JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 17 (2006) 139–145

The copolymer of ε-caprolactone-lactide and tricalcium phosphate does not enhance bone growth in mandibular defect of sheep

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In the field of craniomaxillofacial and orthopaedic surgery there is a constant need for bone or bone substitute. At the present, the most effective way to enhance bone healing clinically is to use autogenous bone grafts. The problems associated with the use of these autografts are donor site morbidity, limited supply and need for a second operative site. Currently there are several different synthetic products commercially available in the market; nevertheless, none of them is ideal for filling bone defects. Therefore, search for new synthetic materials for bone replacement is necessary. A mixture of tricalcium phosphate (TCP) and ε-caprolactone-lactide copolymer P(ε-CL/DL-LA) was prepared and implanted in critical size mandibular bone defects in twelve sheep. Contralateral side was used as a control. Follow-up times for histological and radiological studies were 9, 14, 24 and 52 weeks. We found that the implanted material did not enhance bone formation compared to control site. We also confirmed that defect size was of critical size, since there was no complete healing of the control site either. The results do not encourage us to continue our studies with the mixture of TCP and P(ε-CL/DL-LA) as a filling material for bone defects. Therefore the search for the ideal material is still ongoing.

Introduction

In the field of reconstructive craniomaxillofacial and orthopaedic surgery there is a constant need for new bone. Bone transplantation is required in many different surgical operations due to trauma, congenital deformities, tumors and infections. At the present, the most effective way to enhance bone healing in clinical work is to use autogenous, cancellous bone grafts. The problems associated with the use of these autografts are donor site morbidity, limited availability and often a need for a second operative site. Sometimes also contouring and shaping autogenous grafts is difficult. Therefore an ongoing search for new synthetic materials for bone replacement is necessary.

Biosynthetic bone replacement materials and other agents enhancing bone growth are alternatives to autogenous bone grafting. Some of the earliest biomaterials used as bone substitutes were calciumphosphate compounds, mainly hydroxylapatite and tricalciumphosphate (TCP), which since 1970's have been used in bone replacement [1]. Today different kinds of

calciumphosphate compounds are available as commercial products and used in surgical operations [2, 3]. Most often they are in the form of powder or granules.

Biodegradable polymers can be used when temporary presence of biomaterial is needed in tissue replacement, tissue augmentation, tissue support or in drug delivery systems. Derivatives of α-hydroxyacids, aliphatic polyesters, are the most commonly used polymers in different kind of medical devices in reconstructive surgery. ε-caprolactone-lactide copolymer is one of these polymers. Numerous promising experimental studies have been performed using ε-caprolactone-lactide copolymer as a guidance tube for peripheral nerve regeneration [4–10], as a surgical suture [11], in drug delivery systems [12] in meniscal tissue regeneration [13] and as a cardiac graft [14].

In the present study, β-tricalciumphosphate was mixed with biodegradable polymer, a copolymer of ε-caprolactone-D, L-lactide. This paste was implanted into critical size mandibular bone defects in twelve sheep. The aim of the study was to assess tissue reactions of the mixture during follow-up time of 52 weeks and to compare the healing at the site of implantation with the contralateral control site.

Materials and methods

Experimental animals and implant material

The animal handling and the procedures were performed according to local ethical rules (Helsinki Declaration) and approved by local ethical committee. Twelve adult landrace sheep of both genders, weighing from 52 to 83 kg, were used as experimental animals. The raw material was a copolymer of ε-caprolactone and D,L-lactide acid (P(ε-CL/DL-LA) prepared in the Laboratory of Polymer Technology, Helsinki University of Technology, Espoo, Finland. Polymerization has been explained in detail by Hiljanen-Vainio *et al.* [15]. The initial monomer ratio was 60/40 w/w, and the initial weight average molecular weight (M_w) of the copolymer was 19 400 and the number average molecular weight (M_n) was 14 200. Molecular weights were determined by room-temperature SEC (Water System Interface Module, Waters 700 Satellite Wisp, and four linear PL gel columns: 10^4, 10^5, 10^3, and 100 Å connected series). Tetrahydrofuran was used as the solvent and eluent. The sample was filtered through a 0.5 μm Millex SR filter. The injected volume was 200 μL and the flow rate 1 mL/min. Monodisperse polystyrene standards were used for primary calibration, which means that the Mark-Houwink constants were not used. The copolymer was in paste form. The implanted material was a 27/73 w/w mixture of (P(ε-CL/DL-LA) and β -tricalciumphosphate (β -tri-Calcium phosphate®, Fluka Chemie AG, Buchs, Switzerland). It was prepared in the Institute of Biomaterials, Tampere University of Technology, Tampere, Finland by heating the copolymer slightly and mixing it mechanically with TCP powder. The mixture of (P(ε-CL/DL-LA) and TCP was sterilized by means of gamma radiation (Kolmiset Oy, Ilomantsi, Finland). The minimal dose was 25 kGy.

Operative procedure

Food was withheld for 48 h prior to the surgery, but sheep had free access to drinking water. The sheep were free of any clinical signs of disease. A 0.5 mg dose of atropin (Atropin® 1 mg/ml injekt, Orion, Espoo, Finland) was administered subcutaneously (s.c.) half an hour before the induction of anesthesia. Medetomidine (Domitor® 1 mg/ml, Orion-Farmos, Turku, Finland) 20 μg/kg bodyweight (bwt) was given by intravenous (i.v.) injection. Anesthesia was induced i.v. with propofol (Diprivan® 10 mg/ml, Zeneca Ltd, Macclesfield, England) 3 mg/kg bwt and maintained with 2–2.5 % halothane (Trothane®, I.S.C Chemicals Ltd, Bristol, England). The sheep were intubated and positioned in sternal recumbency with the head extended and fixed in a cushion. A cannula was placed in the saphenous vein. During the operation, 1000 ml of iv fluids (Ringersteril®, Medipolar, Oulu, Finland), metronidatzole (Flagyl® 5 mg/ml, Rhone-Poulenc Rorer A/S, Birkerod, Denmark) 11 mg/kg and benzylpenicillin sodium (Geepenil®, Orion-Farmos, Turku, Finland) 35 000 IU/kg were administered i.v. Both sides of each mandible were shaved and scrubbed with antiseptic solution, chlorhexidine gluconate (Klorhexol® 5 mg/ml, Leiras, Finland).

An incision was made along the inferior border of the right side of mandibular body, and soft tissues and periosteum were reflected down to bone. A 23 mm × 11 mm defect was drilled with the surgical bur through the cortex to cancellous bone. All cortical bone from that area was removed and the mandibular nerve was exposed (Fig. 1) . The defect was filled with the mixture and the incision was closed in layers, using absorbable sutures (Dexon®). The same procedure, without insertion of the material was carried out on the left side of the mandible. About 0,3 g of the material was also implanted subcutaneously for electronmicroscopical studies, which have been published earlier [16].

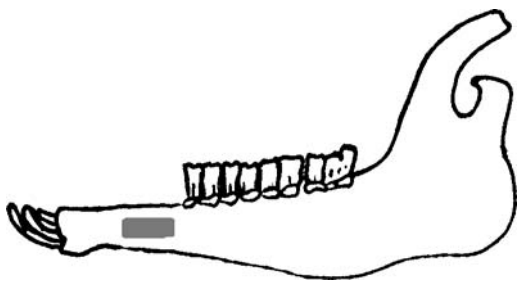

Figure 1 Schematic drawing of the surgical technique in mandible.

Postoperative procedure

Postoperatively, benzylpenicilliumprocaine (Ethacillin® vet injekt 300 00 IU/ml, Intervet, Boxmeer, Holland) 35 000 IU/kg sc and phenylbutazone (Reumuzol® 200 mg/ml vet injekt, Lääkefarmos, Turku, Finland) iv 8 mg/kg were administered once a day for three days. Animals were free to move in their pens and were fed *ad libitum*.

Follow-up times and specimens

Histological studies

The sheep were killed at 9, 14, 24 and 52 weeks postoperatively in groups of three. The mandibles were harvested, cut with oscillating saw and dissected free of soft tissue. For histological studies the bony specimens were fixed in 70% alcohol and embedded in methylmethacrylate [17]. Five micrometer thick sections were cut with Reichert-Jung microtome and stained using the Masson-Goldner method [18] and evaluated by means of light microscopy (Olympus BH-2 microscope with attached Olympus D12 digital camera).

Radiographic studies

All sheep were anesthetized as described above. Extraoral radiographs were taken immediately after operation and after 3, 6 and 9 weeks of follow-up and at the end of follow-up time. Radiographs were taken in lateral position (target-tube distance 115 cm, 48 kV, 8 mAs). The size of the implanted defect was evaluated compared to the size of the non-implanted control side. All radiographs were analyzed by the same person.

Results

No immediate complications were seen postoperatively. There was a fissural mandibular fracture at the site of implantation in one sheep, which was visible radiologically at three weeks after the operation. The animal was closely followed and the fracture healed with heavy callus formation without intervention. This animal was followed 52 weeks and the fracture was considered consolidated already by nine weeks. Otherwise the recovery was uneventful in all other animals.

(a) *Histological results*

The implantation and control sites were clearly visible in all samples.

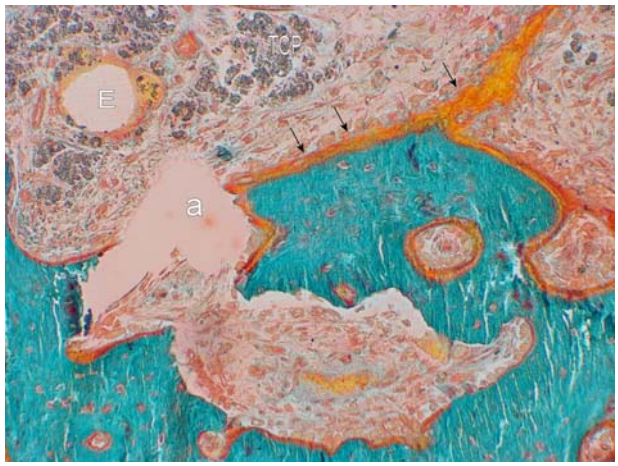

Figure 2 9-week follow-up, site of implantation. Small, empty material space is seen (E). Numerous TCP-particles are discernible in connective tissue. Bone is surrounded by rim of osteoblasts and osteoid (arrow). Artefact (a). Original magnification ×200.

Nine weeks

Site of implantation

Empty spaces, which indicated the dissolved copolymer and small TCP-particles, were observed. Connective capsule encircled the buccal part of the defect area and there was also connective tissue formation between the empty material spaces. A chronic inflammatory reaction was observed with variable amounts of foreign body giant cells, which were lining the empty material spaces. TCP was also found inside of foreign body giant cells. In two samples out of three there was slight formation of bone on the lingual side of the defect area (Fig. 2).

Control site

There were no signs of inflammation. Fat cells were abundant and connective tissue had filled most of the defect area. In two samples out of three there was more bone formation on the lingual side of the defect in this group than in implantation group.

Some osteoblasts were also found adjacent to the rim of osteoid, which was lining the newly formed bone (Fig. 3).

Fourteen weeks

Site of implantation

Empty material spaces were more extensive compared to those at 9 weeks and TCP-particles were abundantly visible. There was also a chronic inflammation with foreign-body giant cells and macrophages engulfing TCP-particles as in previous follow-up. Connective tissue and collagen formation were seen. New bone formation was still ongoing from lingual part of the defect

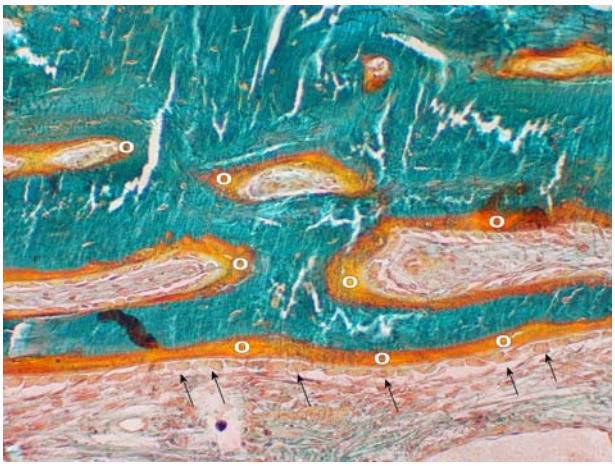

Figure 3 9-week follow-up, control site. Active bone formation is observed by rim of osteoid (o) and osteoblasts (arrow). Original magnification ×200.

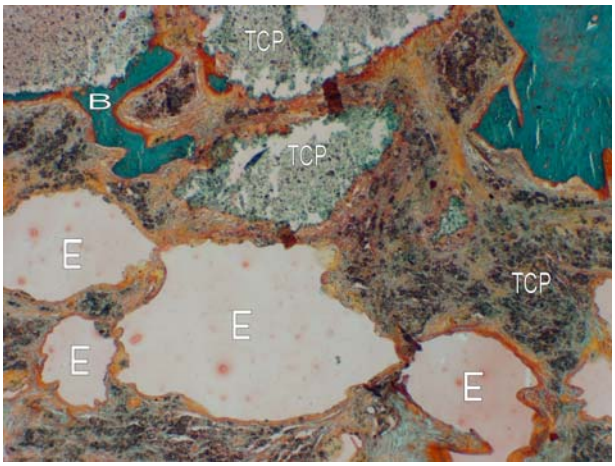

Figure 4 14-week follow-up, site of implantation. Large empty material spaces (E) with multinucleated giant cells are visible. Large areas of connective tissue are occupied with TCP-particles (TCP). Some new bone (B) formation with osteoid can be observed. Original magnification ×100.

and TCP-particles were seen adjacent and in the marrow spaces of new bone (Fig. 4).

Control site

No inflammation was observed. Connective tissue formation was as in previous follow-up and numerous fat cells were present. Compared to the site of implantation more new bone formation and osteoblast activity was seen. The border zone of newly formed woven bone and old lamellar bone was evident.

Twenty-four weeks

Site of implantation

A severe granulomatous inflammation was observed. The amount of foreign body giant cells and

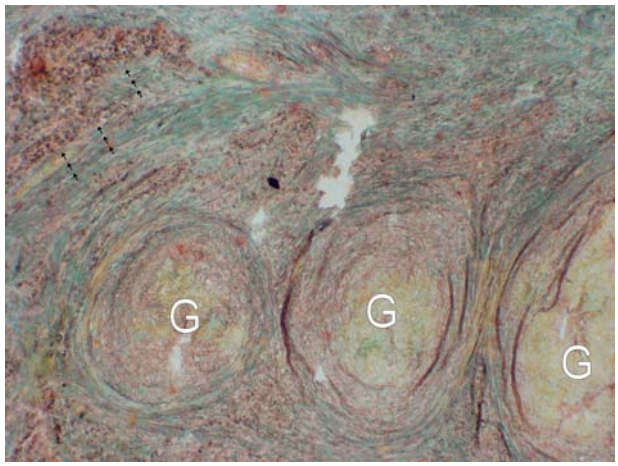

Figure 5 24-week follow-up, site of implantation. Three granulomas (G) surrounded by fibrous connective tissue presenting severe inflammatory reaction. Numerous TCP-particles are visible (repeating end arrows). Original magnification ×40.

macrophages was considerably higher than in previous follow-ups. In one sample out of three there were large, empty material spaces surrounded by multinucleated giant cells and macrophages. TCP-particles were found inside the giant cells. In the second sample large rounded granulomas surrounded by fibrous septae were observed (Fig. 5). In the last sample the granulomas were not discernable, only severe inflammation in the connective tissue. Bone formation with the osteoid line was still ongoing from the lingual side of the defect.

Control site

No inflammation was present. The amount of new bone was enlarged compared to the site of implantation. Fat cells were abundant.

Fifty-two weeks

Site of implantation

Small material spaces surrounded by granulomatous inflammatory reaction were still found. TCP was observed inside the multinucleated giant cells and inside of macrophages (Fig. 6). Bone formation was ceased, there was no increment of new bone compared to previous follow-up and the thin osteoid line was occasionally seen. Connective tissue filled the greatest part of the defect. Fat cells were also present.

Control site

No inflammation was present. The amount of bone was greater than at the site of implantation, but no active new bone formation was found. Connective tissue filled most of the defect site (Fig. 7).

(b) *Radiological results*

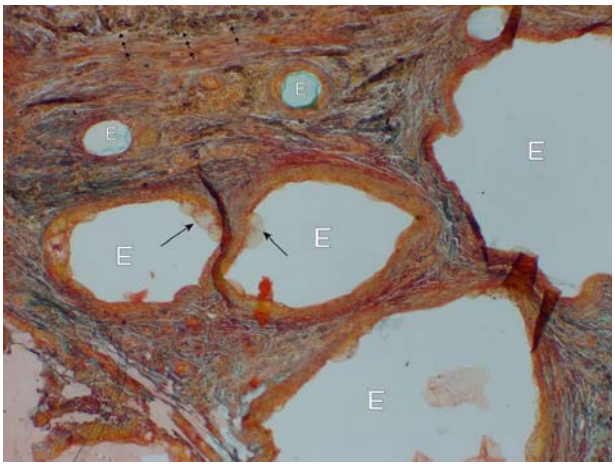

Figure 6 52-week follow up, site of implantation. Empty material spaces (E) surrounded by multinucleated giant cells (arrow) in fibrous connective tissue. TCP-particles are abundantly visible in the upper part of the photomicrograph (repeating end arrows). Original magnification ×40.

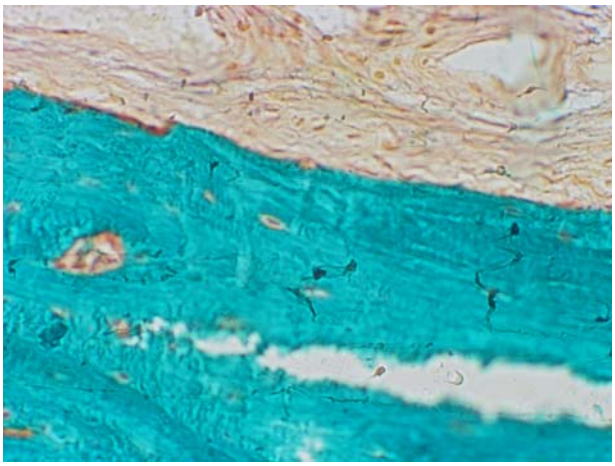

Figure 7 52-week follow-up, control site. No new bone formation, no active osteoblasts are observed. Original magnification ×200.

Due to the limited imaging possibilities, unfortunately, the radiographs could not be analyzed realiably in detail. However, the radiographs clearly showed the same phenomenon seen in histological samples revealing a non healed defect in both sides at all radiological follow-up times. Consequent radiographs of one sheep of 52-week follow-up group are shown in Fig. 8.

Discussion

In our previous study a mixture of P(ε-CL/DL-LA) and TCP was implanted into mandibular bone defect of rabbit. The size of the bone defect at the site of implantation was gradually decreasing during the 15 weeks of observation [19]. The results of the study of Senkouly *et al.* [20] are in line with ours. They implanted a mixture of poly (D,L-lactide/ε-caprolactone) and hydroxyapatite in rat femur and concluded that composite material effectively led to new bone formation. Based on promising results, the present study, where the defect would mimic a large human mandibular defect, was planned. The defect size was 23 × 11 mm, whereas it was 2 × 2 mm in former studies. Surprisingly, the results of present study contradicted with the results from previous ones. There was a slight bone formation ongoing at the site of implantation from the beginning of the follow-up until 24 weeks of follow-up. However, bone formation and osteoblast activity was greater at the contralateral control site than at the site of implantation during the whole observation period. Bone formation had ceased by the 52-week of follow-up at the site of implantation and at the control site. The empty material spaces were still clearly visible as well as non-resorbed TCP-particles. This result was unexpected. It seems that in small bone defects and with short follow-up times, 24 weeks or less, there is some formation of new bone. However, larger defects and longer follow-up times are needed to reveal that bone formation does cease before the defect is completely filled with bone.

There are also promising results from bone growth studies, in which TCP is placed into small and large bone defects. Gatti *et al.* [21] implanted TCP granules in mandibular defects of three sheep. They observed histologically full repair of 4 mm holes four months after implantation and found out that no granules were present in the new bone after one year. Lange *et al.* [22] placed granular tricalciumphosphate into large cancellous defects of tibia and femur of pigs and concluded that TCP is comparable to autogenous cancellous bone graft when placed in moderate metaphyseal bone defects. Based on these findings it seems possible that one reason for the unsatisfactory result of the present study is that biodegradation of the polymer is too slow to allow the resorption of the implanted material in due time. It is also very likely that acidic degradation products of poly (D,L-lactide/ε-caprolactone)-copolymer may prevent or retard bone formation in the proximity of the implanted material resulting poor bone formation. The study by Schliephake *et al.* [23] supports this conclusion. They found out that calvarial defects of rats were not filled with bone after one year of follow up, when glass-ceramic/polylactic acid copolymer was used as an implant material.

Large bone defects are not completely filled with new bone unless soft tissue ingrowth into the site of defect is prevented. Bone defects can be filled with bone graft or they can be covered with different kind of barriers or membranes to prevent soft tissue ingrowth. Connective tissue infiltration into the site of bone defect is an undesirable result, because soft tissue ingrowth inhibits the formation of new bone. During bone healing cells migrate to the defect area with different rates and it is

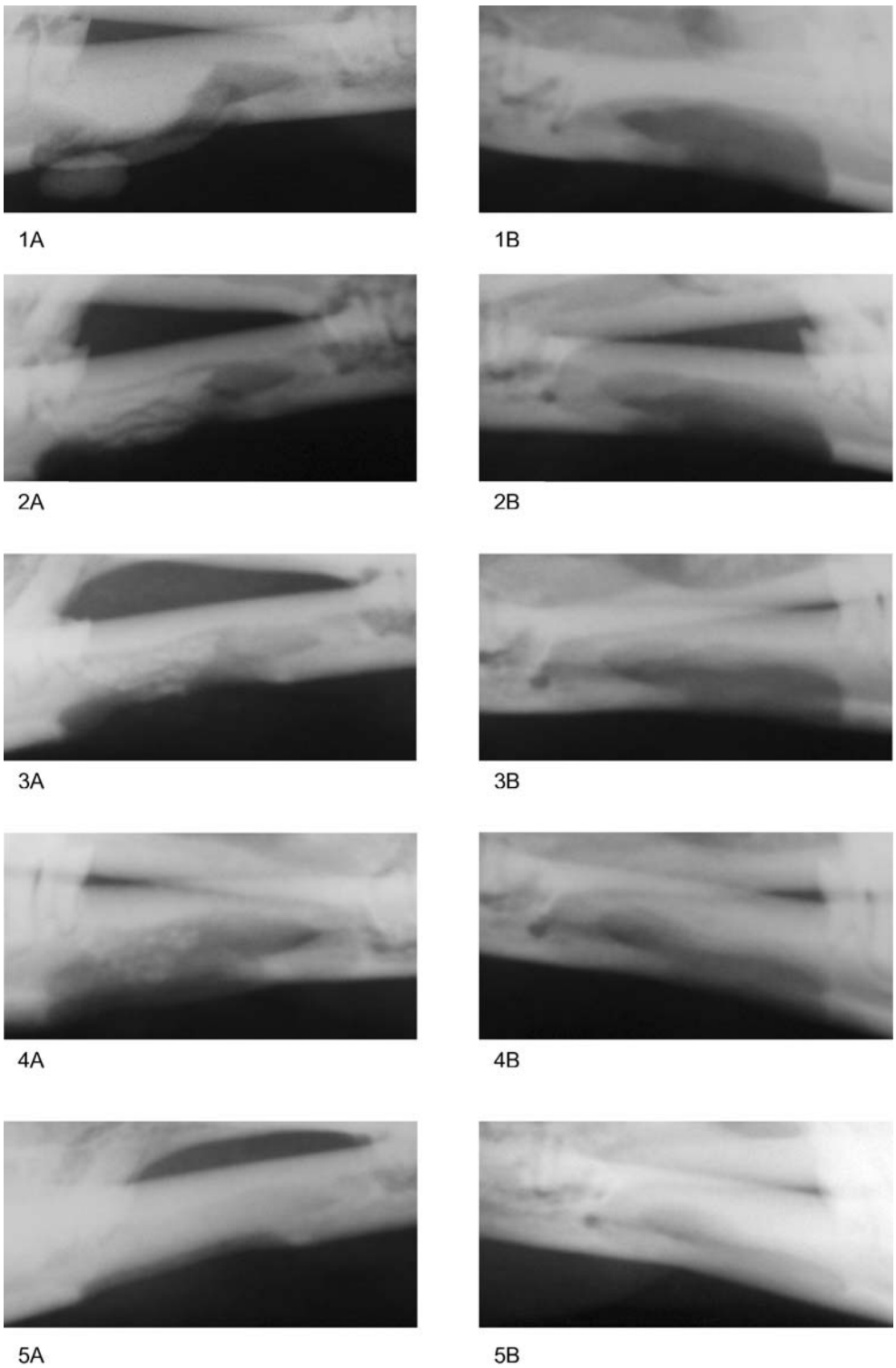

Figure 8 Radiographs of the sites of implantation and control at different follow-up times. At 52 weeks both the control and study defects are still not filled with bone. 1A: after operation, site of defect; 1B: after operation, control site; 2A: 3 week follow-up, site of defect; 2B: 3 week follow-up, control site; 3A: 6 week follow-up, site of defect; 3B: 6 week follow-up, control site; 4A: 9 week follow-up, site of defect; 4B: 9 week follow-up, control site; 5A: 52 week follow-up, site of defect; 5B: 52 week follow-up, control site.

presumed that cells with osteogenic potential are slower than connective tissue cells. At the present study connective tissue filled most of the defect area at the end of the follow-up. This finding is in agreement with the study by Dahlin *et al.* [24]. They operated a rat skull defect, which did not heal spontaneously. The defect was left without bone chips or membrane. At the end of 52-week follow-up only slight remodelling of bone margins was found, mostly the defect was filled with connective tissue.

Biodegradable materials often cause a chronic inflammation at the site of implantation [25]. This phenomenon is related to tissues capacity to clear the degradation products of foreign material and usually it is not significant in practice, but it is considered as a disadvantage. Clinically there were no signs of inflammation at the present study and the healing was uneventful, except a fissural mandibular fracture at the site of implantation in one sheep. Histologically, the mixture of P(ε-CL/DL-LA) and TCP excited a chronic inflammatory reaction in bone from the beginning of the follow up time until the end of follow up, week 52. At 24 week of follow up inflammation was graded as severe. The presence of foreign body giant (FBG) cells and macrophages was evident through the whole period of observation. TCP-particles were seen inside of FBG-cells and macrophages. Empty material spaces of different sizes were found from all samples of the implantation site. No inflammatory reaction was found at the contralateral control site. These findings are in agreement with previous experimental studies performed with P(ε-CL/DL-LA) [4, 5, 7, 8].

Autogenous bone grafts are sometimes difficult to shape. The mixture of P(ε-CL/DL-LA) and TCP was white and opaque in color, the appearance was gummy and the mechanical strength was weak. It could be moulded easily by hands to desired form, which made it excellent to handle and very easy to place into bone defect. Chronic inflammation around the implanted material and a long degradation time, which prevents or retards bone formation and causes soft tissue ingrowth, were disadvantages of the implanted material. The present study does not encourage us to continue our studies with the mixture of P(ε-CL/DL-LA) and TCP as a bone filler and thus search for an ideal material for bone filling is going on. The possible solution could be the use of biomaterial combined with cell therapies or/and growth factors.

Acknowledgments

The author would like to thank M.Sc. Jonna Rinne for revising the language of the manuscript. This work was supported by a grant from the Finnish Cultural Foundation. The study was financially supported from government subsidies.

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Received 2 April
and accepted 28 June 2005