International Orthopaedics (SICOT) (1997) 21: 194–200

International
Orthopaedics

The use of a coral composite implant containing bone morphogenetic protein to repair a segmental tibial defect in sheep

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Accepted: 2 January 1997

Summary. *A composite implant consisting of a coral cylinder, moose bone morphogenetic protein and type IV collagen was used to repair a segmental tibial defect in sheep. Healing, related variance in mechanical strength and immune responses were evaluated. In comparison with a coral control, a larger amount of newly formed external callus was observed in the composite group at 6 weeks. The maximal torque capacity, maximal angular deformation at failure and bone stiffness of a healed osteotomised tibia recovered 113%, 117% and 120% in the coral controls and 67%, 92% and 79% in the composite implants against the corresponding contralateral tibia at 16 weeks respectively. A significantly elevated anti-BMP antibody was detectable in the composite group at 3 and 6 weeks. Augmented bone formation at an early stage and weakened torsional performance at a later stage in the composite implants may indicate the phase-specific osteoinduction and the immune response of xenogenic BMP with time.*

Résumé. *Nous avons étudié l'action d'un composite comprenant un cylindre de corail, une protéine morphogénétique xénogénique extraite de l'os de souris et du collagène de type IV, sur la consolidation la résistance mécanique et la réponse immune d'une perte de substance diaphysaire. L'étude au scanner à 6 semaines a montré une réduction significative du cal dans le groupe composite. A 16 semaines, la résistance à la torsion, la déformation angulaire maximale et la solidité des cals consolidés ont été respectivement de 113%, 117% et 120% dans le groupe controle, et de 67%, 92% et 79% dans le groupe composite, comparées au tibiai contralatéral. Dans le groupe composite, il y a eu une élévation significative des anticorps anti-mBMP. L'augmentation de la formation osseuse au stade précoce et la diminuation des propriétés mécaniques au stade tardif dans le groupe composite, pourraient être l'expression d'une induction séquentielle de l'ostéogenèse et d'une initiation immune des antigènes mBMP xénogéniques après une greffe hétérotopique.*

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Introduction

Coral has proved to be a useful bone substitute in many clinical conditions [11, 17, 18, 23], but there is no conclusive evidence of osteogenicity in these implants. Bone morphogenetic proteins (BMPs) are a family of glycoproteins in bone matrix which play an important role in osteogenesis in embryonic and postnatal life [20]. Extracted human allogenic or recombinant BMPs have been used successfully to repair large bony defects in humans [8, 9, 10], monkeys [2] and sheep [5]. The limited sources and the immunogenicity of natural BMPs are practical disadvantages in their use. With xenogenic bovine BMP (bBMP), an apparently elevated anti-bBMP antibody and involved immune inhibition of the healing of defects in dog's skulls were only found after a second set implantation

Fig. 1. Coral cylinder used as a skeleton of the composite implant.

[16]. We extracted partially purified moose BMP (mBMP) and attempted to confirm Nilsson's observation [16] by a single implantation of a large dose of the xenogenic BMP in a diaphyseal defect.

We used a composite implant of a coral cylinder, partly purified mBMP and type IV collagen and investigated the improvement achieved with this triple bone substitute in the healing of segmental tibial defects in sheep. The principle objectives were to determine the effects of xenogenic BMP-absorbed composite implant and the related immune response on healing and mechanical performance.

Material and methods

Preparation of the implants.

The dimensions of the processed natural coral cylinder (Biocoral^{R}, Inoteb, Saint-Gonnery, France) were a diameter of 15 mm and a length of 16 mm with a plug 3 mm long at each end. A central hole, 4 mm in diameter, was predrilled longitudinally to produce a medullary canal (Fig. 1). Type IV collagen from human placenta (Sigma Chemical Co, St Louis, Mo, USA) was dissolved in 0.25 mol/l hydrochloride containing 0.5 mol/l NaCl, dialysed and centrifuged to remove inpurities. BMP was extracted chemically from the fresh tibia and femur of premature wild moose (Alces alces) by 4 mol/l guanidine hydrochloride (GuHCl), partly purified through a 0.65 μm filter by a tangential flow system and refiltered with a cut-off point of 10 kDa (Minitan Millipore, Maryland, USA) in our laboratory. High performance liquid chromatography showed that partly purified mBMP was the associated protein with 3 fractions (Fig. 2). Lyophilised mBMP and purified type IV collagen were dissolved in 4 mol/l GuHCl and incubated overnight at 20 °C. An aliquot of the mixture was put into individual dialysis tubes with a cut-off point of 10–12 kDa. The coral cylinder was immersed in the mixture in the tubes which were then dialysed together against 10 volumes of 10 mmol/l glycine buffer for 24 hours. The dialysed mixture was centrifuged after the cylinder was transferred into a plastic shape-fit mould. The precipitate containing the remnant of mBMP in the vials was coated on the surface of the corresponding cylinder in the mould. After lyophilisation, each prepared implant contained 100 mg mBMP and 20 mg type IV collagen. Coral cylinders impregnated with 20 mg type IV collagen were prepared as controls. All implants were exposed once to ethylene oxide for 4 hours and then evaporated for 8 hours before implantation.

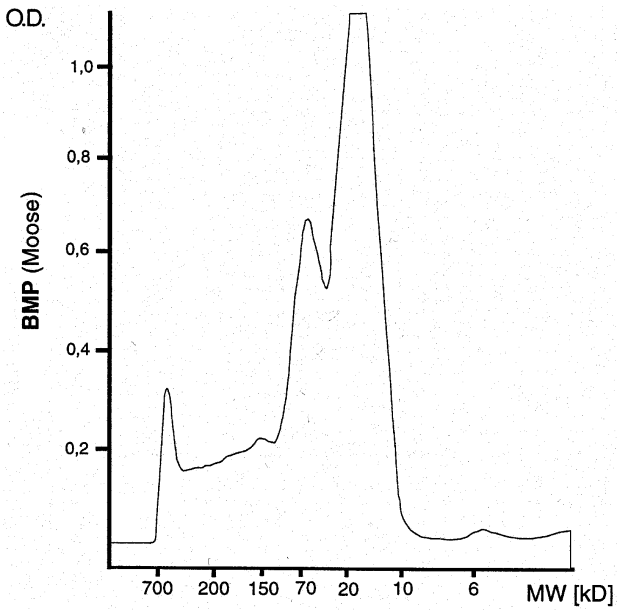

Fig. 2. HPLC chromatographic spectrum of partly purified mBMP in a Sepharyl 200 HPLC column 300 mm long (Pharmacia Diagnostics, Sweden). Eluted fractions were defined with the standard proteins in the absorbency of 280 nm.

Surgical procedure.

A segmental defect was made in the tibia of 11 mature sheep, 7 male and 4 female, with average body weight of 50.6 ± 16.4 kg. The study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Helsinki, and the animals cared for in accordance with the Principles of Laboratory Care (NH publication no: 85-23, revised 1985).

Under general anaesthesia, the middle of the diaphysis of the right tibia was exposed and a defect, 16 mm long, was created subperiosteally with a Gigli saw. The defects were replaced with 6 composite implants (4 in males and 2 in females) and 5 coral controls (3 in males and 2 in females). The tibia was stabilised by 2 overlapping contoured autocompression plates, 4 mm thick, with 8 and 6 holes respectively and with cortical screws on the medial side of the tibia. A single dose of benzylpenicillin, 66,000 IU, was given intravenously one hour before the procedure, and procaine penicillin, 36,000 IU, was given intramuscularly each day for 4 days. The animals were allowed to walk without restriction after the operation. Blood samples were taken from the jugular vein on the day of operation and 3, 6 and 12 weeks afterwards.

Image quantification.

Radiographs were taken at 3, 6, 12 and 16 weeks, and those from 3 to 12 weeks were scanned by a computerised scanner of the Bio-Image System (6 XRS, Millipore Corp, USA). Ana-

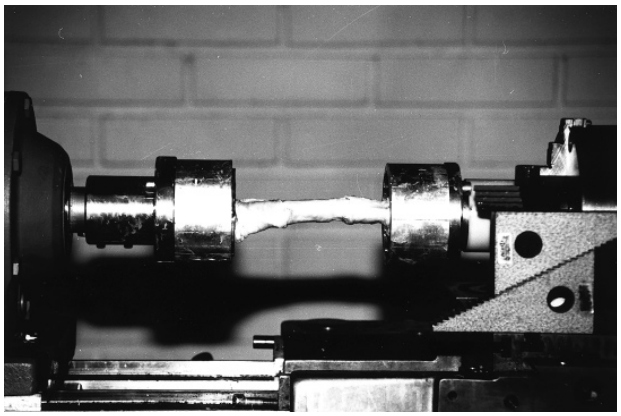

Fig. 3. The mechanical device made for testing torsion.

lysis by 2D system software in a Sunspark Station was carried out to follow quantitative changes in the area and the integrated callus formation. The sum of the area and the integrated intensity bounded on the images of each sample was standardised to one quarter of the total area and integrated intensity of the callus. The unit was defined in mm^2 for the area and optical density for integrated intensity. The tibiae were CT scanned in vitro in consecutive axial sections at 3 mm intervals to quantify the averaged cross-sectional area and density of the callus which was expressed in Hounsfield units [21].

Mechanical testing.

The removed tibiae from both sides were kept moist with physiological saline, sealed in plastic bags and frozen at – 20 °C until tested. This was performed by a torsion test with a constant angular speed 6.5 °/sec and a maximal load of 250 Nm. Total machine errors were below 1% (Fig. 3). The torsion load curves were registered by a plotter.

After being thawed at room temperature, the tibial bone ends were trimmed and embedded in asymmetrical circular aluminium moulds using polyester resin. The orientation of the mould and centring of the specimen were maintained with a specially designed support. The torsion arm was defined as 135 mm. The specimens were saturated with cool saline during preparation and testing. The test was carried out by loading at the defined angular velocity until the tibia failed. The contralateral tibia, in which corresponding holes had been drilled, was used as a paired reference. The percentage of maximal torque capacity (MTC), maximal angle of deformation (MA) and bone stiffness (BS) in the osteotomised compared with the contralateral tibia were calculated. The standard errors, according to a pilot test with intact sheep tibiae, were calculated as: MTC 4.7%; MA 8.4% and BS 8.6%. The fracture lines were recorded and tibiae with composite implant compared with the coral control.

Histology.

Some specimens where the fracture did not pass through the bone-implant interface were sawn transversely into slices 0.4–2.0 mm thick with a diamond saw (Acutome 5, Struers Tech A/S, Copenhagen, Denmark), fixed in 10% neutral formalin and embedded in methylmethacrylate. Undemineralised sections 12–20 μm thick were made by a cutting and grinding method (Exakt-Apparatebau, Hamburg, Germany) and stained with van Gieson for light microscopy.

Anti-BMP assay.

An indirect enzyme-linked immunosorbent assay (ELISA) was performed to determine whether antibody against xenogenic BMP had developed in the sheep. A microtitre plate with 96 wells was coated with purified bovine BMP (0.25 μg/well) diluted in 0.05 mol/l bicarbonate/carbonate buffer at pH 9.6. Uncoated sites were blocked with 1% BSA in PBS. The plate was incubated with sheep serum diluted 1:200 at 4 °C overnight. Donkey anti-sheep immunoglobulin G conjugated with alkaline phosphatase (Sigma Chemical Co, St Louis, Mo, USA) was added to the wells as a second antibody and incubated at 37 °C for 3 hours. A colour reaction was accomplished with the phosphatase substrate. Serum obtained before operation from every sheep was used as a negative control. Optical density was read at 402 nm. The titre of anti-BMP antibody matched with the value of optical density.

Statistical analysis.

Unpaired Student's t-test was used for analysis of quantitative data on the mechanical tests, CAT scans and image analysis. Significance was considered at $p < 0.01$.

Results

There was no infection and all samples were available for analysis 16 weeks after implantation.

Image quantification.

Visible difference in external callus between the composite and coral control groups was demonstrated in radiographs 3 weeks after operation. New bridging callus was heaped up around the composite implant, but was distributed longitudinally in the coral control (Fig. 4). Computer image analysis showed that a significantly increased area and integrated density of bridging callus was present in the composite group at 6 weeks, but not at 3 and 12 weeks, compared to the coral controls (Fig. 5).

Good osteointegration between new callus and the implants was seen in the CAT scan in both groups at 16 weeks. There was no demarcation apparent in the implants and bridging callus. No significant discrepancy in average cross-sectional area and density of callus was found between the coral control and composite group (Table 1). Resorption of the coral substrate was seen in radiographs and tomographs at the circumference and along the inner wall of the central hole of the cylinder after 6 weeks.

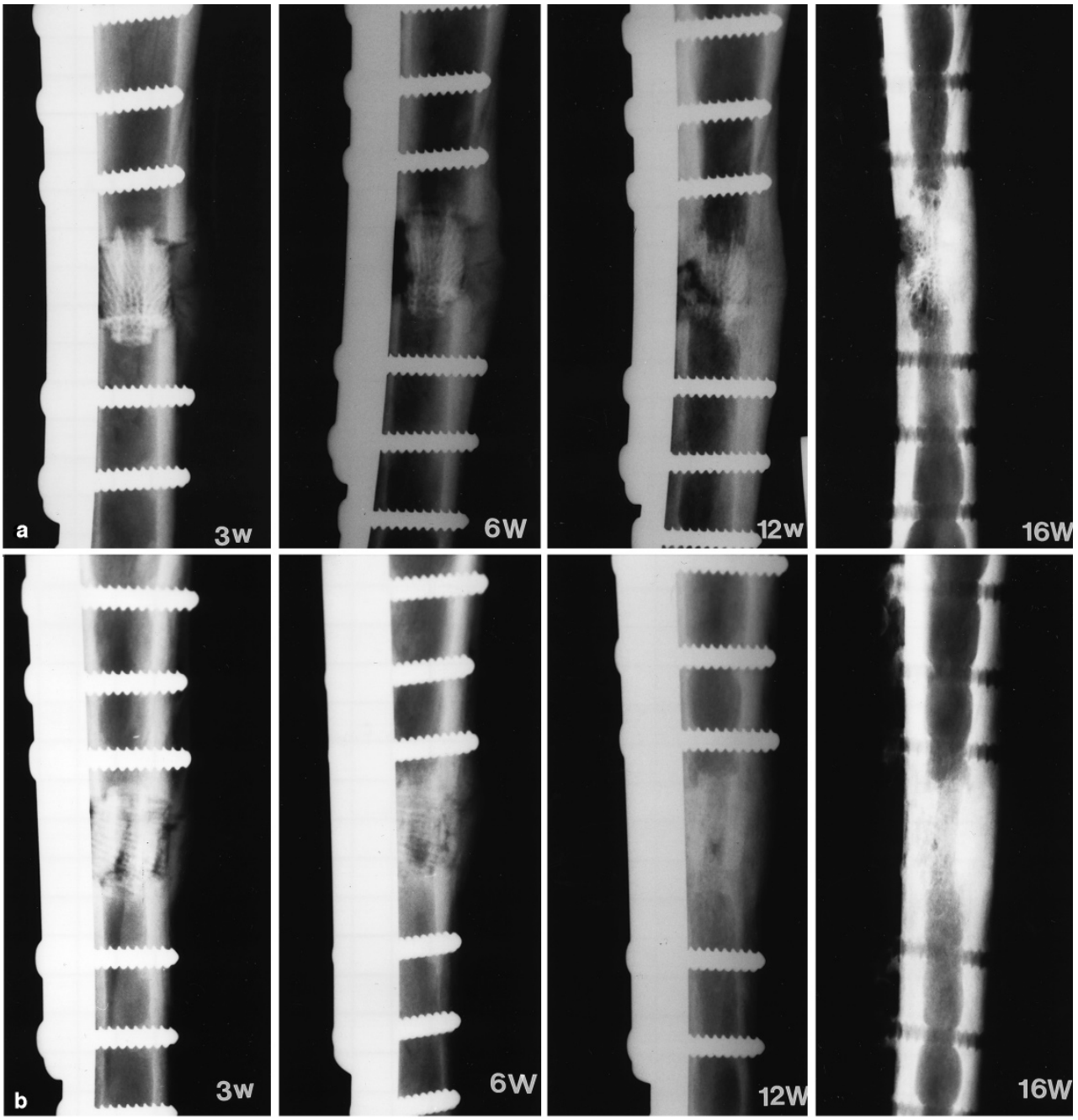

Fig. 4. a Anteroposterior radiograph of the composite and **b** coral control group at 3 (3w), 6 (6w), 12 (12w) and 16 (16w) weeks after implantation. Compared to the control, there is an increase in the extent of callus at 3 and 6 weeks.

Mechanical properties.

The data on the torsion test are summarised in Table 2. The maximal torque capacity, maximal angular deformation at failure and the bone stiffness of the healed tibiae recovered 113%, 117% and 120% in the coral controls and 67%, 92% and 79% in the composite implants compared with the corresponding contralateral tibiae at 16 weeks respectively. Increased mechanical factors were obtained in the controls compared to the composite group. There was, however, no statistically significant difference in the mean values observed. All the specimens failed with a consistent pattern of spiral fracture consistent with the torsion force. The fractures were through the implant-bone interface in 1 out of 5 controls and 1 out of 6 composite implants.

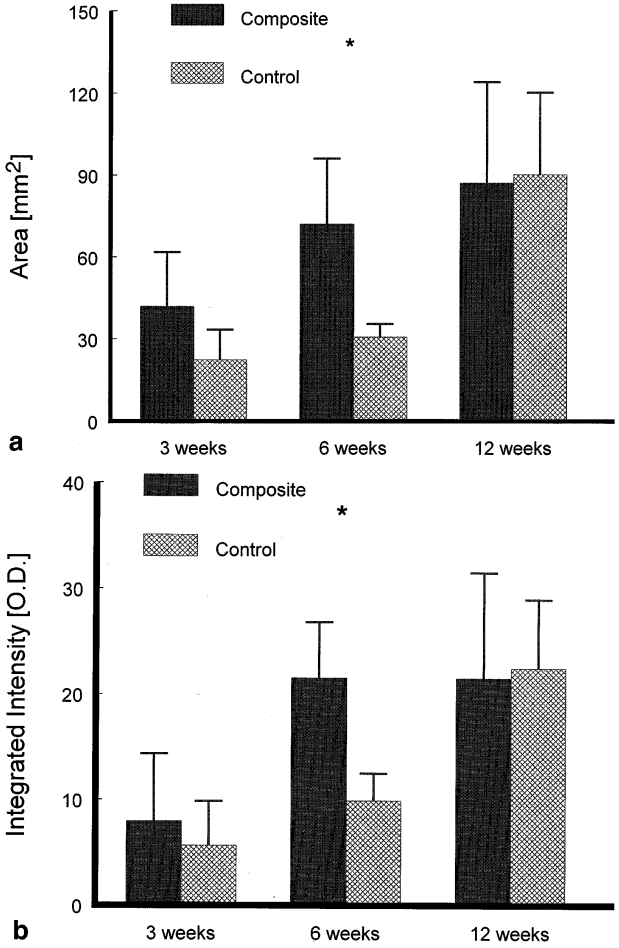

Fig. 5. Histograms showing variation in area in **a** and integrated intensity **b** of bridging callus in the composite and coral control group at 3, 6 and 12 weeks after implantation. There is a significant difference between the 2 groups at 6 weeks.

Table 1. The average cross-sectional area and density of callus on the CAT scanning film at 16 weeks after implantation

	Area (mm^2)	Density (HU)
Composite implant	221 ± 30	1318.24 ± 90
Coral control	311 ± 70	1422.47 ± 134
p-value <	0.04	0.18

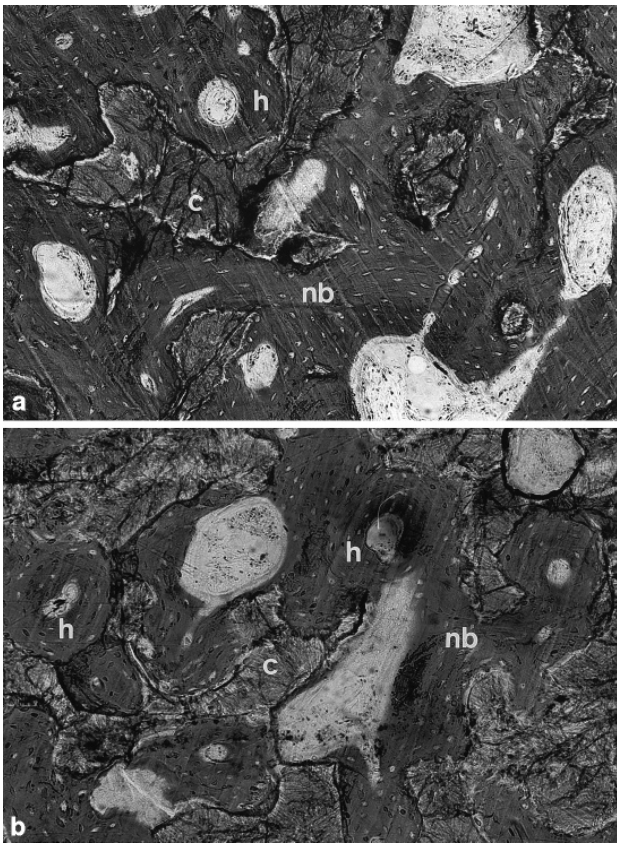

Fig. 6. Photomicrographs showing close osteointegration between the coral substrata (**c**) and new bone (**nb**) in both composite implant and coral control at 16 weeks. Compared with the composite implant **a**, more Haversian units (**h**) were seen in the coral control **b**. (Original ×10, undemineralised).

Histology.

New bone formed through the implant along the inner core and interconnected channels in the composite and coral control groups. The well modelled lamellar bone was structurally normal with no interposed fibrous tissue on the interface between new bone and the coral. There were more Haversian units and less resorption of the coral substrate in the coral controls than in the composite implants (Fig. 6).

Table 2. Mechanical test parameters in healed sheep tibial defects implanted with coral control and composite implant at 16 weeks

	Coral control		Composite implant		p value
	Implant side	% Contralateral	Implant side	% Contralateral	
MTC	40.4 ± 10.2	113 ± 14	26.5 ± 4.6	67 ± 17	< 0.04
MA	18.6 ± 3.8	117 ± 20	15.0 ± 4.2	92 ± 18	< 0.22
BS	2.8 ± 0.7	120 ± 33	2.3 ± 0.7	79 ± 25	< 0.27

MTC: maximal torque capacity (Nm), MA: maximal angle of deformation (degree), BS: bone stiffness (Nm/degree).

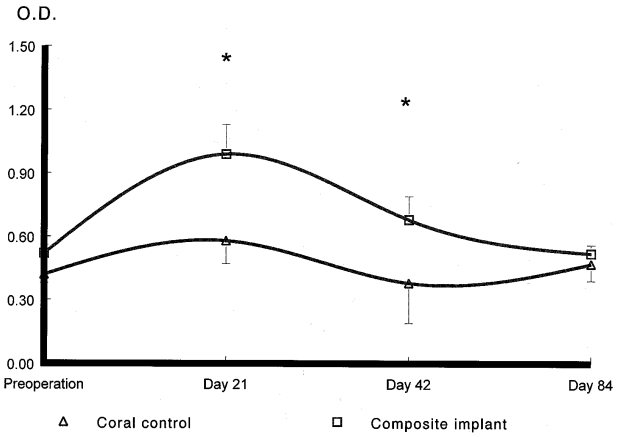

Fig. 7. The fluctuation of anti-BMP antibody in the circulation of the sheep with composite implants and coral controls with time, expressed as means ±SD. OD = optical density in 402 nm. * = statistical significance at $p < 0.01$.

Anti-BMP appearance.

The fluctuation of anti-BMP antibody is shown in Figure 7. A high titre of antibody was measured in the composite group, significantly higher than in the coral controls at 3 and 6 weeks, and returning to the control level at 12 weeks. There was no statistically significant increase in anti-BMP in the coral control group from 3 to 12 weeks compared with the negative control.

Discussion

Using a triple association of TGF-β 1, fibrin glue and coral, Arnaud et al showed that the growth factor was potentiated by coral [1]. Our previous study demonstrated the enhancement of type IV collagen on the osteoinductivity of bBMP [4]. We now tried to combine the mechanical integrity and availability for binding biological factors of coral with the potentiation of type IV collagen on BMPs in order to develop a new composite implant with osteogenic capacity. Our experimental results show that the area of callus was significantly larger and the integrated intensity of new bone significantly higher in the composite than in the coral control group at 6 weeks. This corroborated the observation that the cascade of bone formation induced by BMPs was initially from 7 to 28 days, depending on the animal species and implant sites [13]. In contrast to the coral controls, the dome-like bridging callus around composite implants further demonstrated a local osteoinduction of mBMP at an early stage [15, 22]. Abundant bone regeneration surrounding the composite implant resulted from differentiation and proliferation of inducible cells under the influence of mBMP.

Further knowledge of the immunogenic properties of BMPs has to be sought [19]. In Nilsson's experiments, the first set of implantation of bBMP induced 96% healing of skull defects in dogs, while regeneration of the second set of trephines induced by the same BMP was 34% less [16]. A raised anti-bBMP antibody was present in the circulation after the second set compared with the first. Xenogenic BMPs appeared to be osteoinductive as well as immunogenic. A significantly increased anti-mBMP antibody was measured in the sheep implanted with composite implants in keeping with Nilsson's findings. This demonstrated that, with a large dose of xenogenic BMPs, an immune response is evoked by a single set of implantation [14]. Phase-specific osteoinduction and immunogenesis of mBMP was also found in our previous study. The amount of induced ectopic bone, quantified by ^{45}Calcium incorporation, was significantly less at 20 days than at 10 days after implantation of mBMP in muscle pouches of BALB mice, coinciding with the infiltration of inflammatory cells. This implies that the expression of osteogenesis of mBMP is earlier than that of its immunogen, and that the evoked immune response partly blocked the osteoinductivity of mBMP [6, 12]. Compared to the tibiae with the coral controls, the decreased mechanical factors in those with the composite showed an adverse influence of the immune response on mechanical performance at a later stage in the present study. We assume that this phase-specific effect of mBMP can be attributed to its origin in a wild species and the associated protein aggregates.

In contrast to the composite group, the healed tibia was stronger than the contralateral tibia with drilled holes in the coral control group. The fracture passed through the implant-bone interface in only one of the 5 in the coral group and this was mechanical evidence of close osteointegration. A rigid mixed bone and coral structure persisted with time and strengthened the osteotomised tibia [7]. The dominant calcium carbonate in coral has been considered to be the critical component for the initiation of bone apposition, integration with host bone and biodegradation of the material [3].

The clinical relevance of BMP research concerns the repair of established nonunion of fractures and the filling of large bony defects. Healing of the segmental diaphyseal defects in this study showed that the implant composed of a coral cy-

linder, mBMP and type IV collagen has mechanical integrity, good osteoinductivity and osteogenicity. The implant is easy to handle and could be used for reconstructive surgery [23], although the immunogenicity of naturally occurring BMPs has to be overcome.

Acknowledgements. The authors wish to acknowledge the help of A. Marttinen PhD in the preparation of mBMP and the composite implants, the contribution of S. Hiltunen MD for his cooperation and help in CAT scanning, and the technical assistance of T. Jämsä, Lic. Eng. and Mr A. Nykänen in the torsion testing.

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