Bone growth into a ceramic-filled defect around an implant

THE RESPONSE TO TRANSFORMING GROWTH FACTOR β 1

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Synthetic bone substitutes provide an alternative to autograft but do not give equivalent clinical results. Their performance may be enhanced by adding osteogenic growth factors. In this study, TGF β 1 was absorbed on to a carrier of β tricalcium phosphate and Gelfoam® and used to fill a defect around a tibial implant in a rat model of revision arthroplasty.

We added 0.0, 0.02 μ g, 0.1 μ g or 1.0 μ g of TGF β 1 to the carrier and then implanted it around an hydroxyapatite-coated stainless-steel pin in the proximal tibia of rats. The tibiae were harvested at three, six or 26 weeks and the amount of bone formation and ceramic resorption were assessed.

TGF β 1 had no effect on the amount of bone in the defect, the amount of fluorescent label incorporated or the rate of mineral apposition. The growth factor did not significantly affect the amount of β TCP remaining in the tissue at any of the time points.

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Revision arthroplasty may be technically difficult because of bone loss around the primary implant. The best clinical outcome is achieved by filling these defects with autologous bone, but it is often difficult to harvest adequate amounts of autograft and there may be morbidity at the donor site.^{1,2} The use of synthetic bone substitutes offers an alternative and there has been interest in enhancing the performance of these materials by the addition of osteogenic growth factors.

Most synthetic bone substitutes are composed of calcium phosphate ceramics which have a mineral composition

similar to that of bone and are osteoconductive. Growth factors might add osteoinductive ability allowing the bone substitute to stimulate as well as support bone formation.

Many attempts have been made to enhance bone growth using growth factors such as transforming growth factor β (TGF β),³⁻⁸ insulin-like growth factor-1 (IGF-1),⁹⁻¹² basic fibroblast growth factor (bFGF),^{13,14} prostaglandin E₂ (PGE₂)¹⁵ and bone morphogenetic proteins (BMPs),¹⁶⁻¹⁹ either absorbed on to the filler,^{8,9,13,14} on to the coating of the prosthesis^{3,7} or injected locally,¹⁰⁻¹² with varying degrees of success. TGF β 1 has potent stimulatory effects on the recruitment and differentiation of osteoblasts and on the production of matrix²⁰ and is therefore a potential therapeutic agent for many orthopaedic procedures.

The results from studies using TGF β 1 have been contradictory. Ueda et al²¹ reported a significant increase in the bone mineral density in defects in the rabbit skull after implantation of a collagen sponge loaded with TGF β 1, and Sumner et al⁷ found an increase in the amount of bone forming in a gap around a metal rod coated with TGF β 1. However, although Lind et al^{3,22,23} found a significant increase in bone-implant contact with TGF β 1 absorbed on to a prosthesis coating in dogs and a significant increase in fracture callus in the rabbit, there was no difference in the area of bone and mechanical strength, nor in bone mineral content, cortical thickness or bending stiffness. Moreover, others have found a significant early difference with TGF β 1 which was later lost.²⁴

In this study we investigated the addition of TGF β 1 to a carrier of β tricalcium phosphate (β TCP) implanted in a defect around a tibial implant in a model of revision arthroplasty.²⁵ The initial doses of TGF β 1, chosen from previous reports, were tested at six and 26 weeks, but, after studies of its release and further reports in the literature, a higher dose was tested at three weeks in case an effect could be detected earlier in the healing process.

Materials and Methods

Experimental model. The model has been described previously.²⁵ Briefly, 27.8 mg of β TCP particles (5% porosity, mean equivalent circle diameter of 166 μ m; Plasma Biotol, Tideswell, UK) were mixed with 5.6 mg of Gelfoam®, a medical-grade absorbable gelatin powder (Pharmacia and

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Fig. 1

Diagram showing the dimensions of the pin and the ceramic-filled defect (striped area).

Upjohn, Kalamazoo, Michigan). TGF β 1 (R&D Systems, Abingdon, UK) was reconstituted in 4 mM HCl with 1 mg/ml of rat serum albumin (Sigma Aldrich, Dorset, UK) to the desired concentration. Either 150 μ l of this solution or the carrier alone was absorbed on to the β TCP/Gelfoam $\text{\textregistered}$ and mixed into a paste which was moulded around an hydroxyapatite-coated (Plasma Biotol) stainless-steel pin (R. J. Layland, Rayleigh, UK) in a custom mould and allowed to cure. The resulting implant with its ceramic collar (Fig. 1) was introduced as a press-fit into a defect of the exact dimensions in the proximal tibia.

Release of the growth factor. In order to examine the release of TGF β 1 *in vivo*, [125 I]-labelled growth factor (Amersham Pharmacia Biotech, Bucks, UK) was absorbed on to the β TCP collar and implanted into eight rats. Each animal received 0.02 μ g of TGF β 1 which equated to a dose of 6.25 μ Ci. The radioactivity of the implants was measured using a scintillation counter before implantation and in the harvested whole tibiae after one, two, three and six weeks, using two rats on each occasion.

Experimental groups. Male Sprague-Dawley rats were randomly assigned to one of nine groups each of which had six animals. Groups 1 to 3 received doses of 0.0, 0.1 μ g or 1.0 μ g of TGF β 1 and were killed at three weeks. The other groups received doses of 0.0, 0.02 μ g, or 0.1 μ g of TGF β 1 and were killed at six (groups 4 to 6) or 26 weeks (groups 7 to 9).

Operative procedure. All procedures had received ethical approval and were carried out in accordance with the regulations as laid down in the Animals (Scientific Procedures) Act 1986. All drugs were obtained from National Veterinary Services, Stoke-on-Trent, UK. The animals were anaesthetised with fentanyl-fluanisone (Hypnorm) and midazolam (Hypnovel) in sterile water at a ratio of 1:1:2 and a dose of

2.3 ml/kg body-weight. The right hindlimb was clipped, draped and sprayed with chlorhexidine spray. An incision of 1 cm was made lateral to the patella and a lateral capsulotomy performed allowing medial dislocation of the patella. The tibial plateau was visualised and a hole drilled centrally using a 2 mm hand drill. A defect of the exact dimensions of the implant with the proximal β TCP collar in place was then reamed using a custom-made counterbore. The prosthesis was pressed into place, the patella reduced and the incision closed with interrupted sutures. The animals received post-operative antibiotic cover (Synlux; 150 mg/kg intramuscularly) and both pre-operative (Carpofen; 5 mg/kg subcutaneously) and post-operative analgesia (Buprenorphine; 0.15 mg/kg subcutaneously). They began to weight bear within 12 hours of the operation. They were housed in IVC caging systems (Techniplast UK Ltd, Northants, UK), exposed to a 12-hour light-dark cycle and had access to food and water *ad libitum*.

Fluorochrome injections. Each animal was given an injection of fluorochrome intraperitoneally at one, two and three weeks before being killed in order to label the calcification front. The labels were xylene orange (90 mg/kg), calcein green (5 mg/kg) and alizarin complexone (30 mg/kg). All fluorochromes were obtained from BDH, Dorset, UK.

Tissue processing. After the animal had been killed the right tibia was dissected out and placed in cold 4% w/v paraformaldehyde in 0.1M phosphate buffer, pH 7.4 with 0.1% w/v sucrose and 0.05% v/v glutaraldehyde, for four hours. The specimens were then washed in phosphate-buffered saline (PBS) and dehydrated and defatted in increasing concentrations of alcohol (50% to 100%) followed by acetone under vacuum with several changes of each solution. The specimens were embedded in methylmethacrylate containing 2.5% w/v benzoyl peroxide and 2.5% v/v dibutyl phthalate (Sigma Aldrich). Longitudinal sections of 300 μ m were cut using a low-speed diamond saw (Accutom 5; Struers, Glasgow, UK), then ground and polished to 100 μ m with a graded series of silicone carbide polishing discs using a Rotopol 21 instrument (Struers).

Histological analysis. One section from each specimen was surface-stained with Toluidine Blue (pH 9, 30 minutes at 56°C) and examined under a light microscope (Dialux 20, Leica, Milton Keynes, UK). Images were taken from six regions under the head of the pin which contained the defect using dedicated software (Aquis, Synoptics, Cambridge, UK). Bone, marrow, β TCP, fibrous tissue and the growth plate were identified in each section by their characteristic staining. Each area was highlighted using Adobe Photoshop 5.0 and measured with the freeware image-analysis software, Scion Image (Scion Corporation, www.scion-corp.com). The area of each of these parameters was expressed as a percentage of the total area of tissue in the six regions of interest.

Fluorescence analysis. A section from each tibia was examined under epifluorescence using a DMRB microscope (Leica) using both fluorescein isothiocyanate (FITC) and

Fig. 2

Release of radiolabelled TGF β 1 *in vivo* by scintillation counting. The mean count and SD from the implants at day 0 and the whole dissected tibiae thereafter are shown.

rhodamine filters. Sequential images were captured from the area of the defect as before, using a LC 100C low-light camera (Seescan, Cambridge, UK) and the area of fluorescent label was measured using Scion Image. These

measurements were expressed as the sum of the area of fluorescence seen under both filters in the six fields of interest as a percentage of the total area.

Images were also taken from the areas where two or more labels were visible. The rate of mineral apposition was calculated from these images using a semi-automatic image-analysis package with a digitising tablet, cursor and drawing tube attachment (SUMMASKETCH II; Summagraphics, Fairfield, Connecticut).

Tartrate-resistant acid phosphatase (TRAP) analysis.

One section from each sample was stained for TRAP. All reagents were obtained from Sigma Aldrich unless otherwise stated. The methacrylate was removed from each section using acetone and the sections were rehydrated through graded alcohols to distilled water. They were placed in a reaction mixture (0.1 M citrate buffer pH 4.5 containing 1 mM naphthol AS-BI phosphate and 10 mM sodium tartrate) for seven minutes at 37°C. The reaction was stopped by washing in cold 50 mmol sodium fluoride and the sections were incubated with post coupler (0.1 mM acetate buffer pH 6.2 containing 2.2 mM Fast Garnet GBC) for five minutes at 4°C, rinsed in distilled water and mounted using

Fig. 3

Photomicrographs showing that at three weeks most of the defect is filled with clot material but there is some cellular infiltration at the edges of the collar (a) where osteoclasts can be seen resorbing the β TCP particles (b) and osteoid is laid down around the particles (c). The tissue has an active appearance with 'active marrow' (c) and extensive osteoblast activity on the endosteal surface of the cortical bone (d). Toluidine Blue (x 100). The images are all orientated so that the pin shaft is horizontal and the head of the pin is vertical to the right of the image (C, clot; B, bone; M, marrow; AM, active marrow; I, implanted pin; HA, hydroxyapatite coating; F, fibrous tissue; P, particle; O, osteoid; R, remodelling; arrow; osteoclasts).

Fig. 4

Photomicrographs showing that at six weeks, there is cellular infiltration throughout the defect (a) and although there is fibrous tissue in contact with some parts of the implant (b) there is mostly new bone in direct contact with the HA coating pin (c) and (d) osteoclast activity around the ceramic particles shown by TRAP staining (red). Toluidine Blue (x 100). Abbreviations etc. are as given for Figure 3.

Aquamount (BDH). Images were taken from the area of the defect using Aquis and the total area of TRAP staining was measured using Adobe Photoshop 5.0 and Scion Image.

Statistical analysis. The differences between the groups at each time point were measured using one-way ANOVA with *post-hoc* testing using Bonferroni's correction. This test was also used to determine the effect of time on the parameters measured.

Results

Two animals from the 26-week series died from unknown causes. The first died after six weeks and was excluded from the study, the second died the day before the end of the experiment but was included in the study.

Release of the growth factor. The release of TGF β 1 was exponential with most being lost in the first week after implantation (Fig. 2). The half-life of TGF β 1 *in vivo* was three to four days.

Histological analysis. By three weeks most of the defect had been filled with clot but cellular infiltration could be seen at the periphery of the ceramic collar (Fig. 3a). When there was cellular access to the β TCP, there was evidence of ceramic resorption with multinucleated giant cells visible

on the particle surface (Fig. 3b) and bone formation around the particles (Fig. 3c). Below the level of the collar there was a shell of bone in contact with the hydroxyapatite coating with a large amount of osteoid, particularly on the endosteal surface of the cortical bone (Fig. 3d). In some cases cellular material was present with a more active and less regular appearance than normal marrow with a higher cell density on a fibrous-type matrix. We termed this 'active marrow' (Fig. 3c).

By six weeks, small amounts of residual clot remained in some sections but cellular infiltration was seen throughout the defect (Fig. 4a). In some areas fibrous tissue was adjacent to the implant (Fig. 4b), but mostly there was bone-implant contact (Fig. 4c). Osteoid was seen throughout the section and was also found on the cortical bone surface. TRAP staining showed osteoclasts surrounding the β TCP particles (Fig. 4d).

At 26 weeks the tissue was more organised (Fig. 5a). There was some fibrous tissue which was usually seen on the shoulder between the head of the pin and the stalk (Fig. 5b). However, for the most part the bone was in contact with the HA coating on the implant and the β TCP particles were encased in bone (Fig. 5c). In general, the tissue was quiescent with very little osteoid compared with the earlier sam-

Fig. 5

Photomicrographs showing that the tissue is more organised at 26 weeks (a) but in some cases fibrous tissue persists in the shoulder between the head and stalk of the pin (b) and (c) the ceramic particles are now surrounded by bone with only limited evidence of remodelling occurring on trabecular struts (d). Toluidine Blue (x 100). Abbreviations etc. are as given for Figure 3.

ples, but there was some evidence of bone remodelling, particularly on the trabecular struts (Fig. 5d).

Image analysis. The image analysis on the sections stained with Toluidine Blue showed that there was significantly more clot material in the group which had received 1 µg of TGFβ1 ($p = 0.042$) at three weeks and that this had resulted in significantly less bone ($p = 0.048$) and bone plus marrow ($p = 0.042$) (Fig. 6a). Although there was less βTCP with the higher dose of TGFβ1, this was not significant and there was no difference between the groups for any other measurement. At six weeks there was significantly more active marrow ($p = 0.024$) and less bone plus marrow ($p = 0.035$) in the group receiving 0.02 µg of TGFβ1. The least amount of βTCP and most bone was found with the higher dose of TGFβ1 but this was not significant (Fig. 6b). There was no significant difference between groups for any measurement at 26 weeks (Fig. 6c).

There were significantly fewer particles in the control group at three weeks compared with the higher dose of TGFβ1 ($p = 0.028$) (Table I). There were no other differences between the groups in the median particle size and the number of particles remaining in the tissue at each time point.

Table I. The median ($\pm$ SD; µm) size and the total number of βTCP particles remaining in the six regions of interest at each time point

Weeks		Median size	Particle number
3	0.1 µgTGFβ1	7419.2 $\pm$ 829.3	246.4 $\pm$ 26.3
	1.0 µgTGFβ1	6209.6 $\pm$ 1229.4	273.8 $\pm$ 41.9
	Control	8322.3 $\pm$ 2950.1	177 $\pm$ 52.3*
6	0.02 µgTGFβ1	8394.595 $\pm$ 643.1	198 $\pm$ 39.6
	0.1 µgTGFβ1	7050.86 $\pm$ 1318.1	149 $\pm$ 31.9
	Control	7658.46 $\pm$ 705.2	180 $\pm$ 32.3
26	0.02 µgTGFβ1	8010.67 $\pm$ 489.9	97.5 $\pm$ 33.6
	0.1 µgTGFβ1	7075.9 $\pm$ 1165.5	91 $\pm$ 31.9
	Control	7107.615 $\pm$ 1716.8	70 $\pm$ 24.6

* $p < 0.05$

Fluorescent analysis showed no significant differences between the groups in the total amount of label incorporated or the rate of mineral apposition at six or 26 weeks (Figs 7a and 7b). The control group showed the least TRAP activity at three and six weeks, but again any differences were not significant (Fig. 7c).

Effect of time. When all the groups were assessed together, one-way ANOVA showed that there was a significant reduction in the area, size and number of βTCP particles, the

The mean (SD) amount of each tissue type is given as a percentage of the total area for each treatment group at a) three b) six and c) 26 weeks after implantation (*p < 0.05).

amount of growth plate, activated marrow, clot material, incorporation of fluorescent labelling and the rate of mineral apposition with time, with a corresponding increase in the amount of bone and marrow (Table II). There was also an increase in the amount of fibrous tissue with time. TRAP activity peaked at six weeks and was significantly different from that at three and 26 weeks (Fig. 7c and Table II).

Discussion

Our results indicate that the addition of TGFβ1 made little difference to bone healing and resorption of ceramic in this model. The tissue had a different appearance in the TGFβ1 groups with more active marrow in these groups at three and six weeks, but this did not translate into more bone in the

Fig. 7a

Fig. 7b

Fig. 7c

The mean (SD) a) fluorescent label incorporation b) rate of mineral apposition and c) amount of TRAP activity for each treatment group at three, six or 26 weeks.

defect or less residual ceramic. There was less bone and bone plus marrow and more particles with the highest dose of TGFβ1 at three weeks because there was more clot material in the defect in this group. With less cellular access, the βTCP particles could not be removed and replaced by marrow or bone. It is not clear, however, why

there was more clot material in this group or indeed in the group receiving 0.02 µg of TGFβ1 at six weeks, which accounted for the reduction in bone plus marrow in this group.

The presence of multinucleated giant cells and TRAP-positive cells on the surface of the βTCP particles indicates

Table II. The effect of time on each parameter analysed by one-way ANOVA

Parameter	Trend	p value
Area of β TCP	↓	<0.001
Particle number	↓	<0.001
Median particle size	↓	0.038
Active marrow	↓	0.033
Clot material	↓	<0.001
Growth plate	↓	<0.001
Fluorescence	↓	0.021
MAR	↓	<0.001
TRAP	↑↓	<0.001
Fibrous tissue	↑	0.042
Bone	↑	<0.001
Marrow	↑	<0.001
Bone plus marrow	↑	<0.001

that the ceramic is actively resorbed and does not merely dissolve. Interestingly, there was little difference in the size of particles with time but a definite decrease in their number. This suggests that, once stimulated, the osteoclasts continue resorbing a particle until it is completely removed, ignoring other particles completely. Alternatively, because β TCP is osteoconductive, those particles closest to bone are attractive to osteoblasts and become quickly encased in osteoid and subsequent new bone, as shown by the presence of encased particles at three weeks, whereas osteoclasts have access to other particles deeper in the defect for much longer. TGF β is reported to have an inhibitory effect on the formation and function of osteoclasts²⁶ and to induce their apoptosis.²⁷ It is therefore possible that the TGF β 1 was not evenly distributed throughout the collar and had a non-uniform, inhibitory effect on the resorption of ceramic. In general, however, the results of our study do not support an inhibitory effect of TGF β 1 on resorption and are in agreement with others which showed significantly less carrier remaining²⁴ and more vitronectin-receptor-positive cells²⁸ with TGF β 1.

The endosteal surface of the cortical bone was active in all cases at three and six weeks. This remodelling may have been stimulated by changes in distribution of stress through the tibia after implantation of the prosthesis. The increase in fibrous tissue from three to 26 weeks and its position in the shoulder of the head and stalk of the pin may be due to stress shielding in this area.

The numbers in this study were relatively small and perhaps with more animals per group the power of the study could be increased, allowing some of the differences seen to reach significance. However, the effect of TGF β 1 appears to be marginal and it is unlikely that its use in clinical practice would be of value.

Although others have had a more successful outcome when using TGF β 1 to promote bone formation, significant improvement in the results is often qualified. Lind et al^{3,22} found an increase in bone-implant contact when TGF β 1 was absorbed onto the coating of the prosthesis and implanted in the humerus in dogs, but there was no difference in the

volume of bone in the gap around the prosthesis or in mechanical strength. Blom et al²⁹ reported an increase in bone-area fraction and cement-bone contact, but there was no difference in closure of the defect.

Several studies have reported a positive effect of TGF β 1 on the repair of fractures with an increase in callus.^{4,6,23,30,31} The most consistent of these studies was by Beck et al⁴ who also found an effect on fracture union and mechanical strength of the radius in the rabbit, especially when autologous bone marrow was added to the TGF β 1/carrier mixture before implantation. Therefore, the most appropriate application for TGF β 1 in orthopaedics may be in the promotion of repair of fractures. However, these studies all report radiographic evidence of fracture callus and fracture union but when Tielinen et al,⁶ who found an increase in radiographic fracture callus with a 50 μ g dose of TGF β 1, measured the size of the callus histologically there was no difference from a control group. Given the difficulty of achieving reproducible radiographs, it is unlikely that a difference which could not be confirmed on histological examination would provide any significant clinical advantage. The other studies mentioned above did not measure any histological parameters with the exception of that of Lind et al²³ who found an increase in callus but no difference in cortical thickness, bone mineral content or the diameter of the Haversian canals.

The effect of TGF β 1 may depend on the time and duration of the dose. Several groups have reported an early effect of TGF β 1 on the formation of bone which was later lost. Kamakura et al²⁴ found an effect of TGF β 1 on bone formation and implant resorption at four weeks but could not demonstrate this at eight weeks after operation. Schmitt et al⁸ observed a response to TGF β 1 absorbed onto bone wax polymer and implanted in a rabbit calvarial defect at six weeks but this was no longer present at 12 weeks. Goodman et al²⁸ examined bone ingrowth into a bone harvest chamber in rabbits and found an effect on the percentage of bone and the number of osteoclasts when TGF β 1 was given over a day but not over a week, although these differences were not significant.

In our study a difference between groups may not have been observed because either the dose of TGF β 1 was not high enough, it was given at the wrong time or was given for too long. An effect may have been evident at a time point which was not examined or TGF β 1 may be ineffective only in this particular model. However, our results taken in conjunction with the published literature, suggest that it is questionable whether TGF β 1 will provide sufficiently robust and reproducible clinical benefit to be of significant use in orthopaedics.

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