Bone induction by composite of bioerodible polyorthoester and demineralized bone matrix in rats

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A composite of a local, sustained, drug-release system, Alzamer® bioerodible polyorthoester, and demineralized bone-matrix (DBM) particles implanted in the abdominal muscle of 89 Wistar rats induced cartilage and bone formation at the same rate as DBM when evaluated histologically and by ^{85}Sr uptake. The composite implant was technically easier to use than DBM alone.

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Demineralized bone and dentin induce heterotopic osteogenesis, i.e., chemotaxis of mesenchymal cells, mitosis, differentiation of cartilage, vascular invasion, bone differentiation, and formation of an ossicle filled with bone marrow elements (Urist 1965, Bang and Urist 1967, Reddi and Huggins 1972). Experimentally, demineralized bone matrix (DBM) is usually used as chips or powder. In clinical practice, however, such transplants would be technically difficult to use in bone defects, as the particles may displace peroperatively and postoperatively. Incorporating the DBM in a bioerodible carrier thus seems warranted.

When purified bone inductors and growth factors become available, a delivery system for their sustained release will be needed (Sato and Urist 1985). Alzamer® is a bioerodible polyorthoester poly(2,2-dioxy-cis,trans-1, 4-cyclohexane dimethylene tetrahydrofuran) developed as the vehicle in a sustained drug-release system (Capozza et al. 1978).

The purpose of the present study was to evaluate this bioerodible polyorthoester as an osteoinductor delivery system.

Materials and methods

Design of experiment

The study included two series. In Series I, we evaluated histologically the host-tissue responses and the induction caused by the different implants at Weeks 1, 2, 3, 4, 6, and 8. In Series II, we quantified the induced bone formation by ^{85}Sr uptake in the implants at Week 4.

A total of 89 male Wistar rats were used, 44 in Series I and 45 in Series II. The mean body weights of the rats of Series I and II were respectively 357 (*SD 19*) g and 216 (*9*) g. The animals were fed a standard rat diet and water ad libitum.

The weight of donor and recipient rats and the sterilization procedure of the DBM in Series I were the same as in our earlier studies of heterotopic bone induction by different implants (Pinholt et al. 1990). In Series II, we used younger rats (Syftestad and Urist 1982) and no sterilization of the DBM (Munting et al. 1988, Aspenberg et al. 1990) to augment the inductive ability of the different implants. Further, we used only one type of implant in each rat to avoid the risk of local or systemic interactions of the different implants. We chose 4 weeks as an appropriate time of evaluation, because the results of Series I showed that ossicles were formed by this time.

Implants

Demineralized bone matrix (DBM) was prepared, using a sterile technique, from the femur, tibia, and fibula of male Wistar rats of the same weight as the recipients of the corresponding series. Dissected diaphyses were crushed and the marrow was removed. The cortical bone was cut into chips, demineralized in 0.2 N HCl for 48 hours at 4 °C, and flushed in saline (Bang 1973). The demineralized bone chips were suspended in liquid nitrogen and lyophilized for 22 hours. The DBM used in Series I was sterilized in ethylene oxide gas (Alcon Universal Ltd., Fort Worth, Texas, U.S.A.) for 3 hours, whereas no sterilization of the DBM chips was used in Series II. The DBM was kept

at 4 °C and implanted within 48 hours. The chips measured about 0.5 × 2.0 × 2.0 mm and weighed 0.7 mg.

Bioerodible polyorthoester poly(2,2-dioxy-cis, trans-1,4-cyclohexane dimethylene tetrahydrofuran) (Alzamer®, Alza Corporation, Palo Alto, CA, U.S.A.) was used alone and in composites with DBM. The polyorthoester results from condensation of 2,2-diethoxytetrahydrofuran and cis,trans-1,4-bis (hydroxymethyl)cyclohexane. The polyorthoester can be formulated with different physical properties; in this study, into a soft and moldable form. Spherical composite implants were prepared by manually mixing DBM and polyorthoester at room temperature under sterile conditions immediately before implantation. The DBM chips were partly exposed and partly embedded in the polyorthoester.

Surgical procedure

Anesthesia was induced with 0.15 mL/100 g Hypnorm®-Dormicum® i.m.

The abdomen was shaved and washed with a 3 percent chlorhexidine solution before incision. Pouches were created between the oblique abdominal muscles by blunt dissection. Implants were placed in the pouches. The incision was closed in layers.

Three different types of implants—(A) 15 mg polyorthoester, (B) four DBM chips, and (C) a composite of 15 mg polyorthoester and four DBM chips—were used.

In Series I, every animal received all three implants. Each implant was placed in a separate pouch.

In Series II, the animals were randomized into three groups (A–C) of 15 rats each. One pouch was created and filled with 15 mg polyorthoester, four DBM chips, and a composite of 15 mg polyorthoester and four DBM chips, respectively, in groups A, B, and C.

Evaluation

In Series I, 5 random rats were killed by an ether overdose at 1 and 2 weeks, 4 rats at 3 weeks, and 10 rats at 4, 6, and 8 weeks. Dissected abdominal muscles with implants were immediately tagged for orientation purposes. The specimens were fixed in 4 percent neutral formalin, demineralized in 17 percent formic acid, dehydrated, and embedded in paraffin. Serial sections were cut (5 μ) and stained with Harris' hematoxylin and eosin. Osteoinduction was defined as cartilage formation at Week 1, bone formation at Week 2, and bone and bone marrow formation from Week 3 (Reddi and Huggins 1972).

In Series II, all the animals were killed at 4 weeks after having received 10 μCi/100 g ^{85}Sr as $SrCl_2$ intraperitoneally 4 days earlier. The implant and the right iliac bone of each rat were dissected and weighed. ^{85}Sr uptake was determined in a Packard gamma counter. Two indices were calculated; the osteogenic index (Elves 1974) [(counts/min/mg implant)/(counts/min/mg ilium)] and an index we have called the osteoquantum index in which the weight of the implant has been invalidated [(counts/min implant)/(counts/min/mg ilium)].

Statistics

In Series I, McNemar's test for two correlated dichotomous variables was used for comparing the proportion of bone induction of the different implants. If fewer than 10 animals had different values for the two variables, the binomial distribution was used to find the observed significance level. Because comparing multiple groups introduces a greater probability of committing a type I error, the required P-value (0.05) was divided by the number of comparisons (Bonferroni's correction); $P < 0.016$ (0.05/3) was considered significant.

In Series II, the means of both the osteogenic and osteoquantum indices of the different groups were compared. Bartlett's test for the homogeneity of variances showed that the variances of both indices were significantly different among the groups, also after rescaling the data using log transformation, invalidating the use of the overall F-test for one-way analyses of variance (one-way ANOVA). Thus, pairs of means were compared with the two-sample t-test for independent samples with unequal or equal variances. Bonferroni's correction was used; $P < 0.016$ was regarded as significant. The 95 percent confidence intervals for difference of both the mean osteogenic and osteoquantum indices of the different groups were constructed.

Results

There were no peroperative or postoperative deaths. The animals gained weight and showed no signs of illness.

Histologic evaluation Series I

Cartilage and bone induction were found in most of the implants of both DBM and the composite of DBM and polyorthoester, and the proportions of induction of

Table 1. Cartilage and bone induction histologically in Series I. Number of implants with induction/number of implants recovered from hosts

	Week						
Implant	1	2	3	4	6	8	Total
Polyorthoester	0/5	0/5	0/4	0/10	0/10	0/10	0/44
DBM	5/5	5/5	4/4	8/10	9/10	8/10	39/44[a]
Composite	5/5	3/5	3/4	10/10	10/10	9/10	40/44[a]

[a]Different from polyorthoester ($P < 0.0001$).

the two implants were higher ($P < 0.0001$) than that of polyorthoester alone, of which no implants showed induction (Table 1). No difference in proportion of induction could be detected between composite and DBM ($P = 1.0$).

At Week 1, osteoprogenitor cells encapsulated the DBM, and some chondrocytes were seen within the DBM (Figure 1). At Week 2, cartilage and incipient bone formation were present (Figure 2). At Week 3 (Figure 3), more bone and some bone marrow were seen. At Weeks 4, 6, and 8 (Figure 4), the ossicles were gradually remodeled and bone marrow differentiated. Around the implants of polyorthoester and composite, inflammation with some giant cells was present until Week 2. The polyorthoester was clearly seen until Weeks 3–4, but later only occasional traces could be identified.

^{85}Sr uptake Series II

Both the composite of DBM and polyorthoester and DBM alone showed increased ^{85}Sr uptake compared with polyorthoester, as evaluated both by the osteoquantum index and the osteogenic index ($P < 0.0001$; Table 2). The 95 percent confidence intervals for difference in mean osteoquantum index and mean osteogenic index were 5.9 to 7.3 and 0.51 to 0.69 between DBM and polyorthoester, and 4.8 to 8.1 and

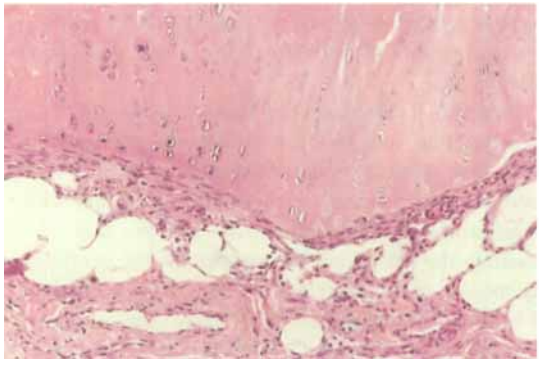

Figure 1. Composite of polyorthoester and DBM at Week 1 showing proliferating and maturing cartilage inside the DBM. Connective tissue with some unresorbed polyorthoester and a slight inflammation are encapsulating the DBM. H&E, field width 0.75 mm.

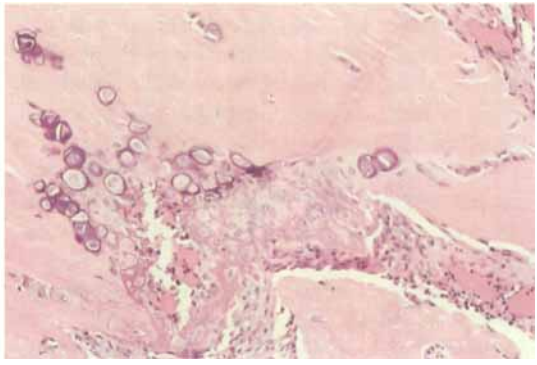

Figure 2. Composite of polyorthoester and DBM at Week 2 showing zones of proliferating, maturing, and calcifying cartilage, and incipient bone formation imitating endochondral osteogenesis in the epiphyseal plate. H&E, field width 0.69 mm.

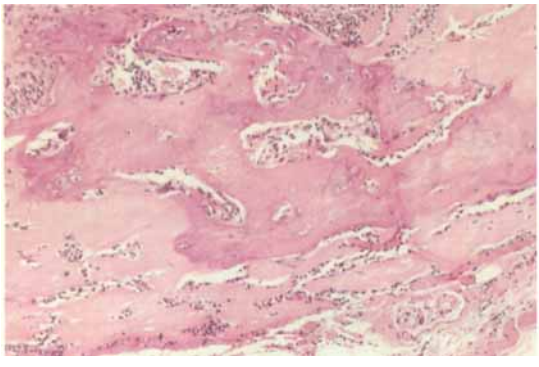

Figure 3. Composite of polyorthoester and DBM at Week 3 showing bone and bone marrow. H&E, field width 0.90 mm.

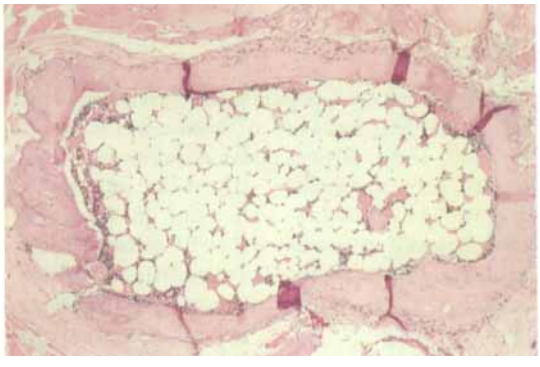

Figure 4. Composite of polyorthoester and DBM at Week 8 showing a mature ovoid ossicle with bone marrow differentiation. H&E, field width 1.61 mm.

Table 2. ^{85}Sr evaluation at Week 4 in Series II. Mean, *SD*

Implant	Osteoquantum index		Osteogenic index	
Polyorthoester	0.31	*0.40*	0.002	*0.004*
DBM	6.88	*1.26*[a]	0.598	*0.172*[a]
Composite	6.77	*3.04*[a]	0.490	*0.214*[a]

[a]Different from polyorthoester ($P < 0.0001$).

0.38 to 0.60 between composite and polyorthoester. There was no significant difference in osteoquantum index and osteogenic index between composite and DBM; $P = 0.90$ and $P = 0.14$, and the 95 percent confidence intervals for difference in means of the two indices were –1.6 to 1.9 and –0.04 to 0.25.

Discussion

Whereas nonsterile demineralized bone may be used in small laboratory animals without inconvenience (Schwarz et al. 1988), the clinical success of this transplant depends on its sterility (Harakas 1984). Sterility may be difficult to maintain during harvesting of cadaver bone and the subsequent processing of the bone, and demineralization in HCl does not seem to assure sterility (Dahners and Hoyle 1989). Thus, ethylene oxide sterilization has been used in many studies by different investigators, both experimentally (Bang 1973, Nilsen 1977, Syftestad and Urist 1982, Schmitz and Hollinger 1988, Pinholt et al. 1990) and clinically (Urist 1968, Ousterhout 1985), and apparently without deleterious effects on osteoinduction. However, recent studies have indicated that such sterilization may reduce the osteoinductive potency of the transplant (Munting et al. 1988, Aspenberg et al. 1990). Thus, although the normal sequence of induction was observed in ethylene oxide-sterilized DBM with or without polyorthoester carrier in Series I (Figure 1–4), we chose to omit such sterilization in Series II to increase the inductive ability and enhance any quantitative differences in ^{85}Sr uptake.

Several materials, such as β-tricalcium phosphate (Urist et al. 1984, Urist et al. 1987), matrix gamma-carboxyglutamic acid-rich protein (Sato and Urist 1985), collagen (Deathergate and Miller 1987), fibrin sealant (Schwarz et al. 1989), plaster of Paris (Yamazaki et al. 1988), copolymer of polylactide-polyglycolide (Schmitz and Hollinger 1988), polylactic acid (Lovell et al. 1989), and polyanhydride (Lucas et al. 1990), have been investigated as biodegradable vehicles.

A delivery system should be biocompatible (Lyman and Searce 1974), have the right physical properties (Deathergate and Miller 1987), provide sustained, controlled release of the active substance (Heller et al. 1981, Urist et al. 1984), and not inhibit osteoinduction. Such inhibition may be caused by a bioincompatible carrier that interferes physiologically with the induction, i.e., by inducing a chronic inflammation (Sela et al. 1986) or by unresorbed carrier physically obstructing bone formation. Whereas the ideal delivery system should be resorbed and replaced by cartilage within 3 weeks and bone within 4 to 6 weeks (Urist et al. 1987), unresorbed β–tricalcium phosphate (Urist et al. 1987), copolymer of polylactide-polyglycolide (Schmitz and Hollinger 1988), and polylactic acid (Lovell et al. 1989) were detected at 4 months, 24 weeks, and 6 months, respectively.

To evaluate the effect of the vehicle on osteoinduction, the composite of osteoinductor and vehicle should be compared with osteoinductor alone. This is difficult to accomplish when purified soluble osteoinductors are used: the osteoinductor is rapidly absorbed before bone is induced (Urist et al. 1984). Thus, in some studies the osteoinductor alone has not been evaluated (Deathergate and Miller 1987, Urist et al. 1987, Lovell et al. 1989), or it has been implanted in gelatin capsules (Urist et al. 1984, Yamazaki et al. 1988).

Bioerodible polyorthoester used here by us seems to have four main advantages when compared with most existing delivery systems. First, we have shown that the polyorthoester does not inhibit osteoinduction, and it causes only a slight inflammation that subsides within 3 weeks. Secondly, our results show that the polyorthoester is mostly resorbed at Week 4 when ossicles have formed, and thus does not physically obstruct the bone formation. The biodegradation of the polyorthoester takes place by hydrolysis to the ultimate products 4-hydroxybutyrate (4HB) and cis,trans-1,4-bis(hydroxymethyl)cyclohexane (CHDM). 4HB is further metabolized in the tricarboxylic acid cycle, with CO_2 and H_2O as the end products. CHDM is excreted in urine (Sendelbeck and Girdis 1985). Thirdly, different formulations of the polyorthoester with different physical properties ranging from gel to solid can be manufactured: in the present study, a soft and moldable form. Polyorthoester of this formulation adheres to wet bone surfaces and provides local hemostasis by physical tamponade (Sudmann et al. 1990, Solheim et al. 1991). Finally, the polyorthoester is designed to be used as a local, sustained, drug-release system (Capozza et al. 1978). It is possible, under certain conditions, to accomplish zero-order drug release from polyorthoesters (Capozza et al. 1978, Heller et al. 1981). The reason has been claimed to be that the rate of drug release is determined by erosion and not by simple diffusion, because the polyorthoester is

hydrophilic and may erode heterogeneously from the surface first (Langer 1986). The polyorthoester may prove to be valuable for sustained release of combinations of bone inductors, growth factors, and drugs.

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