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Mixture of ε-caprolactone-lactide copolymer and tricalcium phosphate: a histological and immunohistochemical study of tissue reactions

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In cranio-maxillofacial surgery, bone transplantation is needed for treatment of bony defects. An autograft, allograft or biomaterial can be used. Autogenous bone grafts are considered to be the best materials available, but there are some disadvantages in their use including donorsite morbidity, need for a second operative site and limited graft supply. A search for new bone-graft materials therefore remains necessary. We prepared a mixture of tricalcium phosphate (TCP), which is a resorbable, non-toxic, osteoconductive ceramic material and ε-caprolactone-lactide copolymer P(ε-CL/DL-LA), a resorbable polymer, and placed it in the dermis and in mandibular bone defects in 13 rabbits. Follow-up times were two, three, seven, eight, 12, 15 and 18 weeks, tissue reactions were assessed, histologically and immunohistochemically. Times of resorption of the material from tissues were reported. We found that the mixture caused a mild inflammatory reaction when placed in bone and severe inflammation when placed in dermis. No highly fluorescent layer of tenascin or fibronectin was found surrounding the implant area. The mixture was excellent to handle and very easy to place into bone defects. The results are promising and have led us to continue development of the mixture. © *1999 Kluwer Academic Publishers*

1. Introduction

In cranio-maxillofacial surgery, bone transplantation is needed for surgical treatment of bony defects resulting from trauma, infection or congenital deformity. Other possible indications for use of a bone graft are contour augmentation and reconstruction of segmental defects. In dentistry there is a need for new bone in periodontology, in cases in which there are deep

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periodontal pockets through loss of bone and in implantology, when an implant is inserted immediately after extraction.

An autograft, allograft or biomaterial can be used. Autogenous bone grafts are considered to be the best materials available and to represent a "gold standard" in relation to treatment. However, there are some disadvantages in their use, including donor site morbidity, need for a second operative site, and limited graft supply, especially in children. Problems connected with use of allografts include transmission of viral infection and poor immunological compatibility. A continuing search for new bone-graft materials is therefore necessary.

Tricalcium phosphate (TCP) is a porous ceramic material, which has the biological properties of resorbability, lack of foreign-body response, osteoconductivity and lack of local and systemic toxicity [1]. It acts as a scaffold for bone ingrowth, undergoing progressive degradation and replacement by bone. It is brittle and weak, with very low tensile and shear strengths, and cannot be used alone in cases in which it has to bear initial structural load [2]. The strength of TCP is comparable to that of cancellous bone, and can be used strictly to replace that [3]. It is most often used in granule or powder forms. Such forms can sometimes be difficult to handle during surgical operations.

The most important polymers used in medical and surgical applications are derivatives of α-hydroxyacids, aliphatic polyesters. ε-caprolactone and D,L-lactide polymers are such derivatives. They can be copolymerized to ε-caprolactone-lactide copolymer, which is available in, for example, solid, wax and paste states [4]. The copolymer has been used in many studies in animals and tissue reactions have been found to be mild and biocompatibility of the copolymer good [5–13].

The aim of our study was to develop a filling material for bone defects. We mixed ε-caprolactone-lactide copolymer paste and TCP powder, and implanted the product into the dermis and mandibular bone of rabbits. Tissue reactions were assessed histologically and immunohistochemically. Times of resorption of the material from the tissues were also determined.

2. Experimental procedure

2.1. Experimental animals and implant material

Thirteen adult rabbits (New Zealand White), of both sexes, weighing from 4500 to 5500 g were used. The raw material used was a copolymer of ε-caprolactone and D,L-lactic acid, P(ε-CL/DL-LA), prepared in the Laboratory of Polymer Technology, Helsinki University of Technology, Espoo, Finland (Fig. 1). Polymerization is explained in detail by Hiljanen-Vainio *et al.* [14]. The initial monomer ratio was 40 : 60 w/w, and the initial molecular weight, Mw, of the copolymer 17 900. It was in paste form. The implanted material was a 50 : 50 w/w mixture of P(ε-CL/DL-LA) and TCP. It was prepared in the laboratory by heating the copolymer slightly and mixing it mechanically with the TCP powder. The mixture of P(ε-CL/DL-LA) and TCP was sterilized by means of γ-radiation (Kolmi-Set Oy, Ilomantsi, Finland). The minimal dose was 25 Gy.

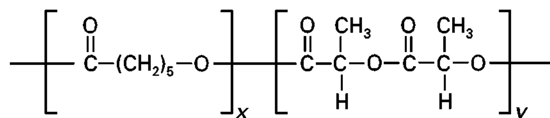

Figure 1 Chemical structure of ε-caprolactone-lactide copolymer.

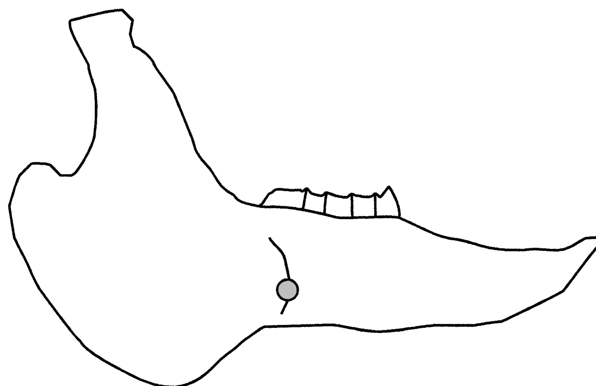

Figure 2 The surgical technique in bone.

2.2. Operative procedure

The rabbits were not fasted before operation. They were anaesthetized with subcutaneous (s.c.) medetomidine (0.3 mg kg^{-1}) and ketamine hydrochloride (50 mg kg^{-1}). They also received procaine benzylpenicillin (30 mg kg^{-1} s.c.) pre-operatively, to prevent infection. Both sides of each mandible were shaved and scrubbed with 80% ethanol. An incision was made along the inferior border of the right side of the mandible body, and soft tissues and the periosteum were reflected down to bone. A 2 mm wide defect was drilled with a dental bur to cortical bone (Fig. 2). The material was inserted into the hole 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 mg of the material was also implanted extra-orally, subcutaneously, in the right cheek.

2.3. Post-operative procedure

All of the animals were fed *ad libitum* after the surgical procedure, and they were free to move in their cages. For post-operative pain control they also received buprenorphine (0.02 mg kg^{-1}) after surgery, and subsequently every 12 h for two days.

2.4. Follow-up times and specimens

Follow-up times were two, three, seven, eight, 12, 15 and 18 weeks (Table I). After the follow-up time, the

TABLE I Follow-up times (weeks) and number of experimental animals

weeks	2	3	7	8	12	15	18
Number of experimental animals	1	1	2	1	3	3	2

animals were sacrificed by administering 60 mg kg^{-1} of pentobarbital, and mandibles were exarticulated. For histological studies the bony specimens were fixed in 70% alcohol and embedded in methylmethacrylate [14]. They were cut into 5-μm sections, stained using the Masson–Goldner method [16], and evaluated by means of light microscopy. The intensity of the inflammatory reaction was graded as mild, moderate or severe throughout the study.

The subcutaneously implanted material was carefully dissected and prepared for histological and immunohistochemical studies. Samples were snapfrozen in precooled isopentane-containing vials in a slurry of ice, and stored at $-70\,^{\circ}$C. For histological studies, the connective-tissue specimens were cut in 6-μm sections, fixed with formaldehyde and stained with haematoxylin–eosin. They were evaluated by means of light microscopy. The intensity of the inflammatory reaction was graded as mild, moderate or severe. For immunohistochemical studies, 5-μm thick cryosections were cut, air-dried, and briefly fixed with cold acetone. Indirect immunofluorescence microscopy was performed using monoclonal antibodies (Mabs) 100EB2 [17] against human tenascin (Tn) and 52DH1 [18] against human cellular fibronectin (Fn). The specimens were incubated with the Mabs for 30 min. Fluorescein isothiocyanate (FITC) coupled with sheep antimouse IgG antiserum (Jackson Laboratories, West Grove, PA) was then applied. The specimens were evaluated using a Leitz Aristoplan® fluorescence microscope equipped with appropriate filters for immunohistochemistry.

3. Results

3.1. Histological study

3.1.1. Bony specimens

3.1.1.1. Three-week follow-up. In the implant area, the site of implantation was clearly visible. The material itself was not visible, but empty spaces, round in shape, were seen. There was slight osteoid formation, and some woven bone was also seen at the border of the defect. The implanted material had provoked a moderate inflammatory reaction. Lymphocytes, plasma cells and macrophages were seen. A foreign-body reaction with a number of giant cells was also seen (Fig. 3).

In the control area, the defect was visible. There was more woven bone at the margin of the defect than in the study group in which implantation took place. Osteoblasts, which were lining the new bone, and some osteoclasts, were also seen. A mild inflammatory reaction was observed.

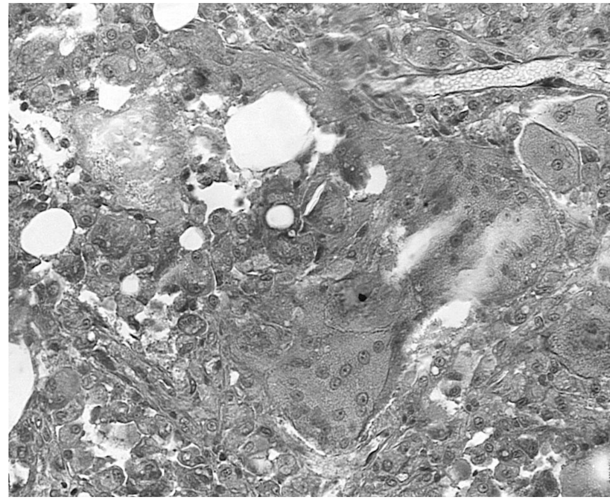

Figure 3 Moderate inflammatory reaction: plasma cells, lymphocytes, macrophages and foreign-body giant cells are seen in a three-week bone specimen. Empty spaces indicate the dissolved material. Masson–Goldner stain. Original magnification × 250.

3.1.1.2. Seven-week follow-up. In the implant area, the site of implantation was clearly visible. Very slight osteoid formation was seen at the edges of the defect with marked osteoblast and also some osteoclast activity lining it. The amount of bone was less than after three weeks of follow-up. More foreign-body giant cells were also seen. The inflammatory reaction was moderate.

In the control area, the defect was no longer clearly evident, even though several slides were prepared from the samples, at different levels.

3.1.1.3. Eight-week follow-up. In the implant area, there was almost no formation of new bone, as little as after seven weeks of follow-up. Empty spaces where material had been implanted were extensive. Inflammatory reaction was moderate, but less than after seven weeks of follow-up.

In the control area, the defect site was no longer evident.

3.1.1.4. Twelve-week follow-up. In the implant area, the area of implantation was still evident. Some woven bone and some lamellar bone was visible at the center and edge of the defect. Formation of osteoid, and osteoblast activity, was noted. The amount of new bone had increased markedly over amounts after shorter follow-up times, and the border of the defect was fading. Empty spaces where material had been were less extensive than before. The inflammatory reaction was moderate in two out of three samples and mild in one sample. The number of foreign-body giant cells was less than after shorter follow-up periods.

In the control area, the defect was no longer evident.

3.1.1.5. Fifteen-week follow-up. In the implant area, the area of implantation had become smaller, reducing in size from the margins. The majority of the new bone

was lamellar, but some woven bone was also visible centrally (Fig. 4). Mononuclear inflammatory reaction was mild, but foreign-body giant cells remained evident.

In the control group, the defect was no longer evident.

3.1.2. Dermal specimens

Follow-up times were two, eight, 12, 15 and 18 weeks. The implantation site was clearly visible in all samples, and there were many small and empty material spaces. A severe, chronic, mononuclear inflammatory reaction was noted in connective tissue around the material (Fig. 5). Macrophages were seen engulfing the material at all follow-up times. No foreign-body giant cells were seen. Eosinophilic leukocytes were seen throughout the observation period. No material was resorbed during follow-up.

In all samples taken from the mandible or dermis at any given follow-up time there was individual variation in intensity of the tissue response.

3.2. Immunohistochemical study

Some tenascin-immunoreactive fibers were detected in dermal connective tissue bordering the implant material even during the second week after operation. The extent and intensity of Tenascin immunoreactivity did not change markedly during 18 weeks of follow-up (Fig. 6).

No highly fluorescent fibronectin-immunoreactive layer was detected during follow-up. Some fibronectin immunoreactivity was also seen in connective tissue close to implant material. The intensity of fibronectin immunoreactivity was relatively low during follow-up (Fig. 7).

4. Discussion

The mixture of P(ε-CL/D,L-LA) and TCP provoked a moderate inflammatory reaction in bone throughout follow-up until week 15, when inflammation was graded as mild. Macrophages, plasma cells, lymphocytes and foreign-body giant cells were seen, as in

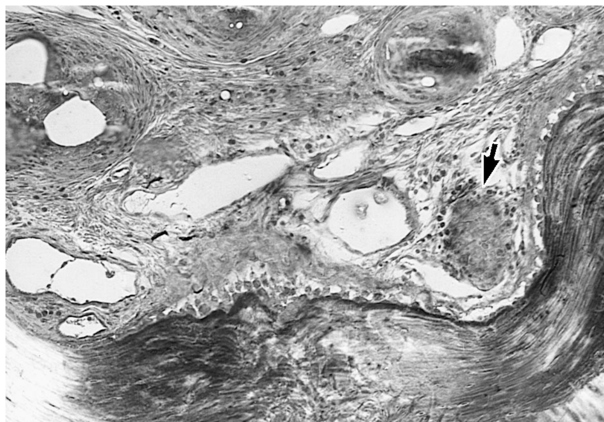

Figure 4 Fifteen weeks of follow-up: most new bone is lamellar, but some woven bone is also visible (arrow). Mononuclear inflammatory reaction is mild. Masson–Goldner stain. Original magnification × 125.

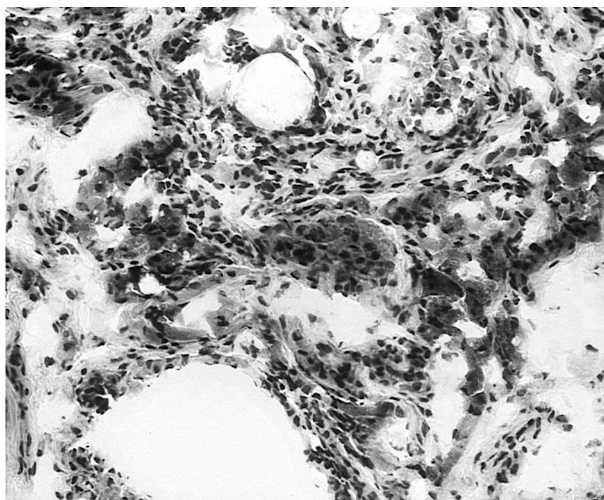

Figure 5 Severe, chronic inflammatory reaction in an 18-week dermal specimen. Spaces devoid of material remain large. H–E stain. Original magnification × 125.

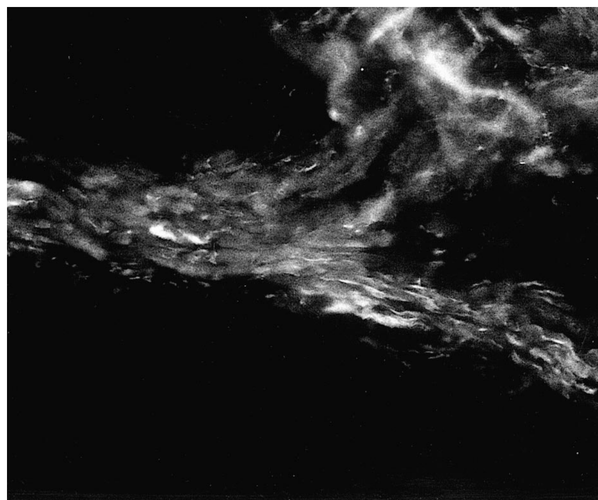

Figure 6 Immunofluorescence micrograph showing tenascin immunoreactivity in connective tissue close to implant in a 12-week sample. Original magnification × 320.

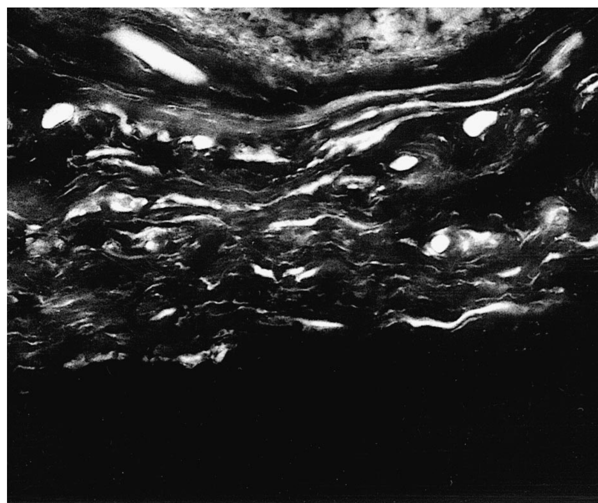

Figure 7 Immunofluorescence micrograph showing fibronectin immunoreactive fibers in the connective tissue close to implant material 15 weeks after operation. Original magnification × 320.

studies with ε-caprolactone-lactide copolymer. TCP is known not to provoke any inflammatory response [3], so the inflammation is most likely a result of the resorption of the copolymer. In a previous study in which P(ε-CL/DL-LA) alone was implanted into bone defects in rat femurs inflammatory response after six months of follow-up was moderate [8]. We therefore decreased the intensity of the inflammatory reaction by addition of TCP to the mixture. Formation of new bone was greater when the mixture of P(ε-CL/DL-LA) and TCP was used than when the copolymer alone was employed to fill bone defects. We found more bone formation after three weeks of follow-up than after seven and eight weeks of follow-up, perhaps because of individual variation and the small numbers of animals used. One problem in cranio-maxillofacial surgery relates to healing of critical-size defects (CSD). A CSD is the smallest intraosseous wound that will not heal spontaneously during the lifetime of an individual [19]. Formation of osteoid in our study was less in the defects where the material had been implanted than in defects in which it had not. This finding was predictable, because the defect was not of critical size. However, we have succeeded in preventing soft tissue growing into the bone defect, which was one purpose of the study.

Subcutaneoulsy implanted material was easy to find on follow-up, because of the TCP in the mixture. The copolymer alone would have been impossible to find in the dermis. In soft-tissue specimens there was connective tissue surrounding the implanted material, and inflammation was severe throughout follow-up. Eosinophilic leukocytes were also found during the whole observation period, which is as in previous studies [6, 12]. They could indicate the existence of some kind of hypersensitivity state. It is also interesting that no foreign-body giant cells were seen in dermal samples, and that no implanted material was resorbed at all during the follow-up.

We also analyzed the dermal expression of two extracellular matrix glycoproteins, tenascin and fibronectin. Tenascin is found during embryogenesis, but is absent from most adult tissue. It is re-expressed during wound healing and oral lichen planus [20, 21], in mammary tumor cells [22], in acute and chronic renal rejection [23] and in odontogenic tumors [24]. Tenascin is detectable in skin wounds more than five days old but it disappears after six–seven weeks [25]. Fibronectin is common in foetal tissues and is found in normal adult tissue, as plasma and tissue. Heikinheimo *et al.* [24] have demonstrated it in odontogenic tumors and it is expressed in many carcinomas [26] and renal rejections [23]. We found tenascin and fibronectin in the encircling connective tissue capsule throughout follow-up. There was no variation in intensity of the fluorescent layer. However, no highly fluorescent layer of tenascin or fibronectin was found facing the implant. This finding is in accordance with results from our histological studies, in which severe inflammation was found around the implanted material. It also explains why tenascin and fibronectin did not disappear by the sixth or seventh week but instead were found throughout follow-up. Kontio *et al.* [27] studied connective-tissue capsules around poly-lactide-implants in rabbits, and they found a highly fluorescent layer of tenascin and fibronectin bordering the implant in the surrounding connective tissue capsule throughout 48 weeks of follow-up. They suggested that implants induced a prolonged tissue response, different from that in conventional wound healing. The substantial expression of tenascin and fibronectin close to the implants could be an early sign of subclinical rejection [27]. If so, the biocompatibility of the mixture of P(ε-CL/DL-LA) and TCP could be better than that of PLA.

Our conclusion is that the mixture of P(ε-CL/DL-LA) and TCP is excellent to handle. It can be molded by hand into various shapes, it is a good haemostatic sealant, and it is very easy to place into a bone defect. However, more studies, of larger bone defects over longer follow-up periods are needed, if the value of the mixture of P(ε-CL/DL-LA) and TCP in bone defects is to be confirmed. The amount of copolymer in the mixture might be reduced, to shorten the resorption time and accelerate the rate of new bone formation.

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