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Effects of Bioerodible Polyorthoester on Heterotopic and Orthotopic Bone Induction in Rats

Thesis

by
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Contents

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Hagavik, June 1992 Eirik Solheim

List of papers

This thesis is based on the following papers, to be referred to by their Roman numerals in the text:

I. Solheim E, Pinholt E M, Bang G, Sudmann E. Comparison of histomorphometry and ^{85}Sr uptake in induced heterotopic bone in rats. Acta Orthop Scand 1992;63(3):334-8.

II. Pinholt E M, Solheim E, Bang G, Sudmann E. Bone induction by composite of bioerodible polyorthoester and demineralized bone matrix in rats. Acta Orthop Scand 1991;62(5):476-80.

III. Solheim E, Pinholt E M, Bang G, Sudmann E. Effect of local hemostatics on bone induction in rats. A comparative study of bioerodible polyorthoester with and without gentamicin, bone wax and fibrin-collagen paste. J Biomed Mater Res 1992;26(6):791-800.

IV. Solheim E, Pinholt E M, Bang G, Sudmann E. Regeneration of calvaria by composite of bioerodible polyorthoester and demineralized bone in rats. J Neurosurg, 1992;76(2):275-9.

V. Solheim E, Pinholt E M, Andersen R, Bang G, Sudmann E. The effect of a composite of polyorthoester and demineralized bone on the healing of large segmental defects of the radius in rats. J Bone Joint Surg (Am), 1992;74-A(10):1456-63.

VI. Solheim E, Pinholt E M, Bang G, Sudmann E. Inhibition of heterotopic osteogenesis in rats by a local bioerodible indomethacin delivery system. J Bone Joint Surg (Am), 1992;74-A(5):705-12.

General introduction

OSTEOINDUCTION BY DEMINERALIZED BONE

Intramuscular implantation of fresh autogenous bone (Levander 1938, Chalmers 1959), HCl demineralized allogeneic bone (Urist 1965, Reddi and Huggins 1972) or purified osteoinductors (Urist et al. 1979) regularly evoke heterotopic osteogenesis in rodents. When heterotopic osteogenesis results from implantation of demineralized bone (DBM) or purified osteoinductors, void of living cells, the process is called osteoinduction.

The major phases of DBM-induced heterotopic osteogenesis in rodents are chemotaxis of mesenchymal cells, mitosis, differentiation of cartilage, vascular invasion, bone differentiation and formation of an ossicle filled with bone marrow elements. Shortly after implantation of DBM there is a blood clot, platelet release and polymorphonuclear leukocytes arrive by chemotaxis. On day 1, osteoprogenitor cells are seen. By day 3, most leukocytes have disappeared and the osteoprogenitor cells are proliferating. On day 5, the proliferating cells differentiate into chondroblasts. By day 7 to 8, there are many chondrocytes producing type II collagen and cartilage specific proteoglycans. Then, there is a capillary invasion leading to hypertrophy of chondrocytes and calcification of cartilage matrix on day 9. On day 10, osteoblasts appear close to the vascular endothelium and new bone is formed by appositional growth on the surface of the calcified matrix and the DBM. Remodeling of the bone by osteoclasts starts by day 12 and an ossicle forms by day 16 to 21 (Urist 1965, Reddi and Huggins 1972).

DBM-induced heterotopic osteogenesis in rodents has been used as an experimental model for investigation of the osteoinduction itself (Urist 1965) as well as the effect of several factors on the osteoinduction: (1) age (Syftestad and Urist 1979, Reddi 1985) and species (Schwartz et al. 1989) of donor and recipient; (2) properties

of the demineralized bone, e.g., demineralization procedure (Delloye et al. 1985, Marinak et al. 1989), particle size (Syftestad and Urist 1979), storage, and sterilization procedures (Munting et al. 1988); (3) dietary factors, e.g., magnesium, (Belanger et al. 1975, Schwartz and Reddi 1979), manganese and copper (Strause et al. 1987), fluoride (Turner et al. 1989), vitamin A (DeSimone and Reddi 1983) and vitamin D (Vandersteenhoven 1988); (4) hormones (Reddi and Sullivan 1980, Burnett and Reddi 1983, Kapur and Reddi 1989); (5) growth factors (Howes et al. 1988, Aspenberg and Lohmander 1989); (6) biphosphonates (Bauer et al. 1986); (7) indomethacin (Törnkvist et al. 1985); and (8) biomaterials (Vandersteenhoven and Spector 1983, Uretzky et al. 1987, Schwarz et al. 1989, Ripamonti et al. 1989).

Experimentally in animals, DBM enhances (1) cranio-maxillofacial reconstructions (Senn 1889, Ray 1957, Mulliken and Glowacki 1980, Glowacki et al. 1981a, Kaban and Glowacki 1981, Pinholt et al. 1990), (2) healing of diaphyseal defects (Narang et al. 1979, Oikarinen and Korhonen 1979, Gupta and Tuli 1982, Einhorn et al. 1984, Aspenberg et al. 1986, Aspenberg et al. 1987, Gepstein et al. 1987, Köhler and Kreicbergs 1987, Hopp et al. 1989) and (3) spinal fusion (Lindholm et al. 1982, Ragni et al. 1987).

DBM have been used clinically for (1) cranio-maxillofacial reconstructions (Kümmell 1891, Glowacki et al. 1981b, Ousterhout 1985), (2) healing of diaphyseal defects (Senn 1889, Miller 1890, Urist 1968, Osepian et al. 1989), (3) pseudarthrosis operation (Urist 1968), (4) arthrodesis of hip (Urist 1968) and (5) spinal fusion (Sharrard and Collins 1961, Urist 1968, Urist and Dawson 1981)

Whereas it has been clearly documented that DBM induces heterotopic bone formation in rodents and enhances bone formation in skeletal sites both in rodents and humans, the results regarding heterotopic bone formation by DBM in primates are conflicting. Aspenberg et al. (1988) found no bone and very little cartilage 40 days postoperatively in squirrel monkeys and concluded that the observed bone formation by DBM implantation in skeletal sites in humans is not necessarily caused by osteoinduction, but may be the result of the implant acting as a osteoconductive scaffold. Further, Aspenberg and Andolf (1989) found that human DBM implanted

intramuscularly in athymic rats induced bone formation and they concluded that the possible decreased ability of such DBM to induce heterotopic bone formation in adult primates may be due to an inability by the recipients to respond to the inductive stimuli of the DBM (Aspenberg and Andolf 1989). However, Hosny and Sharawy (1985) found that intramuscularly implanted DBM in rhesus monkeys induced cartilage and bone although the process was delayed compared to that in rodents, and Ripamonti reported bone formation in 73 out of 96 DBM implants in 24 adult male baboons (1991).

PURIFIED OSTEOINDUCTORS AND GROWTH FACTORS

The existence of osteoinductor(s) and growth factors in transplanted bone and bone extracts has been postulated for a long time (Gallie and Robertson 1929, Levander 1938, Lacroix 1945, Urist 1965). In recent years several osteoinductors and growth factors have been isolated and characterized from bone matrix extracts: (1) bone morphogenetic protein (BMP) (Urist and Mikulski 1979); (2) osteogenin (identical to BMP-3) (Sampath et al. 1987); (3) insulin-like growth factor I (IGF I, identical to somatomedin-C) (Canalis et al. 1988) and insulin-like growth factor II (IGF II, similar to SGF) (Farley and Baylink 1982); (4) transforming growth factor beta (TGF-b, identical to cartilage inducing factor, CIF) (Seyedin et al. 1985, Linkhart et al. 1986); (5) acidic and basic forms of fibroblast growth factor (FGF) (Hauschka et al. 1986); and (6) platelet-derived growth factor (PDGF) (Hauschka et al. 1986).

In the past few years, many osteoinductors and bone growth factors, including subgroups of the factors mentioned above, e.g., BMP 1-7, have become readily available by recombinant DNA technology and knowledge about their biologic effects is evolving (Hauschka et al. 1988, Gospodarowicz 1990, Marden et al. 1990, Ozkaynak et al. 1990, Pfeilschifter et al. 1990, Wozney et al. 1988, Wang et al. 1990, Mohan and Baylink 1991, Reddi and Cunningham 1991). The osteoinductors are necessary to initiate osteogenesis, whereas growth factors may augment different stages of the process, e.g., attracting preosteoblasts, accelerating their

proliferation, and stimulating angiogenesis (Kawamura and Urist 1988, Reddi et al. 1989, Marden et al. 1990).

ORTHOPEDIC ABSORBABLE IMPLANTS

Orthopedic absorbable implants have been developed for different purposes: (1) temporary internal fixation of skeletal parts (Getter et al. 1972, Rokkanen et al. 1985); (2) bone substitutes, i.e., polymers and ceramics, used alone or in combination with bone grafts (Driskell et al. 1972, Hollinger 1983, Higashi et al. 1986, Hollinger and Battistone 1986); (3) bone cement (Gerhart et al. 1989); (4) osteoinductor delivery (Urist et al. 1984, Lovell et al. 1989, Lucas et al. 1990); (5) drug delivery, (Mackey et al. 1982, Tasslet and Inhoff 1988); and (6) local hemostasis (Geary and Frantz 1950, Harris and Capperauld 1980). Implants used for the latter three purposes are discussed below.

OSTEOINDUCTOR DELIVERY SYSTEMS

Demineralized bone matrix (DBM) used as chips or powder is technically difficult to apply for reconstructions and bridging of bone defects as the particles may displace during or after the operation. Incorporating the DBM in a bioerodible carrier thus seems warranted (Glowacki and Mulliken 1985). Further, it has been claimed that effective osteoinductive stimulus in humans require supply of a proper combination of an osteoinductor, i.e., BMP-2 or osteogenin (BMP-3) to induce bone formation and growth factors to augment the process (Kawamura and Urist 1988). A biodegradable delivery system is needed for sustained release of such factors (Takagi and Urist 1982, Sato and Urist 1985, Marden et al. 1990).

The ideal delivery system should fulfill several criteria; it should (1) be biocompatible (Lyman and Searce 1974); (2) have the right physical properties (Yamazaki et al. 1988); (3) provide local hemostasis (Sudmann et al. 1990); (4) provide sustained, controlled release of the active substance (Heller et al. 1981, Rosen et al. 1983, Urist et al. 1984); (5) be resorbed and replaced by preosseous tissues within two weeks, cartilage within three weeks and bone within four to six weeks (Urist et al. 1987); and (6) not inhibit

osteogenesis in any way, neither the different stages of osteoinduction nor osteoconduction.

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

DRUG DELIVERY SYSTEMS

Conventionally, bioactive agents are delivered by periodic ingestion or injection. During this process drug levels reach a maximum and then fall to a minimum. If drug concentrations fall above or below the toxic level or minimum effective level, respectively, alternating periods of toxicity or inefficacy can result. The main goal of a controlled release system is to maintain the drug concentration between these two levels from a single dosage form (Langer 1986). Further, implanted drug delivery systems make possible local application of the drug in the effector tissue or organ. This principle may be especially useful in orthopedic surgery where such systems may be applied during bone transplantation, internal fixation and prosthesis surgery, e.g., to stimulate or inhibit new bone formation, provide infection prophylaxis or deliver other drugs with high local concentration without systemic effects.

In a controlled release system, a bioactive agent is incorporated into a carrier, generally a polymeric material. The rate of release of the substance is determined by the properties of the polymer itself and is only weakly dependent on environmental factors. Controlled release systems can deliver substances slowly and continuously for long periods. Polymers release drugs by four general mechanisms: diffusion, chemical control, solvent activation and magnetism.

The most commonly used biodegradable polymers for drug delivery systems are polylactic acid (Chang 1976), polyglycolic acid and their copolymers (Langer 1986). Other biodegradable

polymers that have been used for controlled drug delivery systems include polymers based on caprolactone (Pitt et al. 1979), various copolymers of amino acids (Sidman et al. 1980), polyanhydrides (Rosen et al. 1983) and polyorthoesters (Heller et al. 1981).

Drug release from bioerodible matrix devices can be controlled by either diffusion or erosion. If erosion of the matrix is much slower than diffusion, the release kinetics are essentially those of a diffusion controlled matrix. If however the drug is immobilized in the matrix so that diffusional release is minimal compared to erosion, the rate of drug release will be erosion controlled (Heller 1986).

Heterogeneous erosion occurs when degradation takes place only at the surface of the polymer, whereas homogeneous erosion is the result of degradation occurring through the polymer matrix. Heterogeneous erosion can lead to zero-order drug release providing diffusional release of the drug is minimal and the overall surface area of the device remains constant (Heller 1986). Hydrophobic polymers are more likely to erode heterogeneously since water is excluded. Hydrophilic polymers such as polylactic acid and polyglycolic acid, in contrast, may absorb water and erode homogeneously leading to progressive looseing of the matrix, changes in permeability and drug diffusion resulting in nonlinear drug release (Heller et al. 1981, Rosen et al. 1983). Polymers known to display hydrolytically controlled surface erosion and where the polymers break down to monomers are polyorthoesters when certain additives are included (Heller et al. 1981) and polyanhydrides (Rosen 1983).

BIOERODIBLE POLYORTHOESTER

Poly(2,2-dioxy-cis,trans-1,4-cyclohexane dimethylene tetrahydrofuran) (Alzamer®, Alza Corporation, Palo Alto), a bioerodible polyorthoester, results from condensation of 2,2-diethoxytetrahydrofuran and cis,trans-1,4-bis(hydroxymethyl)cyclohexane. 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). The polyorthoester has been developed to be used as the vehicle in a sustained drug release system and the drug added is released at a constant rate (Capozza et al. 1978, Benagiano et al. 1979). The polymer can be formulated with different physical properties. Polyorthoester of the formulation used in this work is soft and moldable. The polyorthoester adheres to bone surfaces and probably provides local hemostasis by plugging of spongy bone and secondarily promoting concentration of platelets and coagulation factors (Sudmann et al. 1990, Solheim et al. 1991).

INHIBITION OF BONE FORMATION

Systemic administration of the nonsteroidal anti-inflammatory drugs (NSAIDs), indomethacin, acetyl salisylic acid or ibuprofen, has been shown experimentally in animals and clinically to inhibit both heterotopic and orthotopic osteogenesis (Dahl 1974, Sudmann 1975, Rø et al. 1976, Sudmann et al. 1979, Sudmann and Bang 1979, Allen et al. 1981, Törnkvist et al. 1983, Elmstedt et al. 1985, Solheim et al. 1986, Sodemann et al. 1988). Further, locally administered indomethacin contained in bioerodible polyorthoester gel has been shown to inhibit the healing of closed mid-diaphyseal femur fractures in rats (Engesæter et al. 1992).

Experimentally in rats, Törnkvist (1985) found that indomethacin in doses of 2 or 3 mg/kg inhibited DBM-induced heterotopic osteogenesis, whereas rats treated with 6 mg/kg all died within one week of perforated gastric ulcer. DiCesare (1991) found a dose related inhibition on DBM-induced heterotopic osteogenesis by indomethacin over a range between 0.04 and 4 mg/kg body weight. In order to inhibit DBM-induced heterotopic osteogenesis in rats, indomethacin had to be present before or at the time of implantation of DBM (Törnkvist et al. 1985, Nilsson et al. 1986). A short period of indomethacin treatment at the time of implantation of DBM is sufficient to reduce experimental bone formation, but the inhibitory effect slowly diminishes if the inductive process is continuous (Nilsson et al. 1986).

Inhibition of bone formation may be desirable in some clinical situations: (1) heterotopic ossification following total hip

arthroplasty, (2) post-traumatic partial growth plate arrest, (3) craniosynostosis or (4) bone coalitions. Inhibition of bone formation may be accomplished by administration of NSAIDs or implantation of interpositional materials. In arthroplasties, the NSAIDs indomethacin or ibuprofen has been used either prophylactically at the time of the arthroplasty (Dahl 1974, Almåsbakk and Røysland 1977, Elmstedt et al. 1985, Sodemann et al. 1988) or after surgical removal of ectopic bone to prevent heterotopic ossification (Ritter and Gioe 1982, Kjærsgaard-Andersen and Schmidt 1986). Further, indomethacin inhibits osseous rebridging of the growth plate in rabbits (Sudmann et al. 1982).

Inhibition of osteogenesis by interpositional material has been accomplished clinically or experimentally in animals, by surgical removal of bone and interposition of (1) free fat transplant (Langenskiöld 1975, Merikanto et al. 1987, Olney and Asher 1987), (2) cartilage (Lennox et al. 1983), (3) muscle (Collins 1987), (4) bone wax (Friedenberg and Brashear 1956), (5) methyl methacrylate (Friedenberg 1957) or (6) silicone-rubber (Bright 1974). Implantation of nonresorbable materials may, however, induce a chronic inflammation, predispose for infections and require a second operation for removal of the implant. To prevent systemic adverse effects including inhibition of the osteogenesis in other parts of the skeleton than intended, a local bioerodible indomethacin delivery system providing constant high local concentrations of the drug without systemic effects seems desirable.

LOCAL HEMOSTATICS

In bone surgery, nonabsorbable bone wax (Parker 1892, Horsley 1892) of 88% beeswax and 12% isopropylpalmitat, is being used for local hemostasis (Crenshaw 1987). Bone wax may, however, produce a chronic inflammation with foreign-body reaction (Geary and Frantz 1950), retard bone healing (Howard and Kelley 1969), predispose for infections (Culliford et al. 1976, Robicsek et al. 1978), impair bacterial clearance (Johnson and Fromm 1981) and cause wax embolization (Robicsek et al. 1981). These complications has spurred the development of absorbable local hemostatics

(Bergel 1909, Frantz et al. 1944, Correll and Wise 1945, Battista et al. 1967, Matras et al. 1972, Harris and Capperauld 1978, Silverstein et al. 1980, Sudmann et al. 1990), of which fibrin sealant (Matras et al. 1972), fibrin-collagen paste (Harris and Capperauld 1978) and bioerodible polyorthoester (Sudmann et al. 1990) may be the most suitable for bone surgery as they adhere readily to bleeding bone. The two former materials have been used in clinical orthopedic practice, while the latter has been shown in animal models to be suitable for such use (Sudmann et al. 1990).

AIMS OF THE PRESENT STUDY

The present work was designed to examine a new bioerodible polyorthoester as a DBM-carrier, a local hemostatic and a drug-delivery system in heterotopic and orthotopic models in rats. The specific objectives of the study are defined as listed.

1. To examine the relation between histomorphometry and ^{85}Sr uptake of DBM-induced heterotopic bone (paper I), and thus, establish an accurate method for evaluating heterotopic bone formation by ^{85}Sr analyses.

2. To study the effect of bioerodible polyorthoester on DBM-induced heterotopic (paper II) or orthotopic (papers IV and V) osteogenesis.

3. To compare the host tissue response and the effect on osteoinduction of three local hemostatics for osseous tissue, bone wax, fibrin-collagen paste and bioerodible polyorthoester with or without gentamicin (paper III).

4. To study the effect of a bioerodible local indomethacin delivery system on DBM-induced heterotopic osteogenesis (paper VI).

Summary of results

PAPER I

A method for assessing heterotopic bone formation by ^{85}Sr analyses was established. Five, 10 or 15 mg DBM powder was implanted in 45 male Wistar rats and the relationship between the results of histomorphometry and ^{85}Sr uptake analysis of DBM-induced heterotopic bone was evaluated at 4 weeks postoperatively. Two indices of ^{85}Sr uptake were calculated; the osteogenic index [(counts/min/mg implant)/(counts/min/mg os 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 os ilium)]. The osteoquantum index was found to have a linear relationship to the area of the induced bone with a correlation coefficient of 0.90 ($p<0.0001$). Only weak linear relationships were found between the osteogenic index and the area of the new bone ($r=0.32$, $p=0.03$) and between the osteogenic index and the osteoquantum index ($r=0.33$, $p=0.03$). The osteoquantum index and the area of the induced bone both increased proportionally to the quantity of implanted DBM, whereas the osteogenic index did not change significantly.

PAPER II

Bioerodible polyorthoester was evaluated as an osteoinductor delivery system in 89 male Wistar rats by implantation of DBM, polyorthoester or a composite of the two in the abdominal muscle of the animals. Heterotopic bone formation and tissue reaction were evaluated by light microscopy in 44 rats at weeks 1, 2, 3, 4, 6 and 8 and strontium^{85} uptake in 45 rats at week 4. Composite of polyorthoester and DBM induced cartilage and bone at the same rate as the DBM alone. The composite implant was technically easier to use than the DBM alone. Around the implants of the

polyorthoester and the composite, inflammation with some giant cells was present until week two. The polyorthoester was clearly seen until 3-4 weeks but later only occasional traces could be identified.

PAPER III

The tissue response and effect on DBM-induced heterotopic osteogenesis in the abdominal muscle of 120 male Wistar rats by different local hemostatics were evaluated by light microscopy and ^{85}Sr uptake analyses. Nonabsorbable bone wax of 88 % beeswax and absorbable bovine fibrin-collagen paste both significantly inhibited osteoinduction, whereas bioerodible polyorthoester drug delivery system with or without 4% gentamicin caused no significant inhibition. Bone wax was not absorbed and induced a chronic foreign body reaction. Fibrin-collagen paste induced less inflammation with numerous monocytes and macrophages with engulfed material. Bioerodible polyorthoester caused a very moderate tissue reaction and was mostly resorbed at week 4.

PAPER IV

In 36 male Wistar rats, the healing of critical size calvarial defects without implant or filled with DBM, polyorthoester or a composite of the two former was studied. Defects filled with the composite of polyorthoester and DBM or the DBM alone were bridged by bone at 4 weeks histologically and radiographically, whereas unfilled defects or defects filled with the polyorthoester only, did not heal. The polyorthoester caused a slight inflammation that subsided by 3 weeks. Only traces of the polyorthoester could be detected at 4 weeks. The polyorthoester provided local hemostasis when used either alone or in composites with DBM. The composite implant was moldable and easily contoured and technically easier to use than the DBM alone.

PAPER V

The healing of large osteoperiosteal defects of 50 % of the length of the radius was studied in 60 male Wistar rats. The defects were left with no implant or filled with DBM, polyorthoester or a composite of the two. At 50 days the specimens were evaluated by light microscopy and measurement of bone formation within the original defect on radiographs by Sigma-Scan Measurement System including an electromagnetic digitizing tablet and a personal computer with Sigma scan software. Defects filled with the composite of polyorthoester and DBM or the DBM alone showed regeneration of bone corresponding to respectively 93.6 per cent (SD 44) and 77.6 per cent (SD 30) of the area of the defect. Defects with no implant or defects filled with the polyorthoester only, showed significantly less regeneration. The DBM used alone was rapidly mixed with blood forming a grainy mixture that tended to be displaced, whereas the composite implant was moldable, easy to place in the defect and the tendency for displacement was less. The polyorthoester provided local hemostasis when used either alone or in composites with demineralized bone.

PAPER VI

The effect of a bioerodible system for local delivery of indomethacin on DBM-induced heterotopic bone formation in rat abdominal muscle was evaluated. Two separate series were conducted in a total of 48 Wistar rats. In both series two types of implants were used: (A) control group, polyorthoester and DBM; or (B) experimental group, polyorthoester with 5 % indomethacin and DBM. In the first series, host-tissue responses and osteoinduction were evaluated at 2, 3 and 4 weeks histologically. In the second series, bone formation was quantified by ^{85}Sr uptake at week 4. Blood samples were obtained under anesthesia from 3 random rats of each group in Series I at week 1 and of all rats of group B (polyorthoester with 5 % indomethacin and DBM) prior to death at week 2-4. Plasma was prepared and stored at -70°C until analyses. Indomethacin in plasma was assayed by a specific High Performance Liquid Chromatographic method with UV-detection

at 320 nanometers. The detection limit was 25 nanograms per milliliter. The bioerodible system for local delivery of indomethacin significantly inhibited demineralized bone-induced heterotopic bone formation as evaluated by light microscopy and ^{85}Sr uptake. The polyorthoester, with or without drug, caused little tissue reaction and was mostly resorbed at week 4. Indomethacin could not be detected in any of the blood samples.

General discussion

MATERIAL AND METHODS

Animals

Young male Wistar rats were chosen as the experimental animals in this study. Rats are well standardized, easily available and their short life span permits experimental intervention for a significant fraction of their lifetime. Rats retain a basically non-Haversian lamellar bone structure throughout their life, however, the physiological mechanisms of bone remodeling are similar to that of humans (Simmons 1976). Models for evaluating both heterotopic (Urist 1965, Elves 1974) or orthotopic (Mulliken and Glowacki 1980, Schmitz and Hollinger 1986, Gepstein et al. 1987) osteogenesis have been established in rats.

The animals were fed standard laboratory food and water ad libitum. Anesthesia was induced by intramuscular injection of a combination of 0.075 mg fentanyl, 3.75 mg fluanisone and 1.875 mg midazolam (Hypnorm®-Dormicum®) per kilogram of body weight (Flecknell and Mitchell 1984). The research protocol was approved by The Norwegian State Commission for the Regulation of Animal Experiments. The national guidelines for the care and use of laboratory animals were observed.

Implants

Demineralized bone matrix (DBM) was prepared by sterile technique from the femur, tibia and fibula of male Wistar rats of the same age and weight as the recipients of the corresponding series, except in paper IV where the donor rats were older than the recipients. Dissected diaphyses were crushed and the marrow was removed. The cortical bone was cut into chips and 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. Several protocols for preparing demineralized bone have been proposed (Urist et al. 1975, Reddi and Huggins 1972, Bang 1973). The more extensive processing of the bone in the antigen-extracted autodigested alloimplant (AAA) protocol (Urist et al. 1975) may lead to some loss of inductive potential compared to demineralization and lyophiliazation only, as used in the present work (Delloye et al. 1985, Marinak et al. 1989).

Whereas non-sterile demineralized bone may be used in small laboratory animals without inconvenience (Schwarz et al. 1988), the clinical success of this implant 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), apparently without deleterious effects on the osteoinduction. Thus, in the first part of the present study, the DBM was sterilized in ethylene oxide gas (Alcon Universal Ltd., Fort Worth, Texas, USA) for 3 hours (Bang 1973). The normal histologic sequence of induction was observed in ethylene oxide-sterilized DBM with or without polyorthoester carrier. However, as recent studies has indicated that such sterilization may reduce the osteoinductive potency of the implant (Munting et al. 1988, Aspenberg et al. 1990), no sterilization of the DBM was used in the latter part of the work.

DBM was used either as chips (0.5 x 2.0 x 2.0 mm; weight 0.7 mg) or as a coarse powder (0.1-2.0 mm^2). The size was assessed by area measurements on photomicrographs of random samples. The osteoinductive potential may increase with decreasing particle size (Syftestad and Urist 1979, Glowacki et al. 1981a, Sampath and Reddi 1984). Pulverization to particle size less than approximately 0.1 mm may, however, cause reduction of the osteoinductive potential, probably by denaturation of contained inductive proteins (Syftestad and Urist 1979, Sampath and Reddi 1984).

The demineralized bone was kept at 4°C and implanted within 48 hours. When stored at this temperature, DBM has been

shown to retains its osteoinductivity for a period of up to six months (Hosny et al. 1987).

The total mass of the implanted DBM ranged from 2.8 mg to 15 mg. Whereas Muthukumaran et al. (1988) found a threshold for bone induction at 10 mg DBM, in the present work, typical histologic signs of induction was found in all experiments regardless of the mass of the DBM; and in paper I, we found no qualitative differences of the bone induced by 5, 10 or 15 mg DBM. In the same paper, both the osteoquantum index and the area of the induced bone increased proportionally to the quantity of implanted DBM. These findings agree with those of earlier studies in which the area of induced bone on radiographs was directly proportional to the weight of implanted osteoinductor, bone morphogenetic protein (Kawai and Urist 1988, Mahy and Urist 1988).

In our studies of heterotopic osteogenesis by polyorthoester and DBM, spherical composite implants were made by mixing approximately equal volumes of DBM as chips or powder and polyorthoester manually at room temperature under sterile conditions immediately before implantation. The DBM particles were partly exposed and partly embedded in the polyorthoester.

Heterotopic model

DBM-induced heterotopic osteogenesis in rat abdominal muscle was used as the experimental model for evaluating biocompatibility and effect on osteoinduction by polyorthoester (paper II), local hemostatics (paper III) and polyorthoester indomethacin delivery system (paper VI). This model has several advantages. First, DBM regularly induces heterotopic bone in rodents and the process has been well described (Urist 1965, Reddi and Huggins 1972). Secondly, composites of biomaterials and osteoinductor should first be evaluated by their ability to induce heterotopic bone, as new bone formation in orthotopic sites may be due to osteoblastic activity by the bone proper, i.e., osteoconduction, making quantification of the osteoinductive potential of the composite difficult. Finally, to evaluate the effect of the biomaterial on osteoinduction, the composite of biomaterial and osteoinductor should be compared with the osteoinductor alone. This is, however, difficult to

accomplish when purified, soluble osteoinductors are used as the osteoinductor is rapidly absorbed before bone is induced (Urist et al. 1984).

Heterotopic osteoinduction has been evaluated by both (1) qualitative methods: light microscopy (Urist 1965, Reddi and Huggins 1972), radiography (Urist 1965) and electron microscopy (Nilsen 1977) and (2) quantitative methods: ash weight (Urist et al. 1970), calcium content (Urist et al. 1970), alkaline phosphatase activity of implant (Firschein and Urist 1972), ^{85}Sr uptake analyses (Elves 1974), ^{45}Ca uptake analyses (Reddi 1975), histomorphometry (Hosny and Sharawy 1985, Marinak et al. 1989) and computerized image analyses of the area of induced bone on radiographs (Kawai and Urist 1988).

In the present work, qualitative histologic examination (papers II, III and VI), computer-assisted histometry (paper I) and ^{85}Sr uptake (papers I, II, III and VI) were used to evaluate osteoinduction. In paper II, qualitative histologic examination was performed at 1, 2, 3, 4, 6 and 8 weeks to examine the effect of the carrier on different stages of osteoinduction. It was shown (paper II), in concordance with previous studies (Reddi and Huggins 1972), that mature ossicles were formed by 4 weeks. Thus, 4 weeks postoperatively was chosen as an appropriate point of time for histologic evaluation in paper III. In paper VI, qualitative histologic examination was performed at 2, 3, and 4 weeks to examine the effect of the indomethacin delivery system on both cartilage and bone formation.

^{85}Sr uptake at 4 weeks postoperatively was chosen for quantitative evaluation of osteoinduction in papers II, III and VI. ^{85}Sr has been used for evaluation of bone grafts both as (1) ^{85}Sr uptake in grafts, calculated as the osteogenic index [(counts/min/mg implant)/(counts/min/mg os ilium)] (Elves 1974, Elves 1975, Delloye et al. 1985, Munting et al. 1988) or the total content (cpm/implant) (Yoshikawa et al. 1988) and (2) loss of ^{85}Sr from prelabeled grafts (Rønningen et al. 1985, Solheim et al. 1986).

In paper I, the ^{85}Sr uptake expressed as the novel osteoquantum index [(counts/min implant)/(counts/min/mg os ilium)] showed a linear relationship to the histometric area of the induced bone with a correlation coefficient (r) of 0.90, whereas only weak linear

relationships were found between the osteogenic index and the histometric area of the bone (r=0.32) and between the osteogenic index and the osteoquantum index (r=0.33). Thus, the osteoquantum index was used in papers II, III and VI; in paper II in addition to the osteogenic index. As the weight of the implant is disregarded in the osteoquantum index, this index, in contrast to the osteogenic index, permits evaluation of the effect of biomaterials on osteoinduction in composites without influence of density and biodegradation of the biomaterial as long as the same amount of osteoinductor is used.

Orthotopic models

Both cranial defects and radial defects in rodents are commonly used models for evaluation of autogenous bone substitutes. The potential for regeneration of cranial defects is low, especially in adults. Defects that do not heal during the animals' lifetime are termed critical size defects and it has been proposed that evaluation of cranio-maxillofacial bone repair materials should be initiated in critical size defects of the rat calvaria (Schmitz and Hollinger 1986). Nor do large radial diaphyseal defects in rats heal spontaneously (Gepstein et al. 1987, Alper et al. 1989). The absence of supination and the radio-ulnar synostosis contribute to the stability of the bones and to the retention of the implanted material (Alper et al. 1989).

When DBM is used for promoting healing of bone defects, the mode of action of the implant is more difficult to establish than in the heterotopic model as osteoclasts and osteoblasts are already present and might be stimulated by the surgical trauma.

Regeneration of bone defects by DBM is a multistep process. In paper IV, we wanted to evaluate the effect of the polyorthoester on the different phases of the healing of calvarial defects. Thus, a positive treatment response was defined as osteoinduction, i.e., bone formation within the defect and not in contact with the bone edges at weeks 2 and 3 histologically and bone bridging of the defects at week 4 histologically and radiographically. The treatment response of each rat was recorded as 0 (no induction or bone bridging), 1 (induction or bone bridging in one defect) or 2 (induction or bone bridging in both defects).

This is, of course, a rather crude semi-quantitative evaluation. For statistical analyses, the results of the radiographic analyses should preferably be continuous data of high accuracy. For this purpose, measurements of the area of new bone formation on radiographs by digitizer and area analyses software have been used in heterotopic models (Kawai and Urist 1988, Mahy and Urist 1988). Further, we were primarily interested in the effect of the polyorthoester on the final result of the process. Thus, in paper V, we measured the area of bone formed within the original defect on radiographs by Sigma-Scan Measurement System including an electromagnetic digitizing tablet and a personal computer with Sigma scan software at 50 days postoperatively. In both papers IV and V the host-tissue response and osteoinduction were evaluated by qualitative histologic examination.

RESULTS

Handling properties and local hemostasis

Both in the heterotopic model (paper II) and in the two orthotopic models (papers IV and V) the DBM was rapidly mixed with blood forming a grainy mass that tended to be displaced. In contrast, the composite of DBM and polyorthoester was easy to place in the muscle pouch or bone defect and the tendency for displacement was much less. The polyorthoester and the composite had a plastic consistency and could easily be molded peroperatively according to needs. The bioerodible polyorthoester adhered to bone surfaces and provided local hemostasis, probably by plugging of spongy bone and secondarily promoting concentration of platelets and coagulation factors (Sudmann et al. 1990, Solheim et al. 1991). The evaluation of both handling properties and local hemostatic effect was purely qualitative.

Heterotopic osteoinduction

A requirement for an osteoinductor delivery system is that the carrier does not inhibit osteoinduction. In composites of carrier and osteoinductor, the osteoinduction may be inhibited by the carrier by two mechanisms. First, unabsorbed carrier will physically inhibit bone growth and bony bridging of a defect. According to Urist et al. (1987) the ideal delivery system should be resorbed and replaced by preosseous tissues within two weeks, cartilage within three weeks and bone within four to six weeks. Some existing delivery systems do not fulfill these requirements as they are resorbed more slowly than it takes for the DBM-induced regeneration of the defect to take place, thus, inhibiting or delaying the bone formation (Urist et al. 1987, Schmitz and Hollinger 1988, Lovell et al. 1989). Secondly, osteoinduction may be inhibited by a bioincompatible carrier that interferes physiologically with some part of the multistep cascade of bone induction, e.g., by inducing a chronic inflammation (Sela et al. 1986, Alper et al. 1989).

By qualitative microscopy, no difference in bone induction could be identified between the composite of polyorthoester and DBM (papers II, III and IV). At week one, osteoprogenitor cells

encapsulated the DBM and some chondrocytes were seen. At week two, cartilage and incipient bone formation were present. At week three, more bone was seen and by week four, ossicles with bone marrow had formed. Around the implants of polyorthoester and composite an inflammation with some giant cells was present until week two. The polyorthoester was seen until week 3-4 but thereafter only traces could be identified in some sections. The composite of DBM and polyorthoester showed highly significant ($p<0.0001$) increased ^{85}Sr uptake compared with that of bioerodible polyorthoester alone (paper II), whereas no significant difference in ^{85}Sr uptake was found between the composite and the DBM (papers II and III). Thus, it seems unlikely that bioerodible polyorthoester inhibited bone formation by either of the two above-mentioned mechanisms.

In contrast to the polyorthoester, two local hemostatics in clinical use; ordinary nonabsorbable bone wax of 88% beeswax and fibrin-collagen paste were incompletely absorbed, induced a chronic inflammation and inhibited osteoinduction (paper III). The bone wax caused an inflammation with numerous multinuclear giant cells while fibrin-collagen paste caused an inflammation with accumulation of macrophages and proliferation of fibroblasts. Connective tissue and inflammatory cells were seen uniformly distributed through all parts of the composite implants of DBM and bone wax or fibrin-collagen paste, interspersed between the partly resorbed DBM and the local hemostatic. Thus, the lack of osteoinduction cannot be explained solely as an effect of a physical barrier of the local hemostatic. It seems likely that the chronic inflammation induced may be the cause of the inhibition of osteoinduction. Clinically, the result of the study (paper III) suggest that bone wax and fibrin-collagen paste should be used sparingly, whereas polyorthoester with or without gentamicin seems promising as a local hemostatic for use in bone surgery.

Polyorthoester with 5% indomethacin also significantly inhibited DBM-induced heterotopic osteogenesis (paper VI). However, as opposed to bone wax and fibrin-collagen paste, the drug delivery system was absorbed and induced no persistent inflammation. Further, no indomethacin was detectable in blood samples. The results indicate that the inhibition was caused by a

specific local drug effect, e.g., inhibition of local prostaglandin synthesis.

Orthotopic osteogenesis

The critical size defect of the calvaria varies according to species and age. It has been shown that 2 mm parietal defects in 500 g Wistar rats (Freeman and Turnbull 1973), 4 mm parietal defects in 28 day old CD strain, Charles River Breeding Laboratories (Mulliken and Glowacki 1980) and 8 mm parietal defects in 6 months old Sprague-Dawley rats (Tagaki and Urist 1982) do not heal in 12 weeks, 6 months and 12 weeks respectively. We used 4 mm parietal defects in 8 weeks old Wistar male rats and no defect without implant healed during the observation period, while all defects filled with the composite or DBM were completely bridged by bone histologically and radiographically by 4 weeks postoperatively (paper IV).

In the defects filled with the composite or polyorthoester, the polyorthoester was present and slight inflammation with monocytes and some giant cells was seen at week two (paper IV). The inflammation subsided by week three and only traces of the polyorthoester could be detected at week four. In the defects filled with DBM or without implant, the inflammation was milder and no giant cells were detected at week two.

In the radial defects, no significant difference was found between the area of new bone by DBM alone and the composite of DBM and polyorthoester radiographically (paper V). The area of new bone was significantly less in the defects with no implant or filled with polyorthoester only. Only a few of the defects filled with DBM or the composite were fully bridged by new bone. Typically, union of the new bone with the proximal fragment of the radius had occurred, while a small defect remained distally. In the defects with no implant or filled with polyorthoester only some bone formation was typically seen at the bone edges.

Conclusions

1. The novel osteoquantum index was found to have a linear relationship to the area of the induced bone with a correlation coefficient (r) of 0.90 and high statistical significance ($p<0.0001$). Only weak linear relationships were found between the osteogenic index and the area of the bone ($r=0.32$, $p=0.03$) and between the osteogenic index and the osteoquantum index ($r=0.33$, $p=0.03$) (paper I). The osteoquantum index and the area of the induced bone both increased with increasing mass of implanted DBM, whereas the osteogenic index did not change significantly (paper I).

2. Bioerodible polyorthoester did not inhibit heterotopic osteoinduction, it caused only a slight inflammation that subsided within 3 weeks postoperatively and it was mostly absorbed by week 4 (papers II, III and VI). In contrast, nonabsorbable bone wax and fibrin-collagen paste both were incompletely absorbed, induced a chronic inflammation and inhibited osteoinduction (paper III).

3. The composite of polyorthoester and DBM induced bony healing of large calvarial (paper IV) and radial defects (paper V) as DBM alone. Moreover, the composite was moldable and easily contoured, and technically easier to use than DBM alone. Finally, the polyorthoester provided local hemostasis when used either alone or in composites with demineralized bone (papers IV and V).

4. Polyorthoester with 5% indomethacin significantly inhibited DBM-induced heterotopic osteogenesis, probably by local mechanisms as indomethacin could not be detected in any blood sample (paper VI).

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