NATIVE BOVINE BONE MORPHOGENETIC PROTEIN IN THE HEALING OF SEGMENTAL LONG BONE DEFECTS

TAPIO TUOMINEN

Division of Orthopaedic and Trauma Surgery,
University of Oulu

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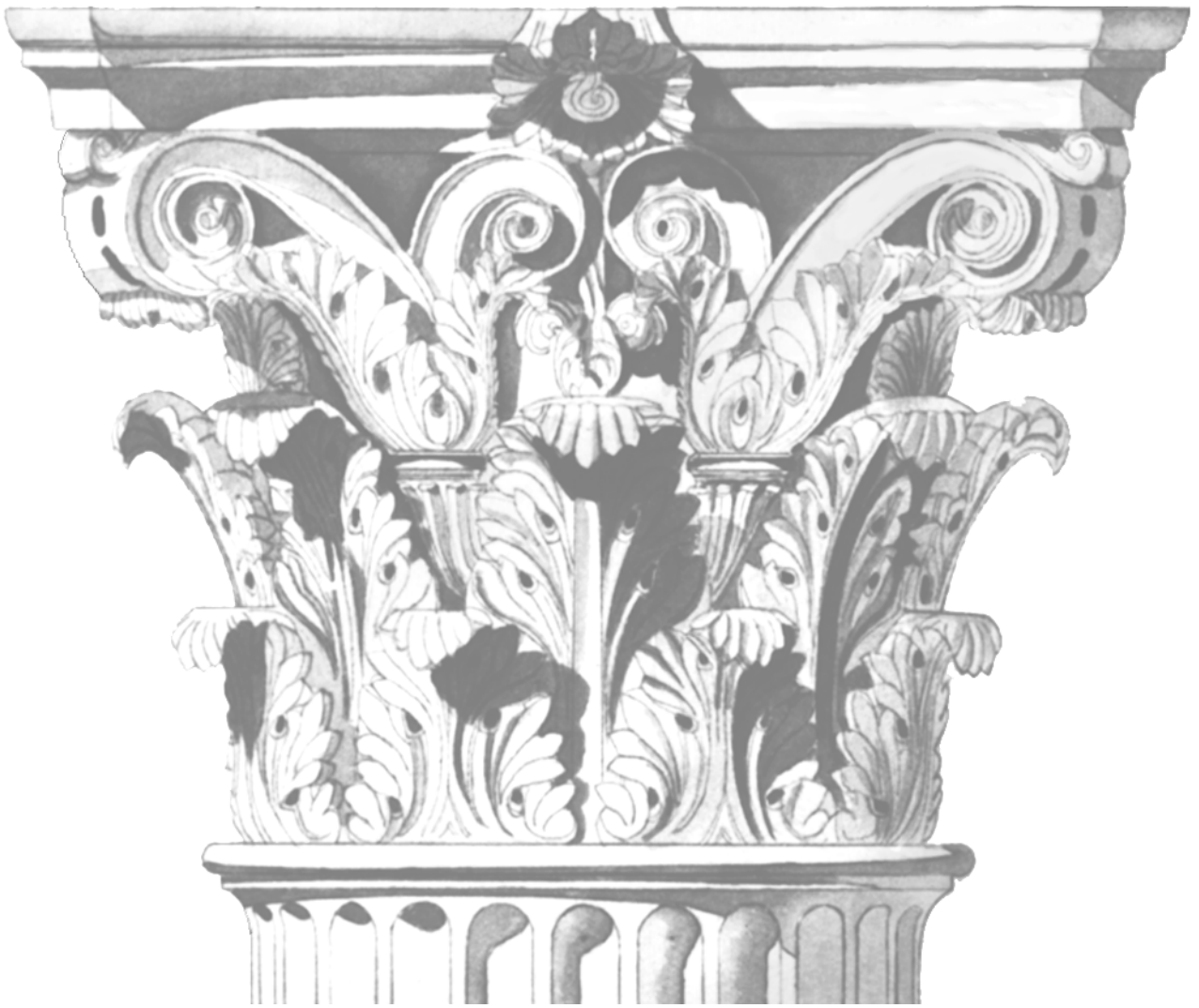

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Abstract

A new animal model was developed to evaluate the effect of bovine native bone morphogenetic protein (BMP) on the healing of segmental, critical-sized bone defects. Laboratory-bred adult beagle dogs were used in the study. A 2 cm corticoperiosteal defect was created using an oscillating saw in mid-ulna, and the defect was treated with bone grafts and implants fixed by an intramedullary Kirschner wire through predrilled holes in the middle of the implant. Plate and screw fixation was also used in some groups. Coral, hydroxyapatite and demineralized xenograft bone were placed in the defects with or without BMP. Autografts and allografts were used as controls. The BMP was extracted from bovine diaphyseal bone.

The follow-up period was 36 weeks. Radiographs were taken at regular intervals during the follow-up period, and bone formation and bone union were evaluated. The radiographs were digitized, and callus was measured and CT scans obtained to define bone density. At the end of the study, the bones were harvested and tested mechanically in a torsion machine until failure. After mechanical testing, the bones were reconstructed and histological sections were made.

With autograft and allograft bone grafts, healing was nearly complete. Hydroxyapatite and demineralized xenograft bone did not result in healing of the bone defect, while coral enhanced bone formation, but the healing was not comparable to autografts or allografts. Hydroxyapatite implants did not resorb during the 36 weeks of follow-up to enhance bone healing, and there was a fibrous capsule around the hydroxyapatite implants in histology. Xenograft bone was resorbed, and very little bone formation and extensive fibrosis were seen at the implant site. Coral was resorbed and gradually replaced by new bone, but did not heal the defect completely. With every implant, added BMP had a positive effect on healing as evaluated either radiographically, mechanically or histologically. Coral was the most optimal carrier material for BMP among the materials tested in this study.

The animal model seems to be suitable for studying the healing of bone defects, as all the animals were physically active from the first postoperative day and did not seem to have problems with motion during the follow-up period. Intramedullary fixation lacks rotational stability, which may have a negative effect on healing. The bones fixed with a plate and screws showed better scores in radiographs and were mechanically stronger, although the study groups were too small to allow definitive conclusions. As a conclusion, none of the transplants or implants were equally efficient as cortical autograft in healing segmental ulnar defects. BMP did not enhance the poor capacity of hydroxyapatite and xenograft bone to heal the bone defect. According to the present findings, the composite implant consisting of coral and BMP seemed to be the best of the composite implants tested.

Keywords: bone morphogenetic protein, segmental bone defect, bone grafting, composite implants

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Tampere, June 2001 Tapio Tuominen

Abbreviations

AAA-bone	Autolyzed antigen-extracted allogeneic bone
ACG	Autogenous cancellous bone graft
BMC	Bone mineral content
BMD	Bone mineral density
BMP	Bone morphogenetic protein
bBMP	Bovine bone morphogenetic protein
BS	Bone torsional stiffness
cBMP	Canine bone morphogenetic protein
CDMP	Cartilage-derived morphogenetic protein
cDNA	Complementary deoxyribonucleic acid
CSA	Cross-sectional area
DBM	Demineralized bone matrix
GDF	Growth and differentation factor
GuHCl	Guanidine hydrochloride
HA	Hydroxyapatite
hBMP	Human bone morphogenetic protein
MA	Maximal angular deformation
MAE	Maximal absorbed energy
mBMP	Moose bone morphogenetic protein
MTC	Maximal torque capacity
NCP	Noncollagenous protein
PDLA	Poly-D-lactide
PDLLA	Poly-DL-lactide
PGA	Polyglycolide
PLA	Polylactide
PLLA	Poly-L-lactide
rhBMP	Recombinant human bone morphogenetic protein
rhOP-1	Recombinant human osteogenic protein-1 = BMP-7
TCP	Tricalcium phosphate
TGF-β	Transforming growth factor β

Definitions

Autograft bone	Bone material taken from the same individual.
Allograft bone	Bone material taken from another individual of the same species.
Bioassay	Evaluation of the effect of an agent (e.g. BMP) on a living organism.
Biodegradation	Breakdown of material in living tissue.
Bone graft	Bone material used to replace bone tissue in a defect.
Carrier	Material used to deliver BMP into the body. The carrier is thought to immobilize the inductive agent and to provide a slow release of the agent.
Chemotaxis	A process whereby certain agent(s) attract cells to a certain site.
Collagen	The single most abundant protein in mammals.
Composite	Composite material is composed of at least two different materials or phases acting together as an implant.
Critical sized defect	A defect in bone which does not heal if left untreated.
Implant	A medical devise made of one or more biomaterials that is intentionally placed within the body, either totally or partially buried beneath the epithelial surface.
Mesenchymal cell	A cell with an ability to differentiate in many different ways.
Osseointegration (or osteointegration)	Direct bone-to-biomaterial interface without interconnecting fibrous tissue.
Osteoblast	A bone-forming cell.
Osteoclast	A bone-resorbing cell.
Osteoconduction	The ability to guide bone formation on a material surface in a bony environment.
Osteoinduction	A process whereby one tissue or a product derived from it causes another undifferentiated tissue to differentiate into bone.
Xenograft bone	Bone material taken from an individual of another species.

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I Tuominen T, Jämsä T, Tuukkanen J, Lindholm TS & Jalovaara P (2000) Fresh tubular long bone autografts and allografts in the healing of canine ulnar defect fixed with intramedullary Kirschner wire. J Musculoskel Res 4:55-62.

II Tuominen T, Jämsä T, Tuukkanen J, Marttinen A, Lindholm TS & Jalovaara P (2001) Bovine bone implant with bovine bone morphogenetic protein in healing a dog ulnar defect. Int Orthop 25:5-8.

III Gao TJ, Tuominen T, Lindholm TS, Kommonen B & Lindholm TC (1997) Morphological and biomechanical difference in healing in segmental tibial defects implanted with Biocoral® or tricalcium phosphate cylinders. Biomaterials 18:219-23.

IV Tuominen T, Jämsä T, Tuukkanen J, Nieminen P, Lindholm TC, Lindholm TS & Jalovaara P (2000) Native bovine bone morphogenetic protein improves the potential of biocoral to heal segmental canine ulnar defects. Int Orthop 5:289-94.

V Tuominen T, Jämsä T, Oksanen J, Tuukkanen J, Gao TJ, Lindholm TS & Jalovaara P (2001) Composite implant composed of hydroxyapatite and bone morphogenetic protein in the healing of a canine ulnar defect. Ann Chir Gynaecol 90:32-36.

VI Tuominen T, Raatikainen T, Leppilahti J & Jalovaara P (2001) Native BMP in treatment of a ulnar pseudoarthrosis. A case report (Submitted).

Contents

1 Introduction

In bone grafting, autogenous bone is considered the golden standard, to which other methods are compared. The limitations of obtaining autograft bone are obvious: the amount is limited and the harvesting of autograft bone causes secondary morbidity at the harvesting site. Allograft bone is widely used, but it has not solved all the problems in bone grafting: the amount of allograft is also limited, the healing capacity is generally lower than with autografts, and allografting also carries a risk for certain diseases, such as hepatitis and HIV. Occasionally, incomplete healing is seen in spite of proper grafting procedures. Thus, other methods have been searched.

Synthetic biomaterials can only be used as filling material without any biological activity in initiating bone regeneration. Stimulation of the regeneration of bone is a challenging idea, which would solve many problems in cases with bone defects. The pioneering work of MR Urist aroused the interest in agents able to induce bone. He demonstrated the ability of demineralized bone matrix (DBM) to induce bone in an ectopic place, when implanted in rabbits and rats intramuscularly (Urist 1965). The importance of this work lies in the carefully controlled demonstration that new bone can be induced independently of the surrounding bone tissue. Later, it was shown that low-molecular weight proteins extracted from demineralized bone matrix had more osteogenic activity (Urist & Iwata 1979). They were called bone morphogenetic proteins (BMPs). After that, BMPs have been extracted from bones of many animals.

The purification methods for native BMPs have improved, and sufficient amounts of BMP have been produced for animal studies to demonstrate the effect of BMP in long bone defects, skull trephines, spinal fusion and fracture treatment. Successful clinical studies have also been conducted. The modern gene technology has provided the possibility to produce recombinant BMPs in almost unlimited amounts. In the future, gene therapy may be available to produce locally BMPs with different vectors. There are, however, many unanswered questions concerning such matters as the carrier materials for BMPs, the risks of gene vectors and the basic mechanisms by which BMPs exert their effect in humans. The aim of BMP studies is to answer these questions and to develop BMPs to be used in clinically different bone defects, such as bone tumor treatment, joint prosthesis surgery, maxillocranio-facial surgery and fracture treatment.

The present study focuses on native bovine bone morphogenetic protein in the treatment of segmental bone defects. During the past two decades, active research interest has focused on this matter. The carrier materials for BMP, the fixation methods, the healing process evaluated in radiograms and the mechanical performance of the treated bones are some of the main issues that still involve many unanswered questions in long bone healing. We developed a canine ulnar defect model with intramedullary Kirschner wire fixation to study these issues. We have studied different biomaterials and their performance in a segmental bone defect model in sheep and dog. Using the dog ulnar defect model, the effect of native bovine BMP has been tested in bone healing by comparing the results to autograft bone grafting. Different carrier materials for BMP, including coral, hydroxyapatite and xenograft bone material, have been evaluated.

2 Review of the literature

2.1 Bone grafting and bone substitutes

In the reconstruction of skeletal defects, it often is necessary to transplant cancellous or cortical bone to restore skeletal integrity and to enhance bone healing. The clinical outcome of the grafting procedure depends on many factors, including the type of graft, the type of fixation and the host site. All bone grafts are resorbed initially, and cancellous grafts generally resorb faster than cortical grafts (Goldberg & Stevenson 1987). The materials used in bone grafting can be broadly divided into autografts, allografts, xenografts, synthetic materials, and combinations of these (Bauer & Muschler 2000). Autogenous graft has been shown to be superior to allograft in many studies, as remodelling and bone healing takes place more slowly in allografts compared to autografts (Friedlander 1987, Goldberg & Stevenson 1987, Gross *et al.* 1991, Kienapfel *et al.* 1992, Johnson & Stein 1988, Virolainen *et al.* 1993). The amount of autogenous bone is limited, and the additional surgical procedure causes increased morbidity in the host, which is why allografts are being used widely. Allograft bone is not without problems, however. Firstly, it includes the risks of viral diseases, such as HIV and hepatitis, and secondly, it may cause immunological reactions that interfere with the bone healing process (Burchardt 1983, Stevenson 1987).

Allografts can be processed in various ways for long-term preservation. Bone banking allows allograft bone to be widely used in clinical orthopaedics (von Versen 1992, Malinin 1992, Tomford & Mankin 1999). Freezing and freeze-drying are associated with reduced immunogenicity, and in the latter case the mechanical strength of the graft is decreased (Friedlaender 1983, Pelker *et al.* 1984, Wolfe & Cook 1994). In spite of wide use of bank bone woldwide, there are still many unanswered questions in allograft immunology, incorporation and remodelling (Garbuz *et al.* 1998, Bauer & Muschler 2000).

Xenograft bone represents an unlimited supply of available material if it could be processed to be safe for transplantation in a human host (Block & Poser 1995). Xenograft bone or xenograft collagen material have been used by some authors as a bone substitute experimentally (Salama & Weissman 1978, Salama 1983, Mehlisch *et al.* 1988, Hashizume *et al.* 1998, Young *et al.* 1999), but the procedure has never gained wider acceptance. Xenograft has the same inherent problems as allografts, and being from a different

species, it may cause even more pronounced immunological problems. Human allograft materials are considered more effective and more widely available compared to xenografts at the present (Bauer & Muschler 2000).

Demineralized bone matrix (DBM) is an interesting option, which has been shown to have an osteoinductive potential (Urist 1965, Oikarinen & Korhonen 1979, Oikarinen 1982, Einhorn 1984, Lindholm TS *et al.* 1988). It is hypothesized that the rigid structure of nondecalcified bone does not permit the release of bone-inducing proteins, which become available when bone is demineralized (Guizzardi *et al.* 1992). Furthermore, demineralization is considered advantageous because it destroys the antigenic surface structures of bone. There is, however, marked variation in the results of various studies with DBM. In some studies DBM has been comparable to autograft bone (Oikarinen 1982, Hopp *et al.* 1989, Guizzardi *et al.* 1992), while in some others it has proven to be ineffective (Schwarz *et al.* 1991). Obviously, the processing techniques are important and should be standardized (Russell & Block 1999). It has also been proposed that DBM should be bioassayed prior to use due to the variation in the osteoinductive effect (Wilkins *et al.* 1999).

Various synthetic materials have been developed as bone substitutes and as alternatives to bone materials. These include natural coral, hydroxyapatite, tricalcium phosphate, bioactive glasses and synthetic polymers. They have been used as filling material in bone defects in experimental animal studies and also clinically (Bucholz *et al.* 1987, Elsinger & Leal 1995, Guillemin *et al.* 1987, Heise *et al.* 1990, Peltola 2001). The incorporation of these materials in the host bone is clearly inferior to autogenous bone grafts. They enhance osteoconduction, which is a three-dimensional process of the growth of capillaries, perivascular tissue, and osteoprogenitor cells of the host into the graft (Goldberg & Stevenson 1987). The synthetic materials are not osteoinductive, however, i.e. they do not induce the formation of new bone.

In the future, graft materials together with osteoinductive agents, i.e. proteins that induce bone, will be available for bone grafting, and their effect will probably be superior to that of autograft bone.

2.2 Osteoinduction

The classic osteoinductive phenomenon was defined well by Huggins (1931), who demonstated that autoimplantation of transitional epithelium of the urinary bladder to abdominal wall muscle in dogs provoked ectopic bone formation.

Levander was one of the first to recognize the phenomenon of osteoinduction by demonstrating that crude alcoholic exctracts of bone induced bone formation when injected into muscle tissue (Levander 1934, 1938).

Spemann had a theory of embryonic induction (Spemann 1938), which process involves interaction between two systems: induction and reaction. The inducing system in osteoinduction includes hypertrophied cartilage, newly formed or demineralized bone matrix, transitional epithelium and osteogenic agents, while the reacting system consists of mesenchymal tissue cells with the competence to become osteoblasts.

In a classic study, Urist (1965) described ectopic bone induction in intramuscular implantation of demineralized bone matrix (DBM) in rabbits and rats. This was a key discovery, which stimulated the search for a bone-inducing substance in the bone matrix. Subsequent investigations demonstrated that low-molecular weight proteins could be extracted from demineralized bone matrix (Urist & Iwata 1979). These proteins showed more osteogenic activity than DBM, and they were called bone morphogenetic proteins (BMPs).

Thus, *osteoinduction can be defined as a process whereby one tissue, or product derived from it, causes a second undifferentiated tissue to differentiate into bone.*

It has become clear that skeletal tissue regeneration requires the interaction of three basic biologic elements: cells, growth and differentiation factors and matrix scaffold (Bruder & Fox 1999, Lane *et al.* 1999b). All these factors are necessary for successful bone regeneration, and several studies have demonstrated the effectiveness of combining all these elements (Takagi & Urist 1982a, Niederwanger & Urist 1996, Arnaud *et al.* 1999, Lane *et al.* 1999a, Noshi *et al.* 2000, Reddi 2000).

In this study, the focus is on the growth and differentiation factors, specifically the bone morphogenetic protein, which are of major experimental interest, the aim being the clinical use of BMPs in skeletal defects.

2.3 Bone morphogenetic protein

2.3.1 TGF-β superfamily

BMPs belong to a group of proteins called TGF-β superfamily, and this gene family currently includes at least 43 members (Wozney & Rosen 1998). The proteins of the TGF-β superfamily regulate many different biological processes, including cell growth, differentiation and embryonic pattern formation (Zhu *et al.* 1999). This group of proteins includes, among others, transforming growth factors (TGF-β1 through TGF-β5), BMPs and growth and differentation factors (GDFs) (Burt & Law 1994). BMP1 is the only BMP that is not a member of the TGF-β superfamily, but is a procollagen C-proteinase, which is the prototype of a family of putative proteases implicated in developmental pattern formation in diverse organisms (Kessler *et al.* 1996, Li *et al.* 1996). BMPs 2–16 are the presently known members of the BMP superfamily (Dube & Celeste 1996a,b, Wozney & Rosen 1998), and they can be divided into different subgroups according to how closely they are related to each other structurally (Table 1). For example, BMP-2 and BMP-4 are 92 % identical, while BMP-5, BMP-6 and BMP-7 are 90 % identical (Wozney 1992).

Bone morphogenetic proteins are dimeric molecules with two chains held together by one disulphide bond. Each monomer consists of about 120 amino acids with seven canonical cysteine residues (Reddi 1998a).

BMPs 2, 4 ,5 ,6 and 7 have been shown to be fundamentally important regulators of skeletal tissue formation and repair (Wozney *et al.* 1990, Cook *et al.* 1994a, Riley *et al.* 1996, Wozney & Rosen 1998, Cook 999). Different BMPs are not identical in their osteoinductive potential. For example, BMP 5 is needed in larger amounts to induce the same amount of bone compared to BMP 2 or 7 (Wozney & Rosen 1998).

Table 1. Bone Morphogenetic Protein Superfamily in Mammals (Reddi 1998).

BMP	Other name	Subfamily	Author
BMP2	BMP2A	BMP 2/4	Wozney 1988
BMP3	Osteogenin	BMP3	Reddi1987, Wozney 1988
BMP3B	GDF10	BMP3	Kangava 1995, Hino 1996, Takao 1996
BMP4	BMP2B	BMP2/4	Wozney 1988, Oida 1994
BMP5	BMP5	OP1/BMP7	Celeste 1990, Wozney 1992
BMP6	Vgr1	OP1/BMP7	Lyons 1989, Celeste 1990
BMP7	OP1	OP1/BMP7	Celeste 1990, Özkaynak 1990
BMP8	OP2	OP1/BMP7	Özkaynak 1992
BMP8B	OP3	OP1/BMP7	Zhao & Hogan 1996
BMP9	GDF2	miscellaneous	Celeste 1994, Song 1995
BMP10	BMP10	miscellaneous	Celeste 1995, He 1995
BMP11	GDF11	miscellaneous	Celeste 1994
BMP12	CDMP3/GDF7	CDMP/GDF	Storm 1994, Celeste 1995, Inada 1996
BMP13	CDMP2/GDF6	CDMP/GDF	Celeste 1995, Dube 1995, Inada 1996
BMP14	CDMP1/GDF5	CDMP/GDF	Fang 1996, Murray 1997
BMP15	BMP15	others	Celeste 1996, Dube 1996
BMP16	BMP16	others	Murray 1997

GDF = Growth and differentiation factor, OP = Osteogenic protein, Vgr = Vegetal related, CDMP = Cartilage-derived morphogenetic protein.

2.3.2 Extracted BMPs

After Urist's pioneering experiments, BMP was extracted from many different species, including rabbit (Urist *et al.* 1979), pig (Wu & Hu 1988), cow (Wang *et al.* 1988), dog (Heckman *et al.* 1991), baboon (Ripamonti *et al.* 1992), reindeer (Jortikka *et al.* 1993b), moose (Viljanen *et al.* 1996) and human (Urist *et al.* 1983).

The separation of BMP is extremely difficult, because it is almost totally insoluble in conventional buffer solutions. Furthermore, when extracted from demineralized bone matrix, it appears as high-molecular-weight protein aggregates. To break down these aggregates, a number of sequential precipitation-solubilization steps are required (Marttinen *et al.* 1992, Jortikka 1993a). Briefly, the extraction process involves the following steps:

1. mechanical stripping of long diaphyseal bones
2. pulverization of bone material
3. demineralization in HCl
4. extraction by GuHCl or urea

5. ultrafiltration
6. chromatography

Native BMP is present in cortical bone in minute amounts, approximately 1–2 μg BMP/kg of cortical bone. Thus, large amounts of bone are needed to produce sufficient amounts of BMP for experiments.

Native bovine BMP is the most frequently used native BMP in animal studies because of the availability of bovine bone and the proven effect of bovine BMP. Bovine BMP has a molecular weight of about 18 KD (Urist *et al.* 1982, Bessho *et al.* 1989, 1991a, 1991b, Shibahara *et al.* 1995). Bovine BMP has also been the origin of recombinant BMPs, since the amino acid sequence has been derived from a highly purified preparation of BMP from bovine bone (Wozney *et al.* 1988).

2.3.3 Recombinant BMPs

With the purification of human osteogenic proteins of sufficient purity to provide amino acid sequence data, complementary DNA clones were isolated, cloned and expressed in host cells. Thus, the human BMPs 1 through 7 were found (Wozney *et al.* 1988, Wozney 1989, Celeste *et al.* 1990). The recombinant BMPs 2, 4 and 7 have been shown to induce bone in many experiments and are now also being tested in clinical studies (Boden 1999).

Although it has been shown that a single rhBMP is able to induce bone formation ectopically (Wang *et al.* 1988), it is interesting that the amount of human rhBMP necessary to produce bone induction *in vivo* is more than 10 times higher than that of highly purified bovine extracted BMP. Recently, Bessho *et al.* (1999) demonstrated this difference in effect between purified human BMP derived from human bone matrix and recombinant human BMP. This fact suggests that native BMP activity is a combination of the activities of different BMPs or represents synergistic activity between them (Wang *et al.* 1990).

2.3.4 Functions of BMPs

The hallmark of bone morphogenetic protein activity *in vivo* is the induction of new bone. The standard method for assaying BMP is its intramuscular implantation into a mouse or rat and the estimation of new bone induction by radiology and histology (Urist & Strates 1971). In rat bioassay, other growth factors and extracts from other connective tissue matrices prepared according to the BMP extraction procedure do not have osteogenic activity, which means that BMPs are the only growth factors with a known ability to stimulate the differentiation of mesenchymal stem cells in the chondro- and osteoblastic direction (Reddi *et al.* 1987, Aldinger 1991, Chen *et al.* 1991, Solheim 1998). BMPs initiate, promote and maintain chondrogenesis and osteogenesis, and BMPs also have many extraskeletal functions, as they regulate the development of several embryonic structures, including the kidney, lung and gut (Hogan 1996, Reddi 1998b). It seems that, in a mature

animal, bone repair after injury is similar to bone formation in an embryo, suggesting analogous mechanisms for the control of bone formation in adult and embryonic skeletons (Rosen & Thies 1992).

Bone morphogenetic proteins exert their effects through receptors, which are members of a larger family of serine threonine kinases, including the receptors for transforming growth factor betas, activins and inhibins (Massagae *et al.* 1994). BMP receptors are of two types, type I and type II. These receptors phosphorylate cellular Smad proteins, which transcriptionally activate target genes (Dewulf *et al.* 1995, Reddi 1998a, Laitinen 1999, Miyazono 1999).

The interactions of BMPs with other agents remain quite obscure. There is some evidence that prostaglandin E1, for example, has promotive effects on the osteogenic activity of rhBMP (Ono *et al.* 1996).

When an osteogenic implant is implanted, it activates a series of cellular events, including chemotaxis of pluripotential mesenchymal cells into the implant site, differentiation of these cells into chondroblasts and osteoblasts, removal of calcified cartilage, and population of new bone with bone marrow elements. The bone morphogenetic proteins induce new bone formation through endochondral ossification, where cartilage forms first and is subsequently replaced by bone (Sampath & Reddi 1981, Wozney & Rosen 1998).

2.3.5 BMP carriers

To enhance osteoinduction, bone morphogenetic proteins must be mixed with an appropriate carrier substance, since the proteins are soluble within biologic fluids. Although there is no absolute need for a delivery system, if a sufficient amount of bone morphogenetic protein is applied, bone formation can be observed (Forslund & Aspenberg 1998, Wozney & Rosen 1998), a carrier system is required to optimize the osteogenic activity of BMP (Lindholm & Gao 1993, Ripamonti 1993). It has been shown that the carrier material may have an effect on the pharmacokinetics of BMP on the basis of different release patterns (de Groot 1998, Winn *et al.* 1999).

Overall, the development of appropriate osteoconductive carriers has not progressed as rapidly as the isolation and synthesis of growth factors. This has significantly slowed down the development of clinically successful biosynthetic composite implants (Lane *et al.* 1999b).

Theoretically, the carrier material will have to meet the following requirements (Aldinger *et al.* 1991):

1. relative insolubility in physiological conditions
2. biodegradability
3. protection against proteolytic activities
4. substrate for cell adhesion and proliferation
5. immunologically inert
6. slow release of BMP through controlled biological degradation
7. mechanical stability in bridging bone defect

Many different carrier materials have been used in a variety of animal models, in which bone morphogenetic proteins have been tested (Cook *et al.* 1994a, Hollinger & Seyfer 1994, Wozney & Rosen 1998, Winn *et al.* 1999), but the optimal carrier material for BMPs still remains to be found. The optimal type of carrier material used will probably depend on the clinical indication to which the morphogenetic protein will be applied (Wozney & Rosen 1998).

The carrier material can be in the form of blocks, granules, paste, solution or as a self-setting cement (Kamegai *et al.* 1994, Ohura *et al.* 1999).

Carrier materials can be classified based on different criteria, such as inorganic versus organic, biological versus non-biological and bioedgradable versus non-biodegradable (Viljanen 1997)

Broadly speaking, the carrier materials for BMP can be divided into five major categories:

1. Demineralized bone matrix
2. Collagenous materials
3. Resorbable synthetic polymers
4. Calcium phosphate materials
5. Others

Demineralized bone matrix (DBM) has been used in many studies as a carrier material for BMPs, extracted with GuHCl to remove endogenous bone inductive activity (Cook *et al.* 1994a,b, Sciadini *et al.* 1997a), or as a commercially available preparation of demineralized freeze-dried human bone powders (Niederwanger & Urist 1996). The experiments of Cook *et al.* (1994a, 1994b) and Sciadini *et al.* (1997a) demonstrated the suitability of demineralized bone matrix as a carrier for BMP in an animal long bone defect model. Toriumi *et al.* (1993) successfully repaired a 3 cm full-thickness mandibular defect in a dog with allogeneic DBM mixed with recombinant BMP-2. In one study, where different carrier materials for rhBMP-2 were compared in canine periodontal defects, DBM and Bio-Oss (sintered bovine bone) performed well compared to collagen, PLA and PGA, although the authors concluded that other impediments to their clinical use still exist (Sigurdsson *et al.* 1996). Immunogenicity remains a problem in demineralized bone matrix.

A tentative way to solve this problem, namely autolyzed, antigen-free, allogeneic bone (AAA), was developed by Urist and co-workers. AAA bone was later used in clinical studies by Johnson *et al.* (1990, 2000) with promising results. AAA cortical bone has undergone antigen extraction without significant alteration of the residual structural integrity of the cortical graft (Johnson *et al.* 1990).

Collagenous materials are superior in compatibility, because collagen is the major protein component of hard and soft tissues. A range of collagenous materials have been used in different studies, including collagen sponges and pastes (Sampath & Reddi 1981, Takaoka *et al.* 1988, Bessho *et al.* 1991a, Takaoka *et al.* 1991, Gao & Lindholm 1993a, Lindholm *et al.* 1992, Cook *et al.* 1995). However, immunogenicity and inferior osteoconduction limit the suitability of this material as an ideal carrier for BMP. The telopeptides of type I collagen are thought to be responsible for causing an immunogenic response when introduced into xenogeneic hosts. To eliminate this problem, Takaoka *et al.* (1991) used filtration to remove telopeptides. Telopeptide-depleted collagen as a carrier for BMP was found to be superior to conventional collagens in ectopic bone formation.

In the recent years, possibly the greatest interest has focused on resorbable synthetic polymers, such as polylactide (PLA) and polyglycolide (PGA), which are members of a large family of poly-alpha-hydroxy-acids. Polylactide is a synthetic thermoplastic polymer of cyclic diesters of lactic acid. Polylactic acid has two optically active stereoisomers, poly-L-lactic acid (PLLA) and poly-D-lactic (PDLA) (Tielinen 2000). The physical properties of the copolymers of L-lactic acid and D-lactic acid (PDLLA) are dependent on the relative amounts of L- and D-monomers. Their advantages include the synthetic nature of the system and the accumulated clinical and regulatory experience of PLA (Wozney & Rosen 1998).

Heckman *et al.* (1991) treated canine radial defects with BMP using both demineralized bone matrix (DBM) and polylactide carriers. The former had no effect on the healing of the defect, but the polylactide-BMP composite led to union in all cases. Some investigators have found that polylactic and polyglycolic acid porous microspheres, when combined with an appropriate dose of rhBMP-2, appear to be equally effective as inactivated demineralized bone matrix (Kenley *et al.* 1994, Muschler *et al.* 1994). Boström *et al.* (1996) used rhBMP-2 with a paste-like polylactide, treating rabbit ulnar defects with success. Hollinger and Leong (1996) suggested that poly-alpha-hydroxy acids are suitable carriers for BMPs based on some preclinical studies. rhBMP-2 was able to heal large segmental defects in sheep, when used with a PDLLA carrier in sheep femur (Kirker-Head *et al.* 1998). Zegzula *et al.* (1997) demonstrated the suitability of PDLLA as a carrier material for rhBMP-2 in rabbit radial diaphyses. In dentistry, synthetic polymers have also proven to be useful as carriers for BMP (Saitoh *et al.* 1994, Alpaslan *et al.* 1996). In an attempt to quantify osteoinductivity, Winn *et al.* used a measure of radioactivity to quantify rhBMP-2 pharmacokinetics, radiomorphometry, histomorphometry and alkaline phosphatase activity. The results showed that deorganified bovine bone resulted in an initial burst release of morphogen, but thereafter appeared to bind irreversibly a fraction of rhBMP-2. Collagen and PDLLA carriers showed a sustained release, and the latter also a dose-dependent release pattern (Winn *et al.* 1999).

Calcium phosphate materials, including coralline, hydroxyapatite, tricalcium phosphate and their composites, have been proposed as potential carrier materials for BMP. They resemble bone tissue structurally and are usually biocompatible, but their variable and often extremely slow biodegradation makes them suboptimal as carriers (Lane *et al.* 1999a).

Hydroxyapatite (HA) is a material that has been used widely in animal studies as a carrier material for various BMPs. It has been used in ectopic muscle implantation, in a skull defect model, under the periosteum of parietal bone and in mandibular bone defects, and a combination HA-BMP proved to be more effective than HA alone in all these studies (Takaoka *et al.* 1988, Damien *et al.* 1990, Horisaka *et al.* 1991, Ono *et al.* 1995, Asahina *et al.* 1997, Koempel *et al.* 1998). The effect of a HA-BMP combination in spinal fusion was demonstrated by Boden *et al.* (1999). The addition of collagen or bone marrow has further enhanced the osteogenic potential of the HA-BMP composite (Yoshida 1999, Noshi 2000). It has been suggested that the geometrical configuration of hydroxyapatite may be an important factor in osteogenesis (Magan & Ripamonti 1996, Kuboki *et al.* 1998).

Natural coral has been used in animal bone defect models with good results (Gao *et al.* 1997, Sciadini *et al.* 1997b), although there was obviously an immunological reaction to natural bovine BMP in the former, which impaired healing at the later stages of the study (Gao *et al.* 1997). In rat cranioplasty, natural coral with BMP was superior to natural coral alone (Arnaud *et al.* 1999).

Tricalcium phosphate has been used as a carrier material either alone or in combination with other materials, especially hydroxyapatite (Urist *et al.* 1984, Stevenson *et al.* 1994, Gao *et al.* 1996a, Boden *et al.* 1999)

The other materials suggested as carrier materials for BMPs constitute a very heterogeneous group of different materials, including bioactive glass, calcium sulphate, carbon, fibrin sealant and titanium (Lindholm & Gao 1993).

2.4 Canine ulnar segmental defect

Canine ulnar segmental defect is a well-established model. The dog ulna is not directly a weight-bearing bone, as the radius gives some support to the ulna, and there has been some controversy about the fixation methods. The ulnar defect model has been used with no fixation at all (Nilsson *et al.* 1986, Delloye *et al.* 1992, Cook *et al.* 1994b) or with an intramedullary Steinmann pin (Moore *et al.* 1987, Grundel *et al.* 1991) or plate fixation (Johnson *et al.* 1989, Schwarz *et al.* 1991).

Key (1934) was the first to use the segmental defect of canine ulna, and his observation was that a defect 1.5 times the ulnar diameter left empty leads to non-union. Key observed that the insertion of boiled bone, bone powders, calcium salts and other non-viable fillers into the defect produced non-union, while an autogeneic bone graft generally produced solid union.

Heiple *et al.* (1963) used the same model to investigate the process of regeneration in defects, concluding that autogeneic bone was superior to allogeneic bone and to demineralized bone matrix.

Autografts have also been found to be superior to allografts and demineralized bone matrix in other studies (Schwarz *et al.* 1991, Delloye *et al.* 1992). In the former, the ulnar defect was temporarily filled with silicone rubber blocks for eight weeks, which were then replaced by bone grafts. After 24 weeks, only the autogeneic bone had led to healing in all instances. Bone regeneration was not significantly better than in the sham group, in which no graft was employed. The results of Delloye *et al.* (1992) showed that autografts achieved a better union score and were mechanically stronger than allografts, but intracortical bone porosity, the percentage of cumulative new bone and the mineral apposition rate were not variables with statistical significance. In an earlier study, they had demonstrated notable variability of healing patterns in canine ulnar segmental defects and the long-term nature of the healing of cortical autografts, which was not completed at 9 months (Delloye *et al.* 1986).

Ceramics alone and in different combinations have been used as bone substitutes in canine ulnar defects. Moore *et al.* (1987) used a mixture of hydroxyapatite-tricalcium phosphate (HA-TCP) ceramic alone and with autograft cancellous bone, comparing these to autograft bone. Autograft and a combination HA-TCP with autograft showed good

bone healing, while HA-TCP alone was not osteoinductive. The authors concluded that morselized HA/TCP promises to be useful as a graft extender when mixed with autogenous cancellous bone.

Grundel *et al.* (1991) used the same model with HA-TCP with autogeneic bone marrow compared to autogeneic bone marrow alone. Both groups resulted in good bone healing, and HA-TCP combined with bone marrow resulted in complete bridging significantly earlier than bone marrow alone.

Guillemin *et al.* (1987) treated small cortical defects (5x8 mm) of canine ulna with natural coral. The results showed continuous resorption of coral implants and filling of defects with new bone by 8 weeks.

2.5 Treatment of a segmental bone defect with BMP

Segmental long bone defects have been used as models for bone reconstruction to evaluate different transplant materials as well as the efficacy of BMP. This model is valid in studying osteoconductive agents when the defect (large enough) does not heal spontaneously (Einhorn 1999). Animal studies with bone defects treated with bone substitute materials or BMP include dog radius (Johnson *et al.* 1996a, Johnson *et al.* 1996b, Heckman *et al.* 1999, Sciadini & Johnson 2000), dog femur (Johnson *et al.* 1996a, Bruder *et al.* 1988), dog fibula (Enneking *et al.* 1975, Burchardt *et al.* 1978), sheep tibia (Marcacci *et al.* 1999), rabbit ulna (Bolander *et al.* 1986, Hopp *et al.* 1989), rabbit radius (Zellin & Linde 1997, Teixeira & Urist 1998, Wheeler *et al.* 1998) and rat femur (Einhorn *et al.* 1984, Nottebaert *et al.* 1989, Ohura *et al.* 1999) and dog ulna (Key 1934, Heiple *et al.* 1963, Delloye *et al.* 1986, 1992, Nilsson *et al.* 1986, Johnson *et al.* 1989, Grundel *et al.* 1991, Schwarz *et al.* 1991, Cook *et al.* 1994b). In evaluating the results, various methods of analysis have been used, the principal methods being radiography, histology and torsion testing (Table 2).

Table 2. Summary of the methods of analysis used in segmental bone defect models treated with BMP.

Implant/carrier	Species	Bone	Defect size	Analysis methods	Authors and year
bBMP	Rat	femur	1.0 cm	radiography, histology	Tagaki & Urist 1982
bBMP	Dog	ulna	2.5 cm	radiography, histomorphometry	Nilsson *et al.* 1986
bBMP/PLA dBMP/PLA	Dog	radius	0.3 cm	radiography, histomorphometry	Heckman *et al.* 1991
rhBMP-2/DBM	Rat	femur	0.5 cm	radiography, torsion test, histology, radioisotope boneimaging	Yasko *et al.* 1992
rhOP-1/collagen	Rabbit	ulna	1.5 cm	radiography, torsion test, histology	Cook *et al.* 1994
rhOP-1 /collagen	Dog	ulna	2.5 cm	radiography, torsion test, histology	Cook *et al.* 1994

Table 2. Continued.

rhOP-1 /collagen	Green monkey	ulna and tibia	2.0 cm	radiography, torsion test, histology	Cook *et al.* 1995
rhBMP 2/PGA	Rabbit	ulna	2.0 cm	radiography, torsion test, histology	Boström *et al.* 1996
sBMP/TCP	Sheep	tibia	1.6 cm	radiography, torsion test, histology	Gao *et al.* 1996
mBMP/coral	Sheep	tibia	1.6 cm	radiography, torsion test, histology	Gao *et al.* 1997
bBMP/DBM	Dog	radius	2.5 cm	radiography, torsion test, histology	Sciadini *et al.* 1997
bBMP/coral	Dog	radius	2.5 cm	radiography, torsion test, histology	Sciadini *et al.* 1997
rhBMP-2 /PLA	Rabbit	radius	2 cm	radiomorphometry, histomorphometry	Zegzula *et al.* 1997
rhBMP-2/PLA	Rabbit	radius	1.0 cm	radiography	Zellin & Linde 1997
rhBMP-2 / PDLLA	Dog	ulna	2 cm	radiography, histomorphometry	Itoh *et al.* 1998
rhOP-1 /collagen	Dog	ulna	2.5 cm	radiography, torsion test, histology	Cook *et al.* 1998
rhBMP-2 / PDLLA/PGA	Sheep	femur	2.5 cm	radiography, histology	Kirker-Head *et al.* 1998
rhBMP-2 / PDLLA/PGA	Rat	femur	0.5 cm	radiography, torsion test,	Lane *et al.* 1998
rhBMP-2/PLA/ PGA	Rabbit	radius	2.0 cm	radiography	Texeira & Urist 1998
rhBMP-2/PLA	Rabbit	radius	2.0 cm	radiomorphometry, torsion test	Wheeler *et al.* 1998
cBMP/PLA	Dog	radius	0.3 cm	radiography, histomorphometry	Heckman *et al.* 1999
rhBMP-2 /PLA/ PGA	Rat	femur	0.5 cm	radiography, histology	Isobe *et al.* 1999
rhBMP-2/TCP-MCPM	Rat	femur	0.5 cm	radiography, torsion test	Ohura *et al.* 1999
rhBMP-2/collagen	Dog	radius	2.5 cm	radiography, histology, biomechanical testing	Sciadini & Johnson 2000

2.5.1 Naturally occurring BMP preparations in segmental bone defects

BMP has been used in bone defect models in order to improve bone healing, and the goal has been to achieve equally good or better results with these osteoinductive composite grafts compared to those obtained with the golden standard, autograft bone. Both extracted, naturally occurring BMPs and recombinant BMPs have been used in numerous studies.

Heckman *et al.* (1991) used both canine and bovine BMP in a relatively small, 3 mm defect in dog radius, using a polylactic acid carrier. The results showed that canine BMP was able to produce a significant increase in new bone formation compared to the controls. In contrast, when bovine BMP was implanted, no significant reparative new bone was found in the defect.

Interestingly, Sciadini *et al.* (1997a), by using the same model, found out that demineralized bone matrix in combination with bovine BMP resulted in good bone union in all cases, the result being comparable to autograft. In a subsequent study using a natural coral carrier, the results obtained with BMP even exceeded the good results of autografting (Sciadini 1997b).

Stevenson *et al.* (1994) reported a treatment of a rat femoral defect using bovine BMP with a mixture of hydroxyapatite and tricalcium phosphate used as a carrier. Ceramic together with BMP significantly enhanced the formation of bone in and around the segmental defect.

Gao *et al.* (1996a) studied sheep BMP with tricalcium and type IV collagen in a sheep tibial segmental defect. After 16 weeks, the BMP group appeared superior in both radiological bone healing and torsional testing of the bone. In a subsequent study, they used moose BMP in the same model and found a larger amount of external callus in the BMP group at 6 weeks. However, after 16 weeks, torsion testing showed lower mechanical strength in the BMP group, and there was also a significantly elevated anti-BMP antibody in serum samples (Gao *et al.* 1997).

Species-specific canine bone morphogenetic protein induced bone formation in a dog radius bone defect with a PLA/PGA carrier in a study where BMP was also compared with transforming growth factor-beta (TGF-β). The latter did not induce bone formation in this model (Heckman *et al.* 1999).

2.5.2 Naturally occurring BMP preparations in canine ulnar defect

Bovine bone extracted BMP has been widely used in bone defect models because of the good availability of bovine bone. Since bovine BMP cross-reacts immunologically with canine and human BMP and induces heterotopic bone formation in comparable doses in muscle pouches of mice, it is useful in comparative animal research (Nilsson *et al.* 1986).

Nilsson *et al.* (1986) used a 2.5 cm segmental canine defect, into which 100 mg of bovine BMP in a capsule was implanted without any carrier material or fixation. On the contralateral side, they implanted a similar capsule with 100 mg of bovine serum albumin. The autograft served as a control in another group of dogs. The results showed that the BMP-treated defects achieved complete regeneration. The incorporation of autogeneic bone in the ulnar defect occurred within the same period as bone regeneration induced by BMP, but the volume of the spindle of callus and the quantity of the BMP-induced new bone were significantly greater than those produced by a cortical bone autograft.

In another study using the same model, however, BMP did not have a positive effect despite the rigid plate fixation (Johnson *et al.* 1989). Xenogeneic, bovine bone morphogenetic protein (bBMP) and associated insoluble noncollagenous proteins (NCP) were implanted in inbred adult beagle dogs with large, 3–4 cm diaphyseal defects in the ulna

with plate fixation. The defects were implanted with either autogeneic cancellous bone grafts (ACG), bBMP/NCP or a composite of ACG and bBMP/NCP with plate fixation. Compared to the restoration of defects implanted with ACG (95%), bone healing occurred in 50 % of the defects implanted with ACG and bBMP/NCP and failed in all defects implanted with bBMP/NCP alone. It was speculated that the interposition of surrounding muscles into the large defect, the species-specific immune response to xenogeneic BMP, the diminished blood supply and the lesser bone regenerative capacity in aged dogs were the reasons for the failure of BMP.

2.5.3 Recombinant BMP in segmental bone defects

Rat segmental femoral defects 5 mm in length were implanted with two doses of rhBMP-2 (1.4 or 11 µg) together with rat DBM carrier (Yasko 1992). Bone formation was demonstrated on the seventh postoperative day in the high-dose rhBMP-2 group, while the low-dose group radiographic showed no evidence of bone in the defects until the third or fourth postoperative week. Radiographic evidence indicated a significant difference between the high-dose and low-dose groups or the bone matrix controls by the ninth week. Mechanically, the healed defects in the high-dose rhBMP-2 group demonstated stiffness comparable to that in the contralateral, intact femora. The established dose-dependent response in the repair of large segmental diaphyseal defects by rhBMP-2 implies that the larger the defect is, the more rhBMP is needed. Isobe *et al.* (1999) used the same model with a PLA/PGA carrier, and the results showed good bone healing with the rhBMP-2/PLA/PGA capsules compared to the control animals (with a PLA/PGA capsule in the defect), which did not heal.

Using a 1.5 cm segmental rabbit ulnar defect model, the dose-dependent bone-inducing capacity of rhBMP was further confirmed (Cook *et al.* 1994a). rhBMP-7 at doses from 3.13 to 400 µg with allogeneic DBM was implanted in the defects and compared with implants of 250 µg of bovine BMP with the same carrier. All of the bovine bone implants and all of the rhBMP-7 implants except those containing 3.13 µg of the substance showed complete radiographic osseous union within eight weeks. Histologically, the defect sites were filled with primarily normal lamellar bone with well-developed marrow tissue.

Using a 2 cm segmental rabbit ulnar defect, Boström *et al.* (1996) were able to demonstrate the same dose-dependent pattern of healing of the defect with rhBMP-2.

Zegzula *et al.* (1997) treated critical-sized rabbit radial diaphyseal defects with rhBMP-2 in a PDLLA carrier. The BMP they used elicited bone formation and healing of the bone defect.

Recombinant BMP has also been tested in a more demanding, large animal bone defect model (Kirker-Head *et al.* 1998). A mid-diaphyseal 2.5 cm defect in the sheep femur was stabilized with a plate and implanted with 2–4 mg of rhBMP-2 with a PDLLA carrier. Union occurred in 3/7 of the bones treated with 2 mg of rhBMP-2, 2/3 of the bones treated with 4 mg of rhBMP-2 and none in the control group (no BMP). In the animals that healed, the new bone mineral content equaled that of the intact femur by 16 weeks, and recanalization of the medullary cavity approached completion at 52 weeks. At

necropsy, the surgically treated femurs were rigidly healed, the carrier material was resorbed completely, and woven and lamellar bone bridged the defect site. No mechanical testing was performed in this study, as the bones were only tested manually, and the bones with clinical and radiographic union were found to be rigidly healed in the manual test.

Cook *et al.* (1995) used a bone defect in primates for assessing recombinant BMP-7. They created 2.0 cm defects in the ulnae and in tibiae of African green monkeys and implanted the defects with 250–2000 µg of rhBMP-7 with a bovine collagen carrier. Five of the six ulnae and four of the five tibiae treated with rhBMP-7 exhibited complete healing at six to eight weeks. Histological evaluation revealed the formation of new cortices with areas of woven and lamellar bone and normal-appearing marrow elements. Mechanical testing revealed an average torsional strength to failure of 92 per cent and 69 per cent of that of the contralateral intact ulnae and tibiae, respectively. In this study, rhBMP-7 implants elicited healing of the defects that was as good as or better than that achieved with autogenous bone grafts.

In a recent study, rhBMP-2 combined with bone marrow with a polylactide carrier was implanted in a rat femoral defect (Lane *et al.* 1999a). The rhBMP-2 and bone marrow composite grafts achieved 100 % union within 6 weeks. The combination was superior compared to each component alone, which strongly supports biologic synergism.

2.5.4 Recombinant BMP in canine ulnar defect

Cook *et al.* (1994b) used a 2.5 cm segmental canine ulnar defect in a study where recombinant human bone morphogenetic protein (rhOP-1) was used at a dose of 1200 µg with a collagen carrier. All defect sites receiving rhOP-1 were completely bridged radiographically by eight weeks. After 12 weeks of implantation, the ulnae had reached mechanical strength comparable to that of a normal ulna.

In a later study by Cook *et al.* (1998), using the same defect model, ulnae treated with rhOP-1 showed complete radiographic healing at 12 weeks in 89 % of the cases. Histology revealed that the defects were filled with lamellar and woven bone that was in continuity with the host bone, and the mechanical strength of these bones reached 65 % of that of intact ulnae.

Good bone healing was also reported in a study where rhBMP-2 was used with a PLA/PGA/gelatin sponge complex (PGS) as a carrier in canine ulnar defects (Itoh *et al.* 1998). All defects treated with rhBMP-2 at a dose of more than 160 µg revealed bone union radiographically at 12 weeks, whereas defects treated with PGS alone did not heal.

2.6 Gene therapy in a bone defect model

Bone morphogenetic protein can induce bone in a bone defect, but the missing ideal carrier system for BMPs limits their clinical application. Modern gene technology has been able to create BMP-producing cells, which can be used to heal bone defects. Gene thera-

py offers several potential advantages over other methods of osteoinduction, and current research suggests that it may be a treatment option for the ortopedic surgeon in the near future (Scaduto & Liebermann 1999).

Fang *et al*. (1996) used a rat bone defect model with a BMP-4 cDNA construct, which was delivered to the defect by loading on a gene-activated matrix, and the 5 mm defects healed. In another study using gene therapy, Lieberman *et al.* (1999) filled a 8 mm rat femoral bone defect with BMP-2 producing bone marrow cells created by means of adenoviral gene transfer. They found solid bony healing of the defect, and bone formation was more prominent in gene therapy treated defects compared to those treated with rhBMP-2 in a DBM carrier. The authors propose that the osteoinductive stimulus associated with the BMP-2-producing bone marrow cells may be enhanced because the BMP-2 protein is released continuously.

Gene therapy may also be a possible future treatment for difficult fractures and nonunions along with other modes of treatment (Niyibizi *et al.* 1998).

Athough the results of gene therapy seem to be promising, there are still many unanswered questions, such are the duration and amount of protein production *in vivo*, the safety of the viral vector, the immunological response to viral proteins, and the fate of BMP-producing cells after implantation. The research in this field is obviously of great interest and we might find answers to these questions in the future.

2.7 Other animal models with BMP

Although not directly included in this study, the skull defect model and the spinal fusion model deserve a brief review here because of their importance in the evaluation of BMPs for future clinical use.

Skull defect has been a favoured model in animal studies because of the easy accessibility of skull bone. Skull bone has a poor blood supply and also a relative deficiency of blood marrow and hence the necessary bone-forming cells (Simmons 1980). Tagaki and Urist were the first to use the skull defect model in 1982. They used rat skull defects and found that bBMP healed defects that did not normally heal otherwise (Tagaki & Urist 1982b). After that, BMP has been shown to induce regeneration of nonhealing calvarial defects in dog (Sato & Urist 1985), sheep (Lindholm *et al.* 1988), rabbit (Damien *et al.* 1990), baboon (Ripamonti 1992, Ripamonti *et al.* 1996), pig (Lindholm *et al.* 1994) and again rat (Murata *et al*. 1999). Other facial bone defects treated with BMP have also been a target of great experimental interest (Boyne 1996, Asahina *et al.* 1997, Higuchi *et al.* 1999, Toriumi *et al.* 1999, Wikesjo *et al.* 1999, Yoshida *et al.* 1999, Yudell *et al.* 2000).

Spinal fusion is another model that has been used in animal studies, and it is also an important clinical entity. Because of the availability problems of autograft bone, different materials have been tested instead of autografts, including collagen, hydroxyapatite, demineralized bone matrix and biodegradable polymers. These are biocompatible materials, but not osteoinductive, and they are thus used as filling material. This might not be sufficient in many clinical settings, where bone formation and bone union are critical. Thus, osteoinductive BMPs have been tested in animal models of spinal fusion. An optimal method for spinal fusion would induce rapid growth of bone at a site via osteoconductive

and osteoinductive implants (Sheehan *et al.* 1996). Thus, many animal studies have been performed with BMP with different carriers (Muschler *et al.* 1994, Sandhu *et al.* 1995, Schimandle *et al.* 1995, Morone *et al.* 1998, Boden *et al.* 1999, David *et al.* 1999, Hecht *et al.* 1999, Martin *et al.* 1999, Meyer *et al.* 1999, Minamide *et al.* 1999, Paramore *et al.* 1999). The results have been encouraging, as BMP clearly induces bone formation in spinal fusion, but more information is needed of the basic mechanisms of BMP function, and there is clearly a problem with the missing ideal carrier material for BMP.

2.8 Clinical use of BMP

The reported clinical cases where BMP has been used as a therapeutic agent are from the fields of cranio-maxillofacial surgery and orthopaedics and traumatology, the latter consisting mainly of long bone defects after a fracture, non-unions of long bones and spinal surgery. Preliminary clinical trials in healing femoral non-unions (Johnson *et al.* 1988a) and traumatic tibial segmental defects (Johnson *et al.* 1988b) have been conducted with naturally occurring human BMP. In the former, 11 of 12 patients developed union and 1 patient did so after repeat stabilization and implantation of hBMP. The BMP was used with a bone matrix water-insoluble noncollagenous protein carrier at a dose of 50 to 100 mg, which was either implanted in the fracture gap in ultra-thin gelatin capsules or incorporated in a strip of polylactide/polyglycolide copolymer and placed as an onlay across the fracture gap (Johnson *et al.* 1988a). In the latter group, 6 patients with traumatic 3 to 17 cm tibial defects developed solid union by implantation of hBMP with autogenous cancellous bone grafts and stabilization. There were no complications connected with the surgical procedure or the implant (Johnson *et al.* 1988b).

In their later studies using the same BMP product, the authors successfully treated 4 patients with deformed nonunions of the distal end of tibia (Johnson *et al.* 1990) and 25 patients with femoral, tibial or humeral non-unions, of whom 24 finally obtained union (Johnson *et al.* 1992).

In a recently published study, 30 patients with non-unions of the femur after a failure of fracture healing were treated with plating, and allogeneic, autolysed, antigen-free cortical human bone was used as a structural alloimplant and as a delivery system for partially purified human bone morphogenetic protein. Twenty-four femora healed within an average of 6 months, four patients were re-operated, and two patients were lost for follow-up. The authors concluded that the human bone morphogenetic protein with allogeneic, autolysed, antigen-free bone allograft is an excellent structural and delivery system that induces host bone formation (Johnson & Urist 2000).

The use of bovine BMP was reported by Bai *et al.* (1996) in non-unions of femoral shaft fractures. Union was obtained in 16/17 patients, and no significant postoperative complications were detected.

Geesink *et al.* studied rhBMP-7 with a collagen type 1 carrier in patients with a critical-sized fibular defect. In the first phase of this study- the critical-sized nature of the defect was established; positive controls (demineralized bone) caused formation of new

bone, while untreated bones showed no bone formation. In the second phase, 4 of the 5 patients who received rhBMP-7 had new bone formation, while those treated with collagen alone had no significant bone formation (Geesink *et al.* 1999).

In spinal surgery, arthrodesis was found to occur more reliably in patients treated with rhBMP-2-filled fusion cages than in controls treated with autogenous bone grafts. There were a total of 14 patients randomized to receive lumbar interbody arthrodesis with a tapered cylindrical threaded fusion cage filled with rhBMP-2/collagen sponge (11 patients) or autogenous iliac crest bone (3 patients). There were no adverse events related to the rhBMP-2 treatment (Boden *et al.* 2000).

In cranio-maxillofacial surgery, rhBMP-2 and rhBMP-7 have been used with some promising results in maxillary sinus augmentation (Boyne *et al.* 1997, Howell *et al.* 1997, Barboza *et al.* 1999, Groeneveld *et al.* 1999), but there was some unexpected variation in the results.

2.9 Other applications under development

Fracture healing may be one of the major applications of bone morphogenetic proteins in the future. Several growth-promoting substances have been identified at the site of skeletal injury and appear to play a physiologic role in fracture healing (Boström *et al.* 1999). BMPs may be capable of healing the cases of delayed union or non-union, which represent 5–10 % of all fractures (Boström & Camancho 1998). It has been suggested that the bone morphogenetic proteins 2 and 4 are important regulators of cell differentiation during fracture repair (Boström *et al.* 1995). On the basis of the *in situ* hybridization technique, BMP 4 seems to be one of the local contributing factors in callus formation in the early phases of fracture healing (Nakase *et al.* 1994). Bax *et al.* (1999) used rhBMP-2 in fractures of the rabbit tibia. In a series of mechanically unstable fractures, those treated with BMP gained union more rapidly, while in stable fractures the effect of BMP was minimal. It was argued that mechanical factors influence the size of the callus of normally healing fractures, and although BMP-2 accelerates the rate of development of the callus and cortical union, it does not affect the amounts of bone and cartilage produced.

Welch *et al.* (1998) treated gout tibial fractures with rhBMP-2. Callus formation was increased significantly in BMP-treated fractures, but strength and stiffness were only moderately increased.

Most of the studies with BMPs deal with the bridging of critical-sized defects and very few with fracture repair. Athough some animal studies have had promising results, the therapeutic efficacy of bone morphogenetic protein in fracture healing remains uncertain and has to be determined in future studies.

The repair of articular cartilage defects is another possible future application of BMPs. So far, very little information is available in this area. It has been reported that articular cartilage defects fill with repair tissue and show good healing with well organized and intact cartilage at early time points postoperatively, but the repair tissue eventually degenerates due to its inability to withstand the biomechanical forces in the joint (Shapiro *et al.* 1993). *In vitro*, rhOP-1 has been shown to stimulate the synthesis of cartilage-specific molecules by human articular chondrocytes (Flechtenmacher *et al.* 1996) and the differ-

entiation of cartilage from perichondrium tissue (Klein-Nulend *et al.* 1998). Grgic *et al.* (1997) demonstrated rabbit articular cartilage regeneration in drill holes treated with BMP-7. Sailor *et al.* (1996) showed that rhBMP-2 maintains the articular chondrocyte phenotype in long-term cell culture. In a study by Lietman *et al.* (1997), BMP-7 stimulated the proteoglycan synthesis in porcine articular cartilage, implicating that BMP-7 may play a role in the process of cartilage repair. In a rabbit femoral cartilage defect model, Sellers *et al.* (2000) showed that rhBMP-2 applied to the defect resulted in an improvement in the histological appearance and composition of the extracellular matrix at one year postoperatively.

3 Aims of the present study

The series of experiments was designed to elucidate the mechanisms of bone defect healing with a bone morphogenetic protein. The ideal carrier material for bone morphogenetic protein is still a matter of debate, and we wanted to test different carrier materials in demanding conditions, i.e. a long bone defect model.

The specific aims of this study were:

1. To create an animal model in which bone healing can be evaluated with different implants and with and without BMP.
2. To compare autograft, allograft and xenograft bone in bone healing using a canine ulnar defect model.
3. To test biocoral, hydroxyapatite and tricalcium phosphate in segmental bone defects.
4. To evaluate the effect of native extracted bovine BMP in healing segmental long bone defects.
5. To test coral, hydroxyapatite and demineralized xenograft bone as carrier materials for bone morphogenetic protein.

4 Materials and methods

4.1 Animals

Twelve adult female sheep were used with an average body weight of 47.37 ± 11.4 kg (study III). This experiment was approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Helsinki.

Laboratory-bred beagle dogs, both male and female, aged one year and weighing 9.0 to 13.2 kilograms were used (studies I–II and IV–V). All the experimental manipulations of the dogs were approved by the Committee on Animal Experimentation of Kuopio University. The animals were kept in large outdoor/indoor runs with shelter before the operation, in separate cages for 1–2 days after the operation and thereafter again in the large runs for the duration of the study. The dog chew was Serti® (Suomen Nestle, Helsinki, Finland).

4.2 Patient

We had one patient, a 18-year-old female without any history of significant diseases, who developed a non-union in her left ulna after a traffic accident and two unsuccessful operations (Study VI).

4.3 BMP

The BMP used was extracted from bovine diaphyseal bone. Fragmented bone was ground in a frozen state into particles less than 1 mm in size. The bone matrix was extracted in 4 M GuHCl at 4°C for 72 hours after the pulverized bone had been demineralized in 0.6 N HCl. The extracted solution was passed through a Millipore filter (pore size: 0.6 µm, Millipore Corporation, MI, USA). The filtrated solution was dialysed against deionized water, and the water-insoluble precipitate was redissolved in 4 M GuHCl. Gelatine pep-

tides were removed by dialysis against 0.25 M citrate buffer, and the precipitate was centrifuged and lyophilized. The water-insoluble bovine BMP was collected (Urist *et al.* 1984, Gao *et al.* 1993b).

4.4 Bone grafts and implants

Autograft bone segment was taken as a 2 cm cortico-periosteal bulk from the left leg and placed on a 2 cm defect in the right ulna in the same dog. In the allograft group, a similar segment was taken from another dog in the same group and changed with each other (Study 1).

The xenogeneic implant, 9 mm in diameter and 20 mm in length, was manufactured from demineralized bovine cancellous bone. Demineralization was performed in 0.6 N HCl (+4º C for 3 days). After that, the implant was placed in 10 % hydrogen peroxide at room temperature for 24 hours. The partially purified BMP, including a combination of several growth factors, was used at a dose of 30 mg per implant, and BMP was adsorbed into the bovine bone implant. The activity of the extracted BMP was tested prior to the implantation in a rat thigh muscle poach model. The implants were sterilized with ethylene oxide (Study II).

The tricalcium phosphate used (β-247, DePuy, Warsaw, IL, USA) was a beta-whitelockite lattice composed of Ca3[PO4]2 (study III). The overall Ca/P molar ratio was approximately 1.5. The substance has a porosity of 55 % of volume, with pore sizes ranging from 200 to 400 microns. Natural coral cylinders (Biocoral®, Inoteb, LeGuernol, Saint-Gonnery, France) were obtained from the calcium carbonate exoskeleton of scleractinian coral. Calcium carbonate in the form of aragonite accounts for more than 97 % of the weight. Coral has a porosity around 50 % of volume, with pore sizes ranging from 150–500 microns and interconnecting fenestration throughout the entire substratum.

Cylinders of both ceramics were shaped into identical dimensions: 15 mm in diameter and 16 mm in length, with a plug of 3 mm at each end. A central longitudinal hole 4 mm in diameter was predrilled to reproduce a medullary canal. The implants were sterilized with ethylene oxide for 4 hours and then deposed for 6 hours preoperatively. These TCP and coral implants were used in the sheep tibial defects (study III).

The coral material used in canine ulnar defects was the same material as above, and implants with a diameter of about 9 mm and length of about 20 mm were used in nine dogs, and a composite implant containing coral and native bovine BMP was used in another group of nine dogs as an ulnar transplant. The coral implant was immersed in the BMP mixture, and after lyophilization, each implant contained 30 mg of bovine bone morphogenetic protein (study IV).

Cylindrical hydroxyapatite implants (BIOLAND, Toulouse, France) about 9 mm in diameter and 20 mm in length were used in a 2 cm segmental defect in the ulna in six dogs, while a composite HA implant containing BMP was used in another group of six dogs(study IV). The porosity of the HA material ranged within 45–50 %, pore size was 340–450 μm, and the theoretical Ca/P ratio was 2.15. BMP was used at a dose of 30 mg

per implant, as in an earlier study with coral implants. BMP was adsorbed on to a collagen sponge (Lyostypt®, Braun-Melsungen AG), which was wrapped around the HA cylinder. The implants were predrilled to produce a medullary canal (Study V).

The implant used in our clinical case (study VI) consisted of a cylindrical coral frame (Biocoral®, Inoteb, Saint-Gonnery, France) with a diameter of 1.1 cm and a length of 1.5 cm, and BMP at a dose of 30 mg was adsorbed on to a collagen sponge (Lyostypt®, Braun-Melsungen Ag) and wrapped around the coral cylinder.

4.5 Surgical procedures

Sheep tibial defects (study III) were made under general anesthesia with 2.5 % halothane. A segmental unilateral defect 16 mm in length was created with a Gigli saw on the midshaft of the right tibia of each animal. Six defects were replaced with TCP and 6 with coral cylinders. The cylinder was secured in the defect through the plugs inserted in the proximal and distal medullary canals of the osteotomized tibia. The tibia was firmly fixed by two overlapping plates with cortical screws, and the muscles and skin were closed in layers. Procaine penicillin (Novo vet., Novo Industri A/S, Copenhagen, Denmark) at a dose of 39.5 mg/kg was administered intramuscurarly to each sheep for 4 days postoperatively to prevent infection. The animals were permitted to walk immediately after surgery.

The canine operations (studies I–II and IV–V) were made under general anesthesia using pentobarbital (Mebunat®, Orion-Farmos, Helsinki, Finland) at a dose of 15 mg/kg intravenously. Xylazine (Rompun Vet®, Bayer, Germany) at 1 mg/kg was used as premedication before the operation. A single dose of prophylactic antibiotics (1 ml/8 kg of Tribrissen Vet, Mallinckrodt Veterinary LTD) was given during the operation.

For the operation, both forelegs were prepared and draped in a sterile fashion. A rubber band was used as a tournique above the elbow joint. A lateral incision was made and the ulna exposed. Using an oscillating saw, an osteotomy through the whole bone, including the periosteum, was made in mid-ulna about 6 cm from the tip of the olecranon, and another osteotomy was made 2 cm distally from that point.

The 2 cm bulk cortico-periosteal segment was removed and used as an autograft or allograft transplant(Study I). A Kirschner wire (1.2 mm thick) was introduced into the medullary canal through the tip of the olecranon for stabilizing the implant in the defect, extending about 3 cm distally from the distal end of the implant (Fig. 1).

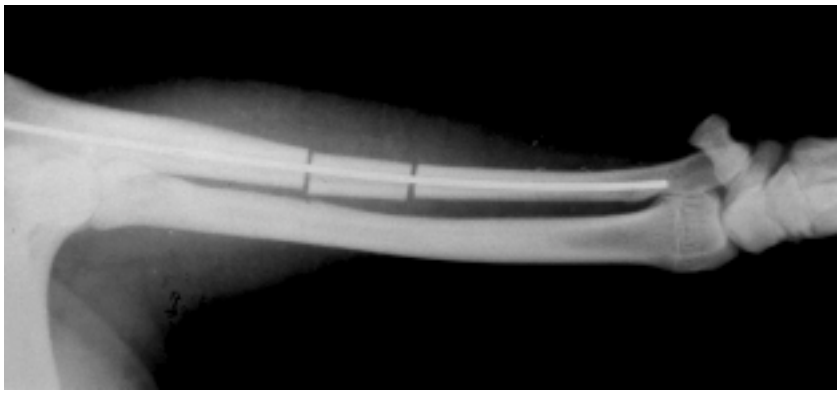


Fig. 1. A radiograph showing the study model. An autograft transplant in the defect fixed with an intramedullary Kirschner wire.

Xenograft implants (study II) were fixed with plates, and the fixation was performed with a 10-hole stainless steel miniplate and screws (Stratec Medical, Oberdorf, Switzerland) by applying 3 screws proximally and distally to the defect, with the 4 middle holes left empty The defect of the left ulna was filled with a pure xenograft implant and that of the right ulna with a xenograft implant with BMP.

The defect of the left ulna was bridged with plain coral and that in the right ulna with an autograft implant obtained from the left side with intramedullary Kirschner wire fication. Similar fixation was used in another group of six dogs with a coral+BMP implant. The Kirschner wires were removed after 9 weeks. A 10-hole miniplate and screws (Stratec Medical, Oberdorf, Switzerland) were used to bridge the defect in three dogs with coral implants. In these dogs, a plain coral implant was used in the left ulna and coral+BMP on the right side (study IV).

In the HA group, a plain HA implant was inserted into the left ulna and a composite implant with HA and BMP on to the right side. The fixation was done with an intramedullary Kirschner wire, which was removed after 9 weeks (Study V).

Pain medication after the operation (study I–II, IV–V) consisted of buprenorfin (Temgesic©, Reckitt&Colman, Hull, U.K.) at 0.01 mg/kg intramuscularly.

The dogs tolerated the operation well, and weight bearing began on the first postoperative day.

The dogs were killed after 20 weeks with an overdose of pentobarbital (Mebunat©) 60 mg/kg intravenously. The ulnae were dissected out and the soft tissue removed.

The bones were wrapped in saline and frozen at –20°C until analysis.

4.5.1 Clinical case

In the clinical case (study VI), a 18-year-old female had sustained a forearm fracture in a traffic accident in 1984. Open reduction and internal fixation of the radius and ulna was performed. The radius fracture healed uneventfully. The reduction of the ulna was not exact, and roentenograms taken at 4 months showed loosening of the proximal screws

and evident nonunion. Repeated fixation of the ulna with an 8-hole plate was performed. The atrophic bone ends of the non-union were excised, and the 2 cm bone defect hereby created was filled with corticocancellous autograft. Three years later, the plates were removed because of some discomfort at the fracture site. There was no statement of the bone union in the surgical records. After this operation, the forearm became painful, and x-rays taken 3 years postoperatively showed pseudoarthrosis of the ulna. The patient was able to manage with her arm and did not seek treatment until after 10 years. The forearm was painful, and a decision for surgery was therefore made. At surgery, the pseudoarthrosis was exposed, the atrophic bone ends were excised, and the defect was substituted by a 2 cm long coral+BMP composite implant.

4.6 Methods of analysis

4.6.1 Radiography

Sequential X-ray views of the osteotomized sheep tibiae were taken at 3, 6, 12 and 16 weeks after surgery. All radiograms from 3 to 12 weeks were scanned by a computerized optical density scanner and analyzed with the Bio Image System (6 XRS, Millipore Corporation, MI, USA) to follow quantitatively the variation in the area and density of external callus formation around the implants.

The position of the transplant and the fixation material in the dog ulna were checked postoperatively with roentgenograms. Bone healing was evaluated with further x-rays by taking both antero-posterior and lateral views at 1, 3, 6, 9, 12, 16, 25, and 36 weeks and after the execution. Bone union, callus formation and bone resorption were estimated independently by two investigators. The cases of disagreement were reviewed together. The interpretation was blinded between the autograft and allograft animals. Bone union was defined as either disappearance or partial bridging of the gap between bone and the transplant.

The radiograms were digitized with a ccd camera (Dage 72E, Dage MTI Inc., Michigan City, USA). The area of callus was evaluated from the lateral view radiograms using a digital image analysis system (MCID/M4, Imaging Research Inc., Brock University, St Catharines, Canada).

The evaluation of bone union (BU) was based on the scoring system proposed by Johnson *et al.* (Johnson *et al.* 1996b), in which proximal union was graded as 0–3 and distal union as 0–3. Thus, the highest possible score for bone union was 6. Bone formation (BF) was also scored, the maximum score being 4. The combined score (BU+BF) refers to the sum score for bone union and bone formation, the maximum score being 10.

In the clinical case, normal a-p and lateral views of the forearm were recorded before and after every operation and at the control visits.

4.6.1.1 Implant resorption

The resorption of TCP and coral was evaluated qualitatively from the x-rays taken of the sheep tibia at 16 weeks.

In the canine ulnar defect study, coral implant was resorbed unevenly, and the resorption of the coral implant was evaluated by scoring at 0–3, where 0 referred to no resorption and 3 to total resorption of the implant.

HA and bovine bone implant were evaluated qualitatively from the x-rays at the end of the study.

4.6.1.2 Callus size

The lateral radiograms were digitized with a ccd camera (Dage 72E, Dage MTI Inc., Michigan City, USA). The area of callus was evaluated from the lateral radiograms using a digital image analysis system (MCID/M4, Imaging Research Inc., Brock University, St Catharines, Canada).

4.6.2 Mechanical testing

The bones were thawed at room temperature for torsional testing. During the testing, the bones were kept moistened to avoid the potential effect of drying (Turner & Burr 1993). The bone ends were embedded in moulds with two-component fiberglass resin using a torsional shaft of 8 cm. After hardening of the resin, the bones were placed in the torque machine and torsionally loaded at a constant angular speed of 6.5 degrees/sec until failure (Jämsä & Jalovaara 1996). Maximal torque capacity (MTC) and maximal angular deformation (MA), bone torsional stiffness (BS) and maximal absorbed energy (MAE) were recorded (Fig. 2).

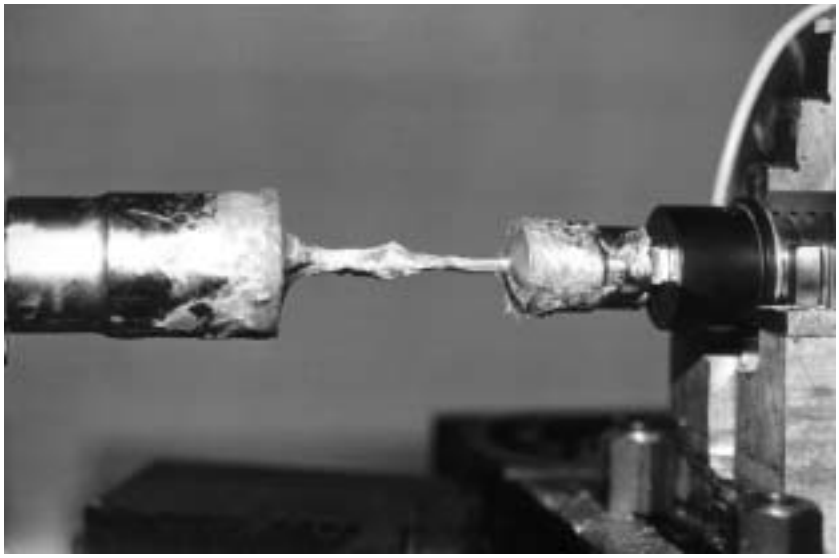


Fig. 2. Torsion testing machine with a bone attached ready to be tested.

4.6.3 Densitometry

Only xenografts and the samples with BMP from the coral and HA groups were available for densitometry testing. All the bones in the xenograft group were tested.

After defreezing, the bones were scanned using a peripheral quantitative computed tomographic (pQCT) system Stratec XCT 960A with the software version 5.20 (Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). A voxel size of 0.295 x 0.295 x 1.25 mm^3 was used. We scanned 5 slices of each sample, one slice in the middle of the implant, two slices at the distal and proximal bone-implant border areas, and two slices outside the implant from the distal and proximal ulna near the bone-implant border, the slice positions being defined from the axial scout view of the pQCT system. Bone mineral content (BMC mg/mm), mean bone mineral density (BMD, mg/mm^3) and cross-sectional bone area (CSA, mm^2) were recorded for each slide as given by the pQCT software. An attenuation threshold of 0.7 cm^{-1} was used to define compact bone.

4.6.4 Histology

Some specimens of sheep bone, in which the fracture line did not pass through the interface between new bone and the ceramic cylinder were sawn transversely into slices 0.4–2.0 mm thick with a diamond saw (Accutome 5, Struers Tech A/S, Copenhagen, Denmark), fixed in 10 % formalin, and then embedded in methylmethacrylate. Undemineralized sections of 12–20 μm were prepared by a cutting and grinding method (Exakt-Apparatebau, Hamburg, Germany) and stained with van Gieson for histological analysis.

After torsional testing of dog bone, the bones were reconstructed and a 4–5 cm long section including the implant site was taken for histological analysis. After fixing in 10 % neutral formaldehyde, the previously frozen samples were decalcified in 0.1 N HCl. The samples were embedded in paraffin, and 6 µm sections were stained with the Masson-Goldner trichrome method. The histological sections were evaluated microscopically and imaged with a color ccd camera (Sony DXC 930P, Japan) using a 1x objective (Nikon, Japan) and a Nikon Optiphot II microscope (Nikon, Japan). The callus was analyzed qualitatively.

4.6.5 Statistical analysis

Student's independent t-test was used to compare the radiographical and mechanical results of the canine autograft and allograft groups (study I).

Student's independent t-test was used for a statistical comparison of the radiomorphometrical quantitation and the mechanical test results between TCP- and coral-implanted sheep tibiae (study III).

Due to the ordinal measurement scale applied to the samples with BMP, a non-parametric Mann-Whitney test was used to compare the scores between the study groups in the studies II and IV–V. The statistical analysis was performed using the SPSS for Windows statistical package (SPSS Inc., ver 7.5.1).

Values of $p < 0.05$ were considered statistically significant.

A specialist was consulted concerning the planning of the statistical analyses.

5 Results

5.1 Coral and tricalcium phosphate healing sheep tibial defects

In sheep tibial defects, the appearance of external callus was noted radiologically at 3 weeks after the operation. The callus bridged the defects at 6 weeks and consolidated at 12 weeks. A quantitative analysis of the area and density of the callus showed radiomorphometrically that the area was larger and the density higher in coral- than TCP-implanted tibiae at 3 weeks ($p < 0.05$). No such difference was noted at 6 and 12 weeks.

The TCP substratum was resorbed more evenly than that of coral. The degradation of coral substratum was more advanced than that of TCP in some samples, but less so in other samples.

In comparison with TCP-implanted tibiae, maximal torque capacity, maximal angular deformation and absorption of energy were significantly enhanced in coral-implanted tibiae in the torsion test at 16 weeks after implantation. A fracture line through one of the conjunctions between the ceramic implant and the tibial stumps occurred in 1/5 of coral-implanted but in 3/6 of the TCP-implanted tibiae.

Histologically, newly formed bone penetrated into the coral and TCP substratum from both the simulated medullary canal and the periphery of the cylinders. The pores and the interconnecting fenestration in full-thickness coral and TCP cylinders (5.5 mm) were occupied by remodelled bone tissue. No interposed fibrous tissue was seen microscopically between the ingrown bone and trabeculae of coral or TCP cylinders. Better osteointegration between new bone and materials was evident with coral compared to TCP cylinders. A greater proportion of Haversian units was demonstrated in coral compared to TCP.

5.2 Healing of canine ulnar defects

5.2.1 Auto- and allografts

In canine ulnar defects treated with auto- and allografts, healing was more rapid at the distal end of the defect, and autografting resulted in faster bone healing than allografting. In the autograft group, 4 of the 6 ulnar defects showed complete union at the end of the study. In two cases, complete union of the distal end of the defect was observed, but there was a slight gap between the proximal ulna and the transplant. 3/6 of the metal pins were broken at 6, 9 and 16 weeks, respectively, but the healing of the defect was complete in these cases.

In the allograft group, 3 of the 6 ulnar defects had healed by the end of the study. In the three cases of failure, the distal end of the defect showed union, but no union had occurred proximally. The pin was broken in three cases at 3, 6 and 16 weeks. Two of them displayed complete healing and one proximal non-union.

The presence of hypertrophic callus in all cases in both groups was well established by 16 weeks. After that, there was a phase of ulnar remodelling in the cases of complete union (Fig. 3).

Upon mechanical testing, all bones fractured with an oblique fracture line. All the bones in the autograft group fractured distally outside the bone graft area, which indicates strong bony union. In the allograft group, 4 of the 6 bones were broken distally outside the graft area and 2 within the graft area, demonstrating weaker union.

The mean values of all mechanical parameters were higher for the autograft group, with maximal torque capacity 1.75 versus 1.11 Nm, maximal angular deformation 42.6 versus 38.2 deg, bone torsional stiffness 0.063 versus 0.049 Nm/deg and maximal absorbed energy 74.2 versus 40.5 Nmdeg. However, the difference was only statistically significant for maximal absorbed energy (MAE, $p=0.02$).

Autograft transplants resulted in solid bone union histologically in 4/6 cases at the end of the study (Fig. 4). In the two remaining cases, complete union with new bone formation at the distal end of the defect was also observed histologically, but the gaps at the proximal ends were filled with cartilaginous callus. In allografts, 3/6 of the cases showed histolocigally complete union with new bone formation at the distal ends. The proximal ends were filled, unlike autografts, with fibrous callus between the transplant and bone.

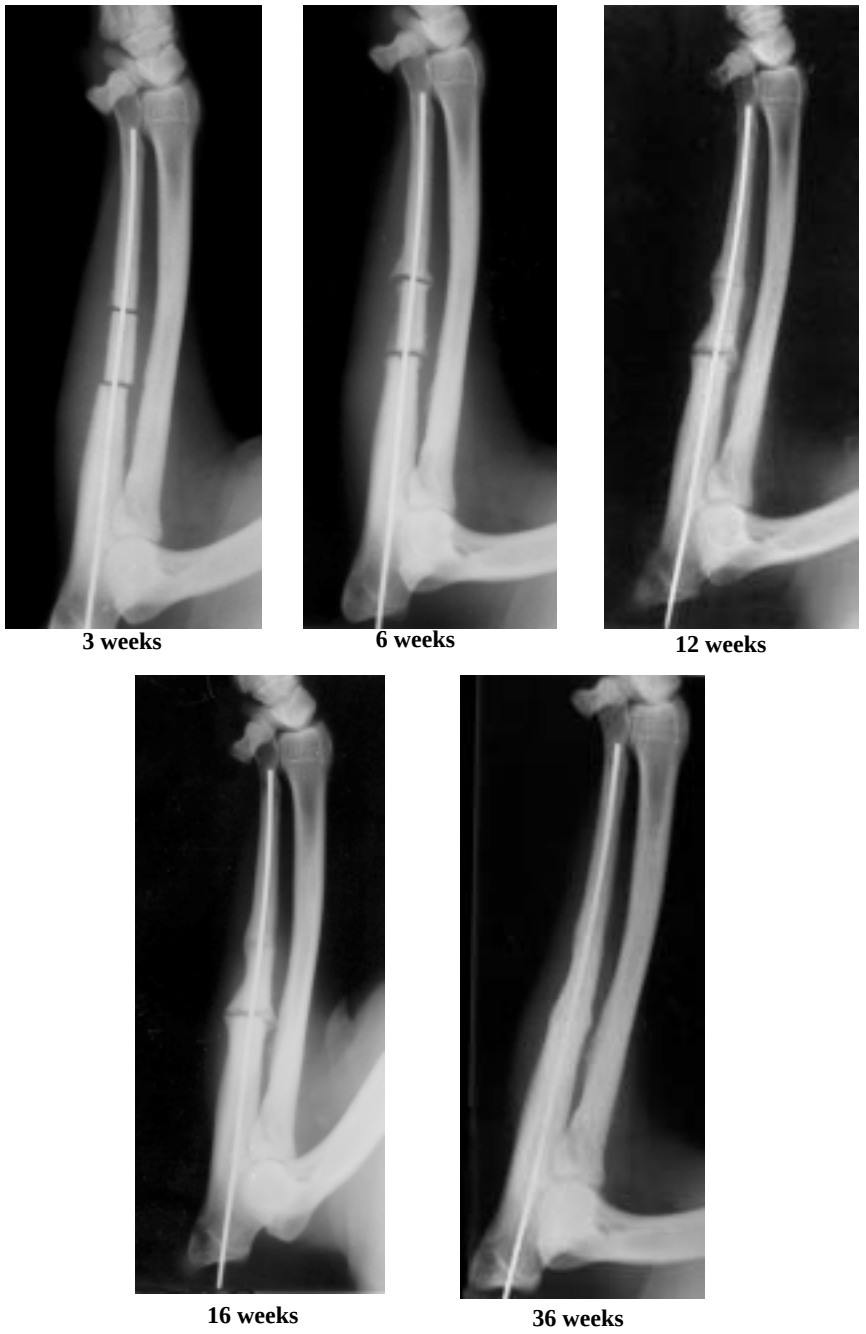


Fig. 3. A series of radiographs of an ulnar defect treated with an autograft transplant leading to solid bone union.

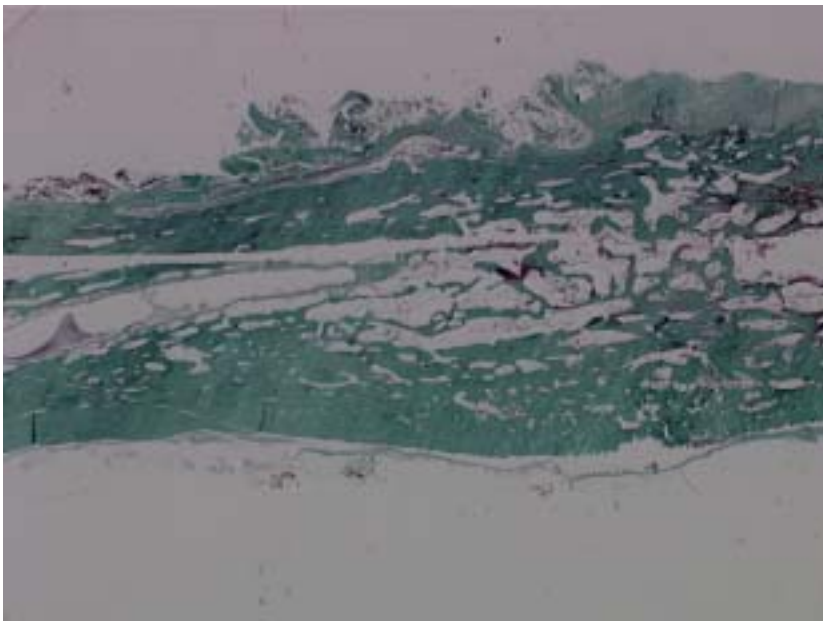


Fig. 4. A histological section of an autograft transplant showing new bone filling the gap and remodellation.

5.2.2 Xenografts and xenograft composite implants

With bovine bone implants, no instances of bone union of the defect were seen in the cases treated with either pure xenograft or xenograft with BMP at the end of the study, the mean score for bone union being equal to zero in both groups. There was some bone formation at the bone ends in all cases of both groups. The xenogeneic implants with BMP induced more bone formation than the implants without BMP evaluated according to Johnson (Johnson *et al.* 1996b), the scores being 1.0 ± 0.7 and 0.5 ± 0.6, respectively, but the difference was not statistically significant ($p = 0.24$) (Table 3).

The partly demineralized bovine bone implants were faintly visible in the first radiographs, but they were later resorbed totally by 16 weeks

When bones with bovine bone implants were tested mechanically in a torsion test, all the bones of both study groups broke at the implant area, indicating weak or absent bony union. The average MTC of the ulnae treated with xenografts impregnated with BMP (0.56 ± 0.34 Nm) was higher than that of the ulnae treated with pure xenografts (0.30 ± 0.24 Nm), but the difference was not statistically significant.

There was a significant difference in the total BMC in the proximal ulna close to the defect in favour of the cases treated with xenografts with BMP ($p = 0.047$). On the other hand, the total BMD of the distal ulna near the defect was lower in the group treated with

xenograft + BMP implants ($p = 0.022$). However, no significant differences in BMD, BMC or CSA were seen between the two xenograft groups in the implant area or at the bone-implant border.

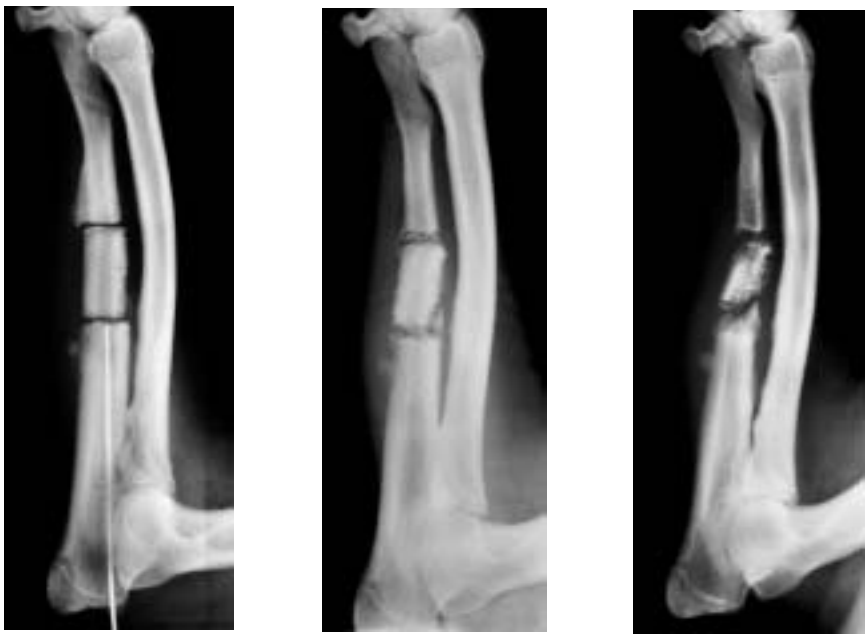
Histologically, fibrotic tissue was found in the implant area in all of the cases treated with xenografts without BMP. There was more remodelling of bone in the cases treated with xenografts with BMP. However, there was also fibrosis between the bone ends in all cases.

5.2.3 Coral implants and coral composite implants

In canine ulnar defects implanted with coral, the bone union caused by the coral+BMP composite graft was better than that caused by coral only, but not as comprehensive as that attained with autografts (Fig. 5). At the end of the study, there was one case implanted with plain coral and fixed with a Kirschner wire with a nearly complete bridge of new bone at the implant site and three cases with no signs of union or bone formation. No signs of union were seen in two cases implanted with plain coral and fixed with a plate. In the coral+BMP group fixed with a Kirschner wire, three cases had nearly complete union. Two of the three ulnae in the coral+BMP group fixed with a plate showed acceptable bone union and marked bone formation, but the third case with a broken plate was without any sign of union.

Statistically, at 16 weeks the combined score for bone union and bone formation in the coral and coral+BMP groups fixed with a Kirshner wire were significantly lower than in the autograft group ($p = 0.002$ and $p = 0.026$, respectively). However, when the groups with plate fixation (coral-P, coral+BMP-P) were also included in the analysis, only plain coral differed significantly from autografts ($p = 0.001$). A significant difference between the coral implants with and without BMP was also found at 16 weeks, the score being better for the BMP group ($p < 0.05$) (Table 3).

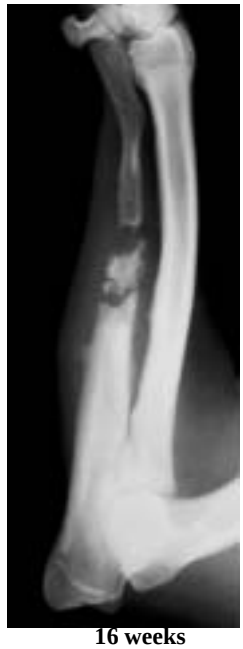
The callus area was significantly larger in the coral+BMP group compared to plain coral at 3 weeks ($p=0.02$), but the difference disappeared after that. The amount of callus was reduced in BMP-treated bones after 3 weeks, while in autografts, for example, the amount of callus continued to increase up to 16 weeks (Fig. 6).



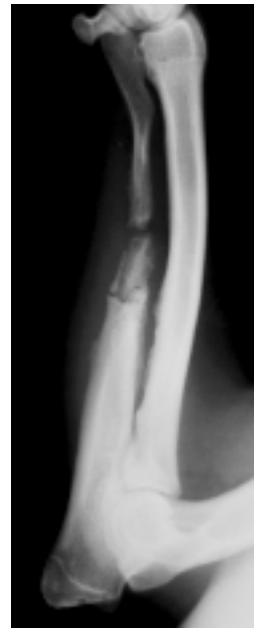
3 weeks

6 weeks

12 weeks



16 weeks



36 weeks

Fig. 5. A series of radiographs showing an ulnar defect treated with coral-BMP composite implant. The implant was resorbed gradually and replaced by bone. The union was not complete despite the good new bone formation. The Kirschner wire came out in this case and was removed after 3 weeks.

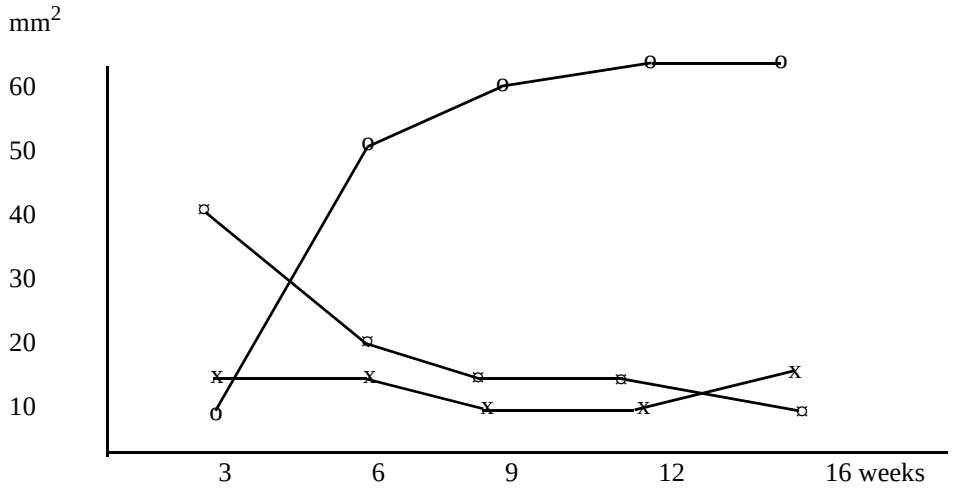


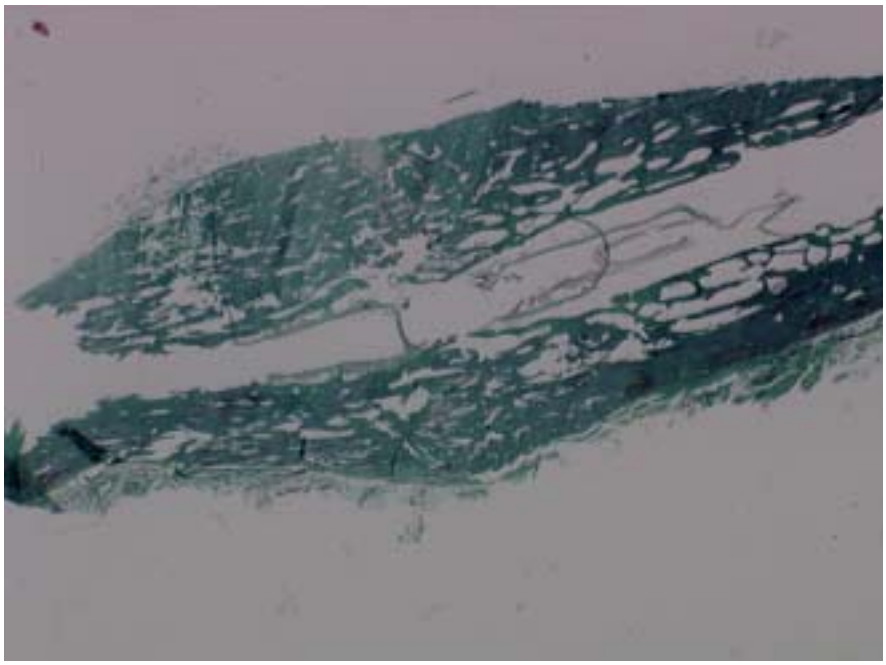
Fig. 6. Area of callus measured in digitized roentgenograms of the autograft, coral and coral composite implants (o = autograft, x = coral implant, ¤ = coral+BMP composite implant).

The mean score of resorption was 1.2 in the plain coral group with Kirschner wire fixation, where only one implant resorbed completely and two showed no resorption at all. Resorption of the implant was significantly different ($p = 0.026$) in the coral+BMP group compared to the plain coral group with Kirschner wire fixation. Five of the six implants were completely resorbed and one almost completely resorbed (mean score 2.8). Resorption was faster in the coral+BMP group, being nearly complete in 12 weeks, compared to 16–28 weeks in the plain coral group. In plate fixation, all the three implants in the coral+BMP group were totally resorbed within 9–12 weeks (score 3.0), and in the plain coral group, two of the three implants were totally resorbed and one almost resorbed (mean score 2.7), the resorption time being here 12–16 weeks.

All the bones that were manually stable were tested mechanically. Thus, all bones with plain coral and Kirschner wire fixation and two bones with coral+BMP and Kirshner wire fixation were left out. All bones with coral implants with or without BMP broke in the implant area in torsional testing. The Mann-Whitney test resulted in a significant difference in the mechanical strength between coral implants with and without BMP ($p = 0.04$). The mechanical strength of the coral implants, even with BMP, was significantly lower than the strength of autografts ($p < 0.01$).

The bones that showed non-union in radiograms were also seen to have a fibrous non-union histologically. In the coral+BMP group with Kirschner wire fixation, there was newly formed bone at the sites where the resorbed implant had been, and bone bridged the defect in 3 cases. In the coral+BMP group with plate fixation, one case showed a broad zone of fibrosis between the bone ends, while two cases also showed bone union histologically (Fig. 7).

A



B

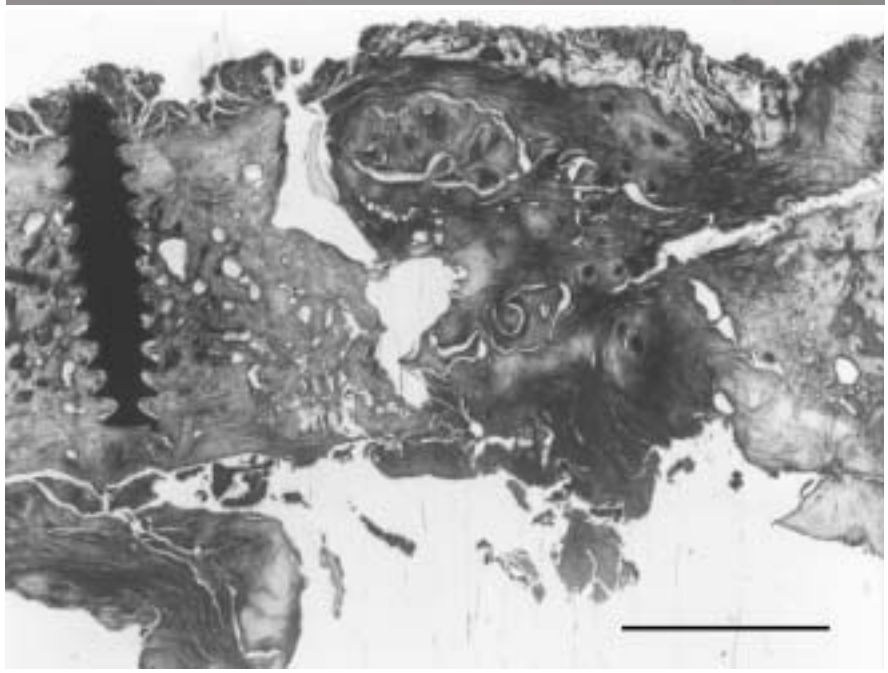


Fig. 7. A. A histological section of a coral implant. Some remnants of the coral material can be seen with a small amount of new bone in the middle. B. A histological section of a coral+BMP composite implant showing more bone formation in the implant area, which was here fixed with screws and plate (Original magnification x4).

Table 3. Summary of scores for bone union and bone formation.

	BU	BF	BU+BF
Autograft	5.5	3.7	9.2
Xenograft	0.0	0.5	0.5
Xenograft+BMP	0.0	1.0	1.0
HA	0.7	0.8	1.5
HA+BMP	1.4	1.0	2.4
Coral	1.5	1.3	2.8
Coral+BMP	3.6	2.2	5.8

5.2.4 Hydroxyapatite implants and hydroxyapatite composite implants

The implantation of canine ulnar defects with hydroxyapatite (HA) showed no completely united cases in the plain HA group (Fig. 8). However, there was some bridging between the implant and the bone at the proximal end of the implant in 2/6 of the cases and at the distal end in 4/6 cases.

In the HA-BMP group, 3/5 of the cases showed some bridging at the proximal end and 4/5 at the distal end. The other cases showed non-union. Even in the cases with bone bridging at both ends, bone formation was scarce and the new bone did not extend over the whole implant area. Bone formation and bone union resulted in a better score for HA+BMP than for HA, but the difference was not statistically significant ($p=0.429$).

The score for bone union and bone formation in the autograft group was significantly better than that in either the HA group ($p=0.002$) or the HA+BMP group ($p=0.004$) (Table 3).

The HA implants did not resorb during the follow-up period. There was only slight fragmentation in some implants, but the shape and density of the implant were generally well preserved.

The callus area was slightly larger in the BMP group after three weeks compared to plain hydroxyapatite, and the difference disappeared later, as the amount of callus clearly decreased in the BMP group.

All the bones that were manually stable were tested. All the bones with HA or HA+BMP implants broke at the implant-bone border, while the fracture line in the bones with autograft implants occurred outside the implant area.

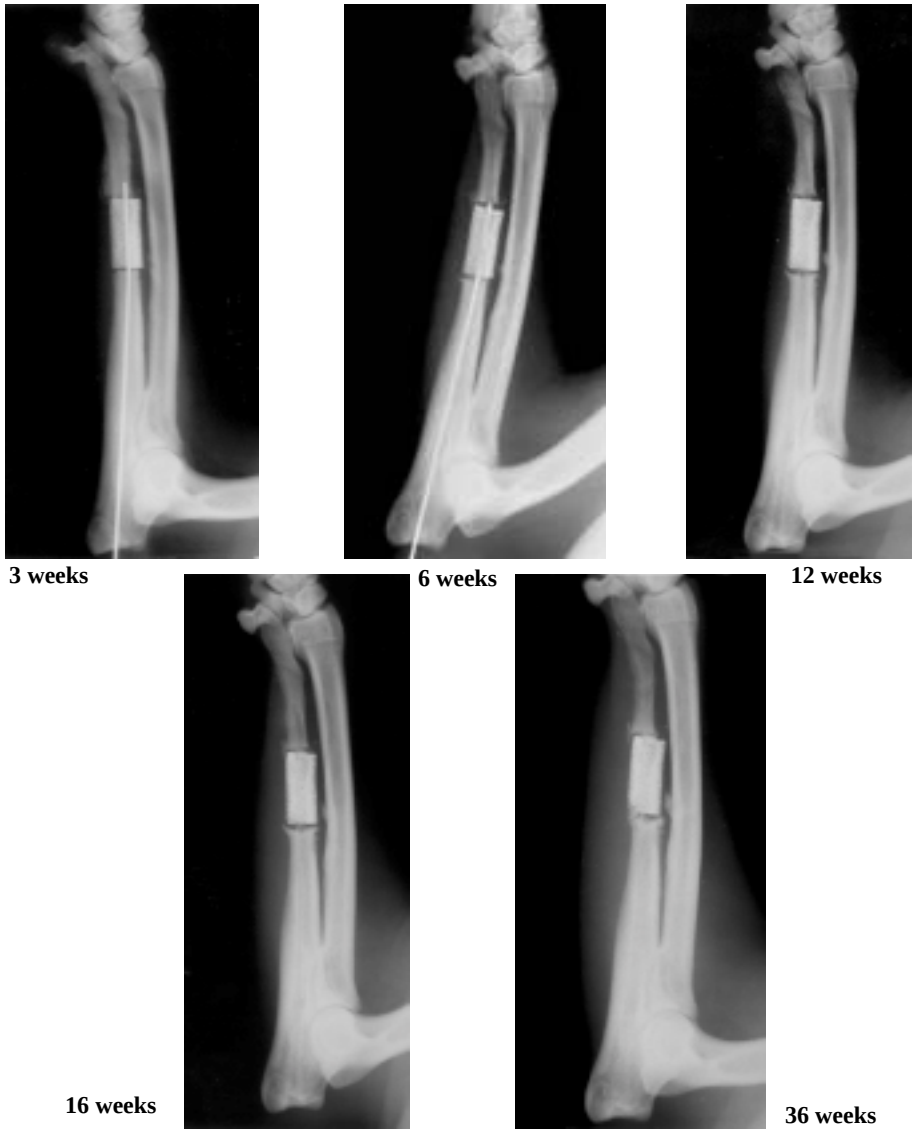


Fig. 8. A series of radiographs of an ulnar defect treated with a hydroxyapatite-BMP composite implant. The implant did not resorb and there is non-union. There is some callus formation, which is not, however, in contact with the hydroxyapatite implant.

In the HA group, only two bones were available for mechanical testing, while the others were manually unstable. In the HA+BMP group, 4 bones were available for mechanical testing. The Mann-Whitney test resulted in a non-significant difference between the HA and HA+BMP implants ($p=0.126$). Autograft bones were mechanically more stable than those in either the HA ($p=0.002$) or the HA+BMP group ($p=0.004$).

The bones that showed non-union and a gap between the bone end and the implant radiologically showed fibrous non-union histologically (Fig. 9). Generally, there was a fibrous capsule between the HA and HA+BMP implants and the bone. There was some bone bridging at the ends of the implant in some cases, but there were no cases with bone bridging over the whole implant area. A close contact between the bone and the implant and ingrowth into the implant could be seen in two cases in the HA+BMP group.

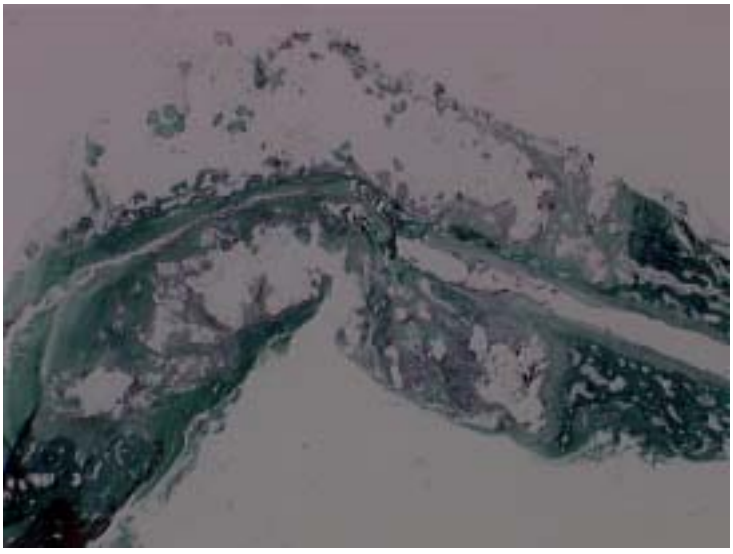


Fig. 9. A histological section of a hydroxyapatite implant showing unresorbable material and some callus, but there is clearly non-union. (Original magnification x4).

5.3 Coral composite implant in the treatment of ulnar pseudoarthrosis (A clinical case)

In the clinical ulnar fracture case, X-rays were obtained after the accident, postoperatively, and 4 months after the operation, when non-union was evident. The ulna was operated again, and 3 years after the operations an x-ray was taken and there was obvious pseudoarthrosis after the screws and plates were removed. Our patient could manage with her hand and did not seek treatment until 10 years after the first operation. At operation, the pseudoarthrosis was revised and a coral+BMP composite implant was inserted into the defect with a bone graft. After that, bone union gradually took place. Complete union could only be seen in the roentgenograms one year after the operation.

6 Discussion

6.1 Methodological considerations

6.1.1 Animal models and fixation methods

Sheep tibial defect was used to test two different bone substitute materials, coral and tricalcium phosphate. Sheep is a large animal, which makes comparison to human bone defects relevant. Sheep tibia has been previously used by Gao *et al.* (1995) and Marcacci *et al.* (1999) in bone substitute material studies. Marcacci *et al.* reported good healing results using hydroxyapatite implants and external fixation (Marcacci *et al.* 1999). However, the experiences of Gao *et al.* (1995) showing that external fixation was not rigid enough to enhance bone healing led us to use plate fixation in this model. The 16 mm defect used in sheep tibia is not a critical-sized defect, which partly explains the good healing outcome.

Dog ulnar defect is a well-established model. Ulna is an easily accessible bone, and it is not essential for weight bearing, because the radius gives stability to the ulna. We used a 2.0 cm segmental defect of mid-ulna, which was about 2–2.5 times the diameter of the bone. This has been shown to be a critical-sized defect, which does not normally heal when left empty (Key 1934, Heiple *et al.* 1963, Nilsson *et al.* 1986). The dog ulnar defect model has been used in various studies with bone grafting, biomaterials or BMP (Nilsson *et al.* 1986, Johnson *et al.* 1989, Grundel *et al.* 1991, Schwarz *et al.* 1991, Delloye *et al.* 1992, Cook *et al.* 1994, Cook *et al.* 1998, Itoh *et al.* 1998).

In some studies, no fixation has been used to stabilize the ulna, and the healing has yet been good or satisfactory (Nilsson *et al.* 1986, Delloye *et al.* 1992, Cook *et al.* 1994b, Cook *et al.* 1998). If fixation has been used, it has been either intramedullary with Steinmann pins (Grundel *et al.* 1991) or plates and screws (Johnson *et al.* 1989, Schwarz *et al.* 1991, Itoh *et al.* 1998).

We used a modification of Grundel's method, an intramedullary Kirschner wire with a diameter of 1.2 mm and plating as an alternative, comparing the results obtained with different fixation methods. The problem with intramedullary pin fixation is that it is not rotationally stable, although it keeps the transplant in the right position axially. We also used

plates and screws, but the plate we used was obviously too weak and broke in the majority of cases after a few weeks. The bone union in this group was good in spite of the loss of fixation. It may have provided initial stability during the first few weeks, however, which is favourable for bone union.

It seems that intramedullary Kirschner wire fixation is not sufficient in this model. It is interesting that some authors have reported good results in canine ulnar defect healing with no fixation at all. The physical activity of the animals after the operation is probably different in different studies. Our dogs were allowed to be free in large runs, being able to run and jump, and they were active throughout the follow-up period.

6.1.2 Healing evaluated from radiographs

We used a-p and lateral radiographic views with standard settings in radiograms. X-rays were taken at standard intervals to evaluate the healing process, and bone union and new bone formation were estimated from each radiograph. In the forearm, the radiological problem with the ulna is the radius, which covers the ulna very effectively in a lateral projection, making the estimation in this projection difficult. The same problem is encountered with the sheep tibia, which is partly overshadowed by the fibula.

In the literature, there are many different scoring systems for bone formation and bone union in experimental bone defect studies (An & Friedman 1999). The aim is to get comparable figures for statistical analysis. The scoring systems vary from 3- to 7-point scales, where bone formation is estimated to fill 0% to 100% of the defect (Delloye 1992, Cook *et al.* 1994b, Boström *et al.* 1996, Kirker-Head *et al.* 1998). Some authors have proposed scoring systems with bone formation, bone union and bone remodelling (Grundel *et al.* 1991, Schwarz *et al.* 1991). Thus, the comparison of the radiological results may be difficult.

Since there is obviously no ideal method for scoring radiograms, we chose a method where both bone formation and bone union are scored. We used the scoring system proposed by Johnson *et al.* (1996b), where bone union and bone formation are separately scored, and the sum of these is the figure representative of radiological healing.

6.1.3 Mechanical testing

A modified torsional testing machine with a maximal loading capacity of 250 Nm was used for the torsion test. The constant angular speed was set at 6.5 degrees/second (Jämsä & Jalovaara 1996). All the bones that were manually stable were tested mechanically.

In the case of a weak union the bone was typically broken at the implant site, while a bone with strong union broke outside the implant area, indicating good bony healing over the implant area. There was large variation even within the same group of bones in mechanical strength, but this is also the case with normal animal bones (Gerhardt *et al.* 1993), and the phenomenon may be due to normal biological variation in the mechanical properties of bone between individuals.

6.1.4 Densitometry

Densitometry showed no significant differences between the xenograft and xenograft composite groups in the implant area or at the bone-implant border in bone mineral density, bone mineral content or cross-sectional area. This also indicates that the BMP added to xenograft bone had no significant effect on the healing of the defect.

6.1.5 Histology

Histology is perhaps the most powerful method of examining the healing of bone defects (An & Friedman 1999). Canine auto- and allografts, which were estimated to have a union radiologically, also showed histological bony union with newly formed bone between bone and transplant. In the sheep model, both biomaterials, i.e. coral and tricalcium phosphate, were well integrated with bone, since there was no interposed fibrous tissue between ingrown bone and the trabeculae of the material.

With other implants, the histological picture was more complicated. The group with coral-BMP composite implant in canine included some cases where the bone histologically bridged over the defect and there was not fibrosis between the bone and the implant. Plain coral did not induce bone formation and there was also fibrosis. With hydroxyapatite- and xenograft-BMP composites, there was no convincing bone formation, either, and hydroxyapatite often resulted in fibrosis between the implant and the bone, while xenograft mostly involved fibrosis at the defect site.

On the basis of histological evidence, coral seems to be the most adequate bone substitute and carrier material for BMP among the biomaterials studied here.

6.2 Bone grafts and implants

Autografts and allografts showed a well-established callus in the bones that achieved union by 16 weeks, and distal union occurred earlier than proximal. All the distal ends of the transplants were ultimately united in both groups. Although the proximal end of the defect displayed roentgenologically a gap in two cases of the autograft group, histological examination showed that the gap was filled with fibrocartilaginous callus and the bone broke outside the defect area. These facts are also in favour of autografting. These results are in good agreement with those of earlier studies (Enneking *et al.* 1975, Burchardt *et al.* 1978, Friedlander 1987, Goldberg & Stevenson 1987, Delloye *et al.* 1992).

Radiographically, defect healing was generally good in autografts and allografts.

In our study, autograft bone was superior to coral implants, even those used with BMP, when measured with x-rays.

Torsion testing showed some differences between autografts and allografts in favour of the former. Similar results have been obtained in most other studies (Goldberg & Stevenson 1987, Delloye *et al.* 1992). In our study, the difference was statistically significant

only for maximal absorbed energy. In torsion testing, the fracture of the bone that gained union took place distally, where the diameter of the bone is smallest. The fracture only occurred in the graft area in two allograft bones, indicating poor bone union.

Histologically, the difference between auto- and allografts was that the cases non-united radiologically were histologically filled with fibrocartilaginous callus in autografts and with fibrous callus in allografts. This suggests that autografts might have united if the observation period had been longer.

In sheep bone, the better mechanical performance in coral- than in TCP-implanted tibiae was attributed to the initial mechanical strength, the appropriate rate of biodegradation of the ceramic and the good osseointegration with the host bone. The degradation of coral substratum is generally thought to be longer than that of TCP *in vivo* (Chiroff *et al.* 1975), which allows the maintenance of stability during the healing period. A persisting mixture of bone-coral structure reinforced the osteotomized legs. The fracture line passing through the implant-bone interface in 1/5 coral versus 3/6 TCP cylinders provides further evidence of intimate osteointegration between the coral substratum and new bone. The principle of this osteointegration was considered to stem mainly from mechanical interlocking through microanchoring (Kotani *et al.* 1991, Neo *et al.* 1992).

Histologically, the sheep bone healing was good with both implant materials and the three-dimensional structure of pores and interconnecting fenestrations in coral might be more favourable for new bone ingrowth (Roux *et al.* 1988). More advanced remodelling bone and Haversian units were histologically evident in coral as compared to TCP-implanted defects. Since bone is a unique tissue proliferating and remodelling according to its own overall biological and biomechanical dictates, newly formed bone would be unlikely to travel those unnatural pathways in sintering TCP.

Natural coral is a resorbable bone substitute. Coral resorbed quickly, unlike hydroxyapatite, and bone ingrowth seemed to maintain the strength of the coral implant even when it began to dissolve (Vuola *et al.* 1998). In this respect, coral seems to be an ideal bone substitute as it resorbs and enhances bone to grow at the defect site. It also makes the assessment of bone union easier, since the coral material resorbed and was gradually replaced by bone.

The hydroxyapatite implant practically failed to resorb at all. Only very slight fragmentation was seen in some implants after 36 weeks' follow-up. This result is similar to the findings reported earlier (Holmes *et al.* 1986, Bucholz *et al.* 1989, Johnson *et al.* 1996b). In fact, Johnson *et al.* (1996b) did not consider hydroxyapatite an ideal graft material, since it does not resorb, thus making the assessment of bone union difficult, as it is radiodense material.

Xenograft bone could not induce any significant bone formation in the defect, probably due to the immunological reactions it causes in the host.

6.3 Effect of BMP

It has been suggested that BMP might enhance bone formation in combination with xenogeneic bone material. In an earlier study, the healing of critical-sized cranial defects in non-human primates was evaluated using autografts and xenogeneic human antigen-

extracted, autolyzed bone impregnated with bovine BMP. The autografts resulted in the greatest volume of new bone formation, but the antigen-extracted, autolyzed bone elicited a significantly greater response than either the bovine BMP derivatives or the controls (Hollinger *et al.* 1990). Minamide *et al.* (1999) studied sintered xenogeneic bone with type I collagen and recombinant human BMP (rhBMP-2) in a rabbit spinal fusion model, and the results showed that xenogeneic bone with rhBMP-2 resulted in a higher fusion rate than autograft. In our study, we used a xenogeneic demineralized bovine bone either alone or with bovine-derived BMP and found that added BMP seemed to increase bone formation, but failed to lead to complete bony union. The immunological reaction with xenograft bone processed the way we did possibly exceeds the effect of BMP.

The resorption of the xenograft implants was very rapid when evaluated roentgenologically. The resorption of the graft might have been too fast to enhance proper ossification even with BMP. This is in line with some earlier findings, where the suitability of xenograft has been considered questionable because of the fast resorption (Burchardt 1983).

The best results obtained in segmental bone defects with coral implant combined with BMP have been consistently better than the golden standard of autogenous cancellous bone graft in terms of the extent of bone formation and the strength of the healed defect. However, our results demonstrated the efficacy of BMP in bone induction, and there was a significant difference in mechanical strength between coral implants with and without BMP ($p=0.04$), although autograft bone was superior to coral implants, even when used with BMP. Roentgenologically, coral with BMP showed better results than plain coral both in union and new bone formation.

As to hydroxyapatite, there are, to our knowledge, no studies where a HA+BMP implant would have been used in demanding bone defects in the weight-bearing bones of larger animals. In this study, the added BMP had only a slight, non-significant positive effect on the HA implant in bone, bone union and mechanical stability. Our sample was small, which may have contributed to the poor statistical strength of the results. Therefore, it is possible that the tendency of a positive effect of BMP on the HA implant might have been more pronounced with a larger number of experimental animals.

Within the hydroxyapatite and bovine bone implant groups, there was some difference between the non-BMP and BMP groups in favour of the latter, but these differences were not statistically significant. Thus, based on mechanical strength, coral was the best carrier material for BMP in this study.

Histologically, the coral implants with Kirschner wire fixation in half of the cases (3/6) resulted in new bone bridging the site of the resorbed implant, while in the other cases there was fibrous tissue and no union histologically. BMP seemed to have an accelerating effect on the resorption of the coral implant. The bovine bone implant failed to unite the bone ends, and histologically there was fibrosis between the bone ends in all cases with or without BMP. There were some remnants of the xenograft and some new bone at the bone ends in the cases with plain xenograft and more implant resorption and more remodellation of bone in those treated with xenograft+BMP.

In a comparison of the results of all the composite implants, coral+BMP composite implant had the best results in the x-ray scoring compared to the hydroxyapatite+BMP and xenograft+BMP composite implants. Although the histological results were not quantified, the coral+BMP composite also resulted in the best bone healing histologically. But

in mechanical testing, surprisingly, the xenograft composite implant had the highest values of MTC. This is probably due to soft tissue growth between the bone ends, which exceeds the strength of weak bony union. It was histologically shown to be filled with fibrous tissue.

On the basis of these experiments, it seems that BMP might have an effect on implant resorption, at least in coral and bovine bone. In these small study groups, BMP accelerated the resorption of both of these implants. It did not, however, have an effect on the resorption of hydroxyapatite, which resorbed very little, if at all. Our study groups were too small to warrant any definitive conclusions in this respect. Whether the implant resorption is an BMP effect remains to be shown in the future studies.

Another matter lacking evidence is the anti-BMP effect. BMP is a protein, and when it is implanted, potential antibody production by the host against it is expected. Some studies have shown either direct (in the form of anti-BMP measurements) or indirect evidence of such an anti-BMP effect (Heckman *et al.* 1991, Gao *et al.* 1996b). Recombinant BMP is considered immunoalert, although solid evidence is lacking (Gao *et al*. 1996c).

In our experiment, the callus area measured from a digitized roentgenogram showed that, both in coral and hydroxyapatite implants with BMP, the callus area was large compared to the implants without BMP and also to autograft at three weeks. After three weeks, however, the area of callus was clearly decreased in the BMP implants and there were no differences during the weeks 6–16. The reason for this remains unclear. One possible explanation could be anti-BMP, which is produced by the host during the first few weeks and which might interfere with the BMP effect. It is likely that single-set implantation of xenogeneic BMP evokes a high concentration of anti-BMP antibody, which apparently inhibits the osteoiductive capacity of BMP or even destroys newly induced bone (Gao *et al*. 1997). The immunogenicity of BMP is still very inadequately known, and more studies are needed to elucidate this matter.

6.4 Future prospects of clinical use of BMP

It seems that although there are some promising results with BMP in clinical use, certain problems still persist. One of the problems is the optimal delivery system for BMP, which remains to be found. Also, there has been variation in the results, as some patients have not responded at all to BMP implants (Geesink *et al.* 1999, Groeneveld 1999). These results suggest that certain factors, which are currently unknown, negatively affect the BMP-dependent bone induction process in humans (Groeneweld & Burger 2000). The dosage of BMP has been under discussion, especially with regard to recombinant BMPs, as the costs of the required milligram doses might be a limiting factor in clinical use, as well as unexpected biological sequelae (Schmitt *et al.* 1999). Many questions remain to be answered before BMPs can be introduced into wider clinical use.

7 Conclusions

1. The canine ulnar defect model seems to be suitable for bone healing studies and studies evaluating the effect of BMP, but the intramedullary fixation method seems to be suboptimal. It seemed to relieve the pain of the animals, but the lack of rotational stability and the pin breakages were uneventful outcomes.
2. Autograft is better than allograft in healing bone in segmental ulnar bone defects, and they both resulted in acceptable healing of the defect. Xenograft seems to be clearly inferior to both auto- and allograft as a bone graft.
3. Coral was superior to both hydroxyapatite and tricalcium phosphate as a bone substitute material in bone defects.
4. Bovine native BMP had a positive effect on bone healing in canine ulnar defects, but it was unable to heal the defect completely with any of the carriers.
5. Bovine native BMP enhanced the efficacy of coral and hydroxyapatite as bone substitute materials. The composite implant of coral and BMP seemed to be best bone substitute in this study. It worked very well even in a clinical case of intractable ulnar non-union. The resorption of coral material seems to be suitable to enhance new bone formation and bone healing.

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