofacial reconstructions [43, 48, 55, 70], healing of diaphyseal defects [7, 29, 32, 45, 69] and spinal fusion [42].

In 1961, Sharrard and Collins reported good results with demineralised bone in the surgical treatment of scoliosis in three children [66]. Urist reported good results by clinical and radiographic evaluation 5 months to 3 years after operations for pseudarthrosis, arthrodesis of the hip and spinal fusion using totally demineralised (n=16) or surface-demineralised (n=10) autogenic bone [84]. Urist and Dawson found approximately 80% excellent and good results and a pseudarthrosis rate of 12% in 40 intertransverse spinal fusions with a composite of demineralised bone and fresh autogenic cancellous bone [86].

Glowacki et al. reported good early healing, according to clinical and radiographic examination and biopsy, of demineralised bone implants used for cranio-maxillofacial reconstruction in 34 patients [31]. Sonis et al. reported a decrease of pocket depth in about half the patients (n=21) with periodontal defects treated with demineralised bone [75]. Kubler et al. found osseous integration and remodelling of the demineralised bone implants with minimal resorption in 19 of 21 patients, 12 to 58 months after cranioplasties [41]. Bodner found that implantation of demineralised bone enhanced bone healing in jaw defects after cyst removal compared to packing with absorbable gelatin sponge [16].

Ousterhout evaluated 25 patients with cranio-maxillofacial defects 6 to 18 months after implantation of commercial demineralised bone. Whereas smaller defects generally healed, resorption of implant and poor bone formation was observed in some cases of larger defects [46]. Toriumi et al. found a high degree of resorption of commercial demineralised bone grafts used for cranio-maxillofacial procedures, mostly corrections of the nose, dorsum and columella [79].

Demineralised bone has been used in maxillofacial surgery, generally with good results, in combination with membranes for guided tissue regeneration [1, 17] and threaded titanium implants to replace teeth [27]. However, in a recent clinical study of commercial demineralised bone used in connection with dental implant replacement and polytetrafluorethylene membranes, demineralised bone close to the host bone underwent a partial remineralisation, whereas demineralised bone distant to the host bone was slowly resorbed and showed no bone formation [26].

Becker et al. found insignificant new bone 21 days after heterotopic implantation of human demineralised bone from commercial sources in athymic mice [13]. Shigeyama et al. found decreased osteoinductive activity and reduced concentration of BMP 2, 4 and 7 in protein extracts of bone from commerical sources [67]. The results of these studies indicate that the osteoinductive potential and the concentration of BMPs may be reduced during the processing of demineralised bone by some commercial sources.

In conclusion, demineralised bone is clinically superior to allogenic fresh or frozen bone. Furthermore,

complications related to harvesting autogenic bone are avoided, and the material is readily available in abundant quantity. Demineralised bone has not been shown to be superior to fresh autogenic cancellous bone. Good results can be expected when demineralised bone is used for filling defects, but the results of onlay grafts are uncertain. This may be due to the appropriate osteoconductive properties of the material, whereas the concentration of BMP may be less than optimal for osteoinduction. The osteoinductive properties are influenced by the age of the donor, the processing technique and the sterilisation procedure.

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