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Porous tricalcium phosphate and transforming growth factor used for anterior spine surgery

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Abstract Harvesting autologous bone graft from the iliac crest is associated with considerable secondary morbidity. Bone graft substitutes such as porous ceramics are increasingly used for spinal surgery. This paper presents the results of an animal study in which β -tricalcium phosphate (β -TCP) bone substitutes were used for anterior spinal surgery in sheep and baboons. The presented baboon study also investigated the effect of impregnating the ceramic material with transforming growth factor (TGF). In the first study, using the sheep model, a stand-alone instrumented anterior fusion was performed. The animals were randomized into three treatment groups: autologous bone, β -TCP granules, and sham group. The results were analyzed biomechanically and histologically at three survival intervals: 8, 16 and 32 weeks. An additional animal group was added later, with ceramic pre-filled implants. In the second study, a baboon model was used to assess the osteointegration of a 15-mm-diameter porous β -TCP block into the vertebral body. The experiment was partially motivated by a new surgical procedure proposed for local bone graft harvest. Three treatment groups were used: β -TCP plug, β -TCP plug impregnated with TGF- β 3, and a sham group with empty defect. The evaluation for all animals included computer tomo-

grams at 3 and 6 months, as well as histology at 6 months. In the sheep model, the mechanical evaluation failed to demonstrate differences between treatment groups. This was because massive anterior bone bridges formed in almost all the animals, masking the effects of individual treatments. Histologically, β -TCP was shown to be a good osteoconductor. While multiple signs of implant micromotion were documented, pre-filling the cages markedly improved the histological fusion outcomes. In the baboon study, the β -TCP plugs were completely osteointegrated at 6 months. For the group that used ceramic plugs impregnated with TGF- β 3, no incremental advantage was seen as a result of this particular application. However, TGF- β 3 is a potent growth factor at a very low dose. Not only does it speed up the ceramic material resorption, but it is also responsible for massive regional new bone formation. More experiments are required to better understand the biological effects of this growth factor in relation to bone formation, and to be able to take clinical advantage of them.

Keywords Tricalcium phosphate · Bone substitute · Transforming growth factor β · Interbody spinal fusion · Anterior surgery · Sheep · Baboon

Introduction

Porous synthetic ceramics

Bone transplantation is a frequent procedure in spinal surgery. Autologous bone is most frequently harvested from the iliac crest, but this requires additional surgery to be performed, adds cost, and is associated with an increase in postoperative morbidity [1, 23, 27]. In addition, autologous bone graft is only available in limited quantities. Bone allograft, on the other hand, would be available in larger quantities, but bears the potential risk of infectious disease transmission [15] or immunologic response, with possible graft resorption.

Ceramic graft materials have now become available in a wide variety of chemical compositions [5]. They are attractive, because there is an infinite supply, the sterilization and shelf storage of the materials is easy, and there is no risk of disease transmission or immunogenic response. Today, the most frequently used inorganic ceramic materials in a clinical setting are tricalcium phosphate (TCP) and hydroxyapatite (HA). They have somewhat different ratios of calcium and phosphorus, but in essence they mimic closely the inorganic phase of bone, which makes up 60–70% of mammalian bone material. Both HA and TCP can act as a scaffold for osteogenic cells. It is important that the scaffold material is open porous [25]. The ability of host bone to gradually in-grow into the synthetic graft material depends on this open porosity. The graft resorption depends on its mineral composition. Whereas HA hardly resorbs at all [8, 21], TCP slowly degrades in vivo, and eventually gets completely replaced with remodeled host bone [6, 8].

Transforming growth factors

Transforming growth factor- β s are a family of multifunctional cytokines that modulate various cellular processes [10, 11]. The TGF- β family includes TGF- β s, activin, and bone morphogenetic proteins (BMPs). TGF- β s play a major role in tissue repair, their main function being to control cell proliferation and differentiation [12, 16]. Five isoforms of TGF- β are known, which show a high level of sequence homology and have similar biological activities, but different potencies [7]. The therapeutic potential of the isoforms expressed in mammals – TGF- β 1, TGF- β 2, and TGF- β 3 – have been investigated for a variety of indications such as wound healing, prevention of oral mucositis, and bone and cartilage repair.

The use of growth hormones from the TGF- β family for the biochemical enhancement of bone healing and bone formation is a field of sustained research. TGF- β 1 was shown to induce bone closure of skull defects [3] and to stimulate bone on-growth, and enhances bone healing

in tricalcium-phosphate-coated implants [13, 24], accelerating the healing in the vicinity of the implant [14].

One important element in the bone healing, as well as the wound healing effects of TGF, may be the strong angiogenesis-inducing properties of these molecules. At the site of injury, angiogenic factors are immediately released from macrophages and tissue. Endothelial cells stimulated by the angiogenic factors migrate and form new blood vessels at the injured site [19].

Anterior spinal surgery animal studies

In this paper, we present two animal studies that both used porous β -TCP bone graft substitute in an anterior surgical procedure of the lumbar spine. The first one was done using the sheep model for an anterior stand-alone interbody fusion, performed with a titanium implant spacer filled with granular β -TCP material. We later added to the anterior fusion model a ceramic pre-filled titanium cage, in order to improve postoperative filling with graft substitute. The preliminary results of this modified model are also presented in this paper.

The second experiment used the baboon model for a partial vertebral defect model. It was motivated by a formerly proposed and biomechanically validated surgical procedure for regional autologous bone harvest from a vertebral body next to the fusion [20]. The goal was to avoid the usual iliac crest bone harvest, but still be able to use autologous bone graft, the material of first choice for obtaining successful spinal fusion. The proposed procedure uses the small amount of bone that can be obtained from a neighboring vertebral body, to fill the cage with autologous graft. The resulting defect, mechanically more stable than the instrumented disc space, can then easily be plugged with a bone substitute. An appropriately sized porous β -TCP block was used to fill the cylindrical defect. The block's osteointegration was then followed over time. A second treatment group was included in this baboon study, utilizing the same β -TCP scaffold, but impregnated with TGF- β 3.

Materials and methods

Instrumented anterior interbody spinal fusion

Fifty-four skeletally mature female Dorset sheep of approximately 45 kg body weight were used for this study. They were divided into three survival groups of equal size, which were sacrificed at 8 weeks, 16 weeks and 32 weeks, respectively. A retroperitoneal approach to the anterior spine was used for exposure of three consecutive disc levels. The step by step surgical technique for retroperitoneal spinal surgery in sheep has been described earlier in great detail [2]. The L2/L3 and L4/L5 disc levels were both prepared for implant insertion by removing the disc material and cartilage from the bony endplates using sharp preparation. The L3/L4 disc remained intact. A custom sized and shaped titanium cage [21] was specifically designed and produced for this study, in ac-

Fig. 1 Mounted sheep specimen to assess the segmental stability in axial rotation, showing the upper (a) and lower (b) custom-made three-clamp fixation grips, and the specimen with ends embedded in acrylic cement (c). The grips can freely angulate in all directions, and can easily be blocked in any starting position

cordance with the distinct sheep anatomy (convex-concave shaped disc space). It had a hollow central part filled with different graft materials, dependent on the particular treatment group. The animals for each of the three survival groups were assigned to the treatment groups in a block randomized fashion. The first treatment group had the cage filled with autologous cortico-cancellous mercerized bone from the sheep's iliac crest (gold standard), the second one used 0.7–1.4-mm-diameter β -TCP porous granules (chronOS, Mathys, Bettlach, Switzerland) to fill the cage, and the third group had an empty cage placed (sham). There were a total of 12 instrumented levels available per survival interval and treatment group for subsequent analysis.

All sheep underwent regular radiographic controls following surgery to search for possible implant migration, failure or bone osteolysis. After sacrifice, the lumbar spine was harvested en bloc and stored at minus 20°C until mechanical testing. To quantify the instrumented levels' bending stiffness in all three cardinal planes, excess soft tissue was removed from the vertebral body ends, and single motion segments were potted in shallow methylmethacrylate fixation blocks (Fig. 1). Once attached to the testing machine (MTS Mini Bionix 856, MTS, Eden Prairie Minn.), three cycles of stepwise loading in 1-Nm steps, from –6 Nm to +6 Nm, were applied to each direction: axial rotation, flexion/extension, and axial bending. The load-displacement curves were plotted for each sample, and the ranges of motion and neutral zones were measured from the third loading cycle. The mechanical testing was followed

by microscopic assessment of all instrumented levels using acrylic specimen embedding for non-decalcified bone sections. Cuts in the mid-sagittal direction through the cage were obtained using a diamond saw, grounded, hand polished, and stained with toluidine blue. A qualitative five-step grading system was used for fusion assessment of each level.

As part of a later experiment, six additional sheep were operated on using an identical surgical protocol. The cage filling technique for the ceramic bone substitute, however, was modified. Based on the experience we had with granular β -TCP material, a pre-filled cage was used instead. The thermal treatment used to fabricate the porous TCP ceramics was applied with the titanium implant embedded in the solution. This technique produced a “ready-to-use” titanium implant, completely filled with a molded TCP insert. A ceramic pre-filled cage was placed in each animal, along with an autologous control, with the segmental levels block randomized to either treatment method. The survival period for this particular experiment was set at 20 weeks. No biomechanical evaluation was performed, only a histological work-up.

Vertebral body block filling

Nine male baboons (*Papio anubis*) of approximately 25 kg body weight were operated using a retroperitoneal surgical approach. The animals were randomized into three treatment groups of equal size. The anterior aspects of the L2 and L4 vertebrae were exposed. A manual core drilling tool, guided over a centrally placed K-wire, was used to remove a bone cylinder of 15 mm diameter and approximately 15 mm in length from the L2 and L4 vertebrae. The posterior vertebral body wall always remained intact. A total of 18 bone plugs were removed. For each animal, both levels underwent identical treatment. Based on the treatment group chosen, the defect was either left unplugged (sham group) or plugged with either a 15-mm-diameter cylindrical porous β -TCP implant or a β -TCP implant loaded with TGF- β 3. In the present study, the TCP blocks were impregnated with a TGF- β 3 solution and then dried under vacuum. The loading was performed under aseptic conditions. The final concentration of TGF- β 3 adsorbed onto the dry TCP block was 50 μ g/ml.

On the anesthetized animal, computed tomographic (CT) scans were obtained at 3 months postoperatively. In addition, bi-planar plain radiographs were obtained at 6 weeks, and 3 and 6 months postoperatively. The baboons were sacrificed at 6 months, and the CT examination was repeated post mortem. The L2 and L4 vertebral bodies were then processed for non-decalcified bone histology. Several frontal cuts through the defect were obtained for each vertebra, grounded and stained with toluidine blue.

Results

Instrumented anterior interbody spinal fusion

The radiographic follow-ups on the sheep rarely showed signs of anterior implant migration. No mechanical implant failure or significant occurrence of bone osteolysis was found. A prominent finding was large anterior bone bridges that characteristically formed at the instrumented levels, especially in the 16-week, and even more notably in the 32-week, survival groups.

For the mechanical testing, the trends found for the ranges of motion (ROM) and the neutral zones (NZ) were very similar, and therefore only the results for ROM are presented (Fig. 2). In all three motion directions tested,

Fig. 2 Segmental ranges of motion (ROM) measured for each survival group in **A** axial rotation, **B** flexion/extension, and **C** lateral bending. Significant differences ($P < 0.05$) found between treatment groups are marked with an *asterisk*

the average ROM significantly decreased (ANOVA, $P < 0.0001$) for each consecutive survival interval. However, except for the 8-week results in axial rotation, no signifi-

Table 1 Histological grades assessed for the three survival groups. The number of observations is listed for each treatment group, and the mean grade is calculated (*grading*: I fibrous tissue, II fibro-cartilage, III uncalcified bone matrix, IV woven or parallel-fibered bone, V remodeled cancellous bone)

Group/grade	Sham	Autologous	β-TCP
8 weeks			
I	6	3	3
II	1	2	4
III	3	3	2
IV	2	1	3
V	—	3	—
mean	2.1	2.9	2.4
16 weeks			
I	2	—	2
II	3	1	1
III	7	5	3
IV	1	5	5
V	—	1	2
mean	2.5	3.5	3.3
32 weeks			
I	—	—	—
II	1	—	1
III	4	—	4
IV	4	4	4
V	4	9	4
mean	3.8	4.7	3.8

cant differences (ANOVA, $P > 0.05$) were found between treatment groups. In axial rotation, the 8-week group with autologous bone had a significantly smaller ROM compared to both the sham and the β-TCP groups (post-hoc tests, Bonferoni corrected, $P < 0.05$).

The histology grading results are summarized in Table 1. A higher grade indicates a better fusion result. At 8 weeks, the grades for the autologous bone group were somewhat better compared to sham and β-TCP groups. At 16 weeks, however, the results of the β-TCP groups were almost as good as the autologous bone group, and better than the sham group. Between 16 and 32 weeks, both the autologous bone and the sham groups showed a remarkable improvement, but the β-TCP group remained almost unchanged. At 32 weeks, the autologous bone clearly demonstrated the best results.

Two additional observations were of interest. Firstly, the cages filled with β-TCP granules often had significant filling defects. But wherever granules were remaining, bone in-growth into the porous ceramic material was regularly seen, starting at 8 weeks following surgery (Fig. 3A). Secondly, more than half the specimens had a fibro-cartilagenous layer running horizontally through a large portion of the cage's inner portion, often spanning the entire cage cross-section. The struts of the cage were often not in direct contact with bone, but separated from bone by a fibrous membrane encapsulating the implant. This finding

Fig. 3A–C Non-decalcified sagittal cut through the cage middle portion, polished to 70 μm , stained with toluidine blue and visualized at very low power magnification. **A** Cage filled with β -TCP granules at 8 weeks, with posterior filling defects (*a*) and woven and parallel fibered bone invading the porous ceramic granules (*arrows*). **B** β -TCP granules at 32 weeks: the implant strut is encapsulated in fibrous tissue (*a*), and a continuous fibro-cartilagenous tissue layer runs across the cage (*arrows*). **C** Autologous bone at 32 weeks, showing the implant (*a*), and the fibro-cartilagenous tissue layer (*arrows*)

Fig. 4 TCP pre-filled cage at 20 weeks, showing the implant (*a*) and unresorbed porous TCP, completely osteointegrated (*arrows*). New bone is evident, mostly in direct contact with the implant surface (non-decalcified ground section, stained with toluidine blue, low power magnification)

was seen to some extent in all three treatment groups, but mostly in the 16- and 32-week survival groups, and mostly for β -TCP and autologous bone (Fig. 3B,C).

The ceramic pre-filled cages showed a marked improvement in histological fusion outcome compared to the ones filled with granular material (Fig. 4). The fibro-cartilagenous tissue layer running across the cage inside, the fibrous membranes encapsulating the implant, as well as the substantial filling defects observed for granular β -TCP, all were no longer a prevalent finding.

Vertebral body block filling

As confirmed on plain radiographs, none of the vertebral bodies showed a fracture or had a radiologically documented height loss. The CT data recorded at 3 and 6 months were reconstructed for coronal and sagittal projections. The sham group regularly showed minimal bone growth into the defect starting from its posterior wall. Nevertheless, most of the vertebral defect was still filled with soft tissue only. The β -TCP plugs appeared well osteointegrated at 3 and 6 months, with direct bone contact to the entire ceramic plug surface, and its boundaries starting to blur (Fig. 5A). However, the ceramic plug material was clearly visible on CT throughout the 6-months period.

At 3 months, the CT scans of the ceramic plugs impregnated with TGF- β 3 suggested a prominent peripheral resorption within the original graft boundaries (Fig. 5B). At 6 months, this resorption zone was filled with dense bone, and the original graft material appeared largely resorbed (Fig. 5B). The baboons that received TGF- β 3 impregnated plugs showed massive subperiosteal new bone formation along the entire anterior aspect of the vertebral body, but no new bone formation occurred in the spinal canal or the neuroforaminal regions.

Fig. 5A,B Computed tomographic scan of baboon lumbar spine with a 15-mm-diameter cylindrical defect, plugged with porous ceramic implant at 3 and 6 months following surgery. **A** β -TCP plug with no growth factor. At 6 months the plug was osteointegrated and there was no bone lysis (*black arrows*). **B** β -TCP plug loaded with TGF- β 3. At 3 months, large “lytic zones” are evident in the ceramic implant periphery (*black arrows*), which are completely filled with dense bone at 6 months. Massive subperiosteal new bone formation had started at 3 months; by 6 months, dense bone with neo-cortex has formed (*white arrows*)

Light microscopy (Fig. 6 A) demonstrated that all β -TCP plugs were osteointegrated, with plenty of direct bone apposition found on the porous mineral surface right to the center of the plugs. However, abundant ceramic material was still left in place. The ceramic plugs loaded with TGF- β 3 (Fig. 6B), on the other hand, showed in the peripheral areas of the plug a complete resorption of the ceramic material, replaced with remodeled cancellous bone. Surprisingly, in the central area there was often poorly osteointegrated ceramic material left that showed no signs of resorption.

Discussion

Instrumented anterior interbody spinal fusions have been performed previously in sheep [18]. However, to our knowledge, we are the first to present a sheep model for an anterior spinal fusion instrumented with a non-threaded cage design, in essence preserving the vertebral endplates. A cage design customized to the sheep anatomy was a

clear necessity. The sheep model, nevertheless, is not ideal for studying anterior interbody spinal fusion, since surgically altered motion segments habitually respond with massive anterior bone bridges being formed as early as 2 months following surgery. Mechanical testing, consequently, failed to determine effects of different graft material placed inside the cage. The tests rather measured the mechanical effect of those progressively forming anterior bone bridges, regardless of the graft material placed inside the cage.

Histological examination provided greater insight into treatment-related effects. The small β -TCP granules often did not fill the cage well, and may have fallen out of the cage during surgical insertion or soon after. β -TCP was found to be a very osteoconductive material, with bone apposition on the ceramic surface found regularly at 8 weeks. Due to the filling defects, though, the fusion mass forming inside the cage was incomplete.

It also became evident that the instrumented anterior spinal fusion studied in the sheep model was not a mechanically stable construct. Frequent absence of direct

Fig. 6A,B Frontal non-decalcified grounded histological sections through ceramic graft beds 6 months following implantation, stained with toluidine blue, at very low power magnification. **A** β -TCP plug with no growth factor. Original plug boundaries (white arrows) are still clearly visible, the β -TCP block is partially resorbed. **B** β -TCP plug loaded with TGF- β 3. Original plug boundaries (white arrows) have blurred out, the β -TCP block is peripherally resorbed, but a large unresorbed and poorly osteointegrated central portion is left

bone apposition on the titanium implant surface, as well as a frequently observed fibrous tissue layer encapsulating the implant, were indicators of implant micromotion. The fibro-cartilagenous tissue layers seen in all treatment

groups were also interpreted as a sign of segmental instability. Alternative interpretations of this phenomenon are a nutritional limitation becoming pertinent in the cage's central area, or the interposition of soft tissue between bone in-growing from either side of the cage. The alternative interpretations were considered less likely, but could not be excluded.

The lack of initial segmental stability of stand-alone anterior lumbar interbody fusion, compared to posterior fusion or circumferential fusion, can clearly be reproduced in human cadaveric studies [22, 26], and is increasingly supported by clinical outcomes, which are unfavorable for stand-alone anterior interbody fusion [9, 17]. Our histological findings in the sheep model corroborate this conclusion.

Using a ceramic pre-filled cage is an interesting option. It appears from our animal study that pre-filling the cage with porous TCP can improve the fusion outcome. Bone substitute filling defects as well as the fibro-cartilagenous tissue layers running through the cage could be avoided. In addition, pre-filled cages can easily be shelved at room temperature and potentially can shorten the surgery time. Whether pre-filled cages will show handling benefits and improve fusion outcome in practice, will have to be established in clinical studies.

The baboon study was primarily designed to validate the proposed local bone graft harvesting procedure, following anatomical and biomechanical studies in a biological experiment. However, it also constitutes the first biological experiment in a non-human primate combining β -TCP as a carrier with TGF- β 3 in a spinal application. The β -TCP plug used without growth factor has already produced very encouraging results. The vertebra's mechanical integrity, disturbed by the iatrogenic bone defect created by locally harvested bone, was considered after 6 months to be functionally completely restored. The plugs were all fully osteointegrated, and the β -TCP block was gradually resorbed. This experiment demonstrates the capacity for porous β -TCP also to serve as an adequate osteoconductor for sizable bone substitute blocks. In this case, up to 7.5 mm successful bone in-growth into a monolithic block (radially toward the center) was confirmed.

For our specific surgical model, the marginal benefits of loading the ceramic plug with a growth factor were not clearly evident. Addition of TGF- β 3 certainly affected the ceramic block resorption over time, and also resulted within 3 months in substantial regional new bone formation in the prevertebral space. This new bone, as confirmed histologically at 6 months, was well remodeled and mechanically competent. However, the ceramic plug with no growth factor addition was considered adequate for the purpose of restoring the vertebral body's integrity. In fact, the surgical procedure for harvesting a local bone plug for the adjacent vertebral body, and plugging this defect with a porous ceramic spacer, has now been used clinically, with satisfactory results.

The application of TGF- β 3 for bone substitution, nevertheless, appears to be a promising direction to be further investigated. The regional new bone formation in the prevertebral space clearly is to be attributed to TGF- β 3, even though the dose used in this study was relatively low. Animal studies showed that in many cases TGF- β induces bone healing at much lower concentrations than other growth hormones tested, including BMPs [13]. In recent human studies showing that recombinant BMP-2 applied in a collagen sponge induced bone formation inside interbody fusion cages, the local concentration of BMP-2 used was 1.5 mg/ml [4].

Impregnating the porous ceramic blocks with TGF- β 3 also affected the ceramic material's resorption pattern. Whereas in the plug periphery the resorption was much faster, it was slower in the central areas when compared to the non-impregnated blocks. The large peripheral resorption zone already evident on the 3-month CT scans had, at 6 months, been replaced with dense bone. We interpreted this observation as an effect of the early and massive angiogenesis mediated by TGF- β 3, which may have permitted the faster resorption in the plug's periphery, to the ex-

tent that it "sealed off" the center of the plug from a timely bone in-growth.

More studies will be required to define the optimal spatial and temporal release characteristics of TGF- β 3. This growth factor hormone has proven to be biologically highly potent in the non-human primate, but other surgical applications need to be further explored.

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