Effects of TGF β on bone ingrowth in the presence of polyethylene particles

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We implanted bone harvest chambers (BHCs) bilaterally in ten mature male New Zealand white rabbits. Polyethylene particles ($0.3 \pm 0.1 \mu\text{m}$ in diameter, 6.4×10^{12} particles/ml) were implanted for two, four or six weeks bilaterally in the BHCs, with subsequent removal of the ingrown tissue after each treatment. In addition to the particles, one side also received $1.5 \mu\text{g}$ of recombinant transforming growth factor $\beta 1$ (TGF $\beta 1$).

At two weeks, the bone area as a percentage of total area was less in chambers containing TGF β compared with those with particles alone ($7.8 \pm 1.3\%$ v $16.9 \pm 2.7\%$ respectively; 95% confidence interval (CI) for difference -14.0 to -4.30; $p = 0.002$). At four weeks, the percentage area of bone was greater in chambers containing TGF β compared with those with particles alone ($31.2 \pm 3.4\%$ v $22.5 \pm 2.0\%$ respectively; 95% CI for difference 1.0 to 16.4; $p = 0.03$). There were no statistical differences at six weeks, despite a higher mean value with TGF β treatment ($38.2 \pm 3.9\%$ v $28.8 \pm 3.5\%$; 95% CI for difference -4.6 to 23.3; $p = 0.16$). The number of vitronectin-receptor-positive cells (osteoclast-like cells) was greater in the treatment group with TGF β compared with that with particles alone; most of these positive cells were located in the interstitium, rather than adjacent to bone.

TGF $\beta 1$ is a pleotropic growth factor which can modulate cellular events in the musculoskeletal system

in a time- and concentration-dependent manner. Our data suggest that there is an early window at between two and six weeks, in which TGF β may favourably affect bone ingrowth in the BHC model. Exogenous growth factors such as TGF β may be a useful adjunct in obtaining osseointegration and bone ingrowth, especially in revisions when there is compromised bone stock and residual particulate debris.

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In revision total hip replacement, the bone bed is often suboptimal for implantation of a prosthesis. Residual foreign-body membrane and particulate debris, as well as sclerotic, devascularised bone, may not provide a biological environment which is conducive to long-term stabilisation of a prosthesis. Press-fit fully porous-coated cementless stems have therefore become popular for revision because of difficulties with penetration of cement. Bone ingrowth and subsequent osseointegration of cementless components, however, may also be difficult in the presence of a compromised bone bed containing residual granuloma and particles. Bioactive, osteoconductive surface coatings such as hydroxyapatite have shown promise in facilitating bone ingrowth and apposition in both primary and revision arthroplasty. Another approach is to use osteoinductive growth factors.

Transforming growth factor beta one (TGF $\beta 1$) is a pleotropic substance which can modulate cellular events in a time- and concentration-dependent manner.¹⁻³ It has diverse effects on the growth, differentiation, and function of many types of cell, especially those of the musculoskeletal system.¹⁻³ TGF $\beta 1$ has been shown to stimulate osteoblast-like cells to proliferate and synthesise collagen in vitro, to induce chondrogenesis and osteogenesis by mesenchymal cells, and to modulate the development of bones and teeth, the healing of fractures and haematopoiesis.¹⁻³

Exogenous TGF $\beta 1$ has been used in an attempt to facilitate osseous integration of orthopaedic implants. Sumner et al⁴ demonstrated a threefold increase in bone ingrowth in titanium-fibre-metal-coated rods treated with $120 \mu\text{g}$ of TGF $\beta 1$ in hydroxyapatite and tricalcium phosphate implanted in a canine humerus model. The model used a 3 mm

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gap around the implant. Lind et al⁵ showed improved mechanical fixation of and increased bone apposition to non-weight-bearing titanium alloy rods surrounded by a 2 mm gap implanted into canine femora with 0.3 or 3.0 µg of recombinant TGFβ1 in tricalcium phosphate. In another study using hydroxyapatite-coated titanium rods implanted in the canine humerus with a 2 mm gap, 0.3 µg and 3.0 µg of recombinant TGFβ1 increased bone ongrowth but not bone volume in the gap. Mechanical push-out testing of the implants was also not affected by the addition of TGFβ1.⁶ Bone ongrowth was significantly increased but mechanical push-out tests showed no differences in a related model in which weight-bearing rods with tricalcium phosphate containing 0.3 µg were implanted in canine femora utilising a gap of 0.75 mm.⁷ Aufdemorte et al⁸ demonstrated increased osteoblastic activity, but no increase in the volume of trabecular bone, 22 days after removal of bone specimens from the analytical bone chamber implanted in baboons, with 1 or 10 µg of recombinant human TGFβ1. In many of the above studies, increasing the dose of TGFβ1 had a mitigating effect on the histological or biomechanical properties of the interface of the implant and retrieved tissue.^{4-6,8}

We have extensively used an in vivo model of a bone harvest chamber implanted in the tibia of the rabbit to study the effects of particulate debris on tissue differentiation.⁹⁻¹¹ Polyethylene particles have previously been shown to inhibit net bone formation in this model. We hypothesised that the use of an adjunctive growth factor, TGFβ1, could enhance tissue differentiation into bone in the presence of polyethylene particles. We therefore performed a histomorphometric analysis of tissue retrieved from the chamber at two, four and six weeks after operation in the presence of polyethylene particles, with and without the addition of TGFβ1.

Materials and Methods

Bone harvest chamber. The bone harvest chamber (BHC) is a device made of commercially pure titanium and is implanted into the proximal part of the medial tibial metaphysis of rabbits.⁹⁻¹¹ The outer cylinder is screwed into bone, so that the two, 1 mm round holes in the cylinder are at the level of the cortex. The cylinder undergoes osseointegration with the surrounding bone. The inner, removable core fits into the cylinder, and contains a groove 1×1×5 mm which is continuous with the holes in the outer cylinder. This provides a canal through the chamber for tissue ingrowth. The tissue within the chamber may be removed repeatedly through a small incision by disassembling the device without disturbing the outer cylinder and surrounding bone. In this way, the effects of different treatments on bone ingrowth and tissue differentiation can be tested sequentially over time.

Polyethylene particles. We obtained medical grade ultra-high-molecular-weight polyethylene particles from DePuy

(Warsaw, Indiana). These were suspended in ethanol and filtered to obtain particles less than 1 µm in diameter. The ethanol was evaporated and the particles were washed with saline and resuspended in 1% sodium hyaluronate (Healon; Pharmacia AB, Uppsala, Sweden), the carrier medium. The mean (±SD) diameter of the particles was 0.3 ± 0.1 µm as measured by laser microscopy. We used a concentration of 6.4×10¹² particles/ml. When corrected for particle size, this concentration corresponded to the same order of magnitude in volume as that in previous studies which used larger polyethylene particles in a different model in rabbits.^{9,10}

Operative techniques. Institutional guidelines for the care and use of laboratory animals were strictly followed. Under general endotracheal anaesthesia the chambers were implanted bilaterally in ten mature, male, New Zealand white rabbits aged 6 to 12 months and weighing 3.5 to 4.2 kg, as previously described.^{9,10} One dose of cephalosporin was given as prophylaxis for infection. After a six-week period for osseointegration, the contents of the chamber were removed and discarded. Polyethylene particles were implanted for two, four or six weeks bilaterally with subsequent removal of the ingrown tissue after each treatment (Table I). In addition to the particles, one of the two chambers in each animal received 1.5 µg of recombinant TGFβ1, provided by Bristol Myers Squibb (Seattle, Washington). The chamber receiving the TGFβ1 was alternated in subsequent treatments in the same animal. After each treatment period with particles, there was a three-week 'rest' period with no particles or TGFβ1, followed by removal.

Tissue processing. Specimens were snap frozen in liquid nitrogen and stored at -70°C until processed. Serial sections 6 µm in size were cut using a cryostat (Cambridge Instruments, Buffalo, New York) and mounted on microscope slides which had been prebaked for three hours at 250°C. One section was stained with haematoxylin and eosin for general morphological characterisation. The sec-

Table I. Implantation protocol for polyethylene particles and TGFβ in the chamber

Week	Treatment
0	Osseointegration, no particles, no TGFβ*
6	PE/PE + TGFβ†
12	No particles, no TGFβ
15	PE/PE + TGFβ
19	No particles, no TGFβ
22	PE/PE + TGFβ
24	No particles, no TGFβ‡
27	Kill

* the first six-week period in which the outer cylinder of the chamber undergoes osseointegration with the surrounding bone

† a time period, before removal, in which polyethylene particles are placed in one chamber, and polyethylene particles and TGFβ in the contralateral chamber in the rabbit tibia

‡ a three-week time period in which neither particles nor TGFβ are placed in the chambers bilaterally

Fig. 1a

Fig. 1b

Two-week harvest. Figure 1a – Photomicrograph from a chamber with PE particles. The harvested tissue is composed of vascularised fibrous tissue and longitudinally orientated woven bone. Figure 1b – Photomicrograph of a chamber with PE particles and TGF β . The harvested tissue is composed of a more cellular vascularised fibrous tissue, scant woven bone and numerous polygonal mesenchymal cells. Brownish coloured, haemosiderin-containing cells are seen in the centre (haematoxylin and eosin $\times 66$).

tions were stained for tartrate-resistant acid phosphatase (Sigma Chemical Co, St Louis, Missouri) to identify osteoclast-like cells, and counterstained with Toluidine Blue to identify bone and cell nuclei. Sections of rabbit spleen containing numerous acid-phosphatase-positive macrophages, which did not stain with the addition of tartrate, served as a control group. Immunoperoxidase staining using monoclonal antibodies identified cells expressing the vitronectin receptor (osteoclast-like cells) and CD11b (macrophages). Both tartrate-resistant acid phosphatase and the vitronectin receptor are relatively specific markers for osteoclasts.

Immunohistochemistry. Frozen sections were fixed in absolute acetone at -20°C for one hour. They were incubated at room temperature with monoclonal antibodies to identify macrophages (CD11b; mouse anti-rabbit Mac-1; Biosource International, Camarillo, California) and osteoclast-like cells exhibiting the vitronectin receptor (CD51; mouse anti-human monoclonal antibody to integrin $\alpha\text{V}\beta 3$; Chemicon, Temecula, California). The sections were then incubated with rabbit anti-mouse immunoglobulins conjugated to peroxidase (Dako, Carpinteria, California). They were washed in phosphate-buffered saline for ten minutes after each antibody step. Positively-stained cells were visualised with diaminobenzidine, 0.01% H_2O_2 , 0.3% sodium azide in 0.05M Tris buffer, pH 7.6. The slides were counterstained with Mayers haematoxylin, dehydrated and mounted.

Assessment and statistical analysis. Histomorphometric analysis was performed on multiple serial, longitudinal sections which allowed the entire length of the specimen to be visualised on one slide.⁹⁻¹¹ A computerised, interactive image-analysis system using NIH Image interfaced with a MacIntosh Quadra computer (Apple, Cupertino, California) and a real-time video light-microscopic system were used to perform the morphometric analysis. The areas or

points for measurement on the entire section were outlined manually using a mousepad. All measurements were performed in a 'blind' fashion without knowledge of the treatment.

The histomorphometric analyses were performed to determine total tissue area, total bone area, the ratio of bone area divided by the total area expressed as a percentage, and the total number of vitronectin-receptor-positive cells abutting the bone and away from the bone in the entire section.

Statistical analysis used the StatView software program (Abacus Concepts, Berkeley, California). Parametric statistics were used including the analysis of variance, and the paired Student's *t*-test. A significance level of 0.05 was used.

Results

Histomorphological findings. At two weeks, the harvested tissue was composed of vascularised fibrous tissue, scant trabecular woven bone, and large, polygonal mesenchymal cells with prominent nuclei (Fig. 1). Faintly staining CD11b-positive macrophages were scattered throughout the tissue, but polyethylene particles were rarely seen, probably because of the small particle size. Vitronectin-receptor-positive cells were plentiful, most being in the interstitial tissue rather than immediately adjacent to bone. Many more of these cells were seen in the sections from chambers receiving TGF β 1 compared with those without. By four weeks, there was an increase in trabecular woven bone compared with the two-week specimens (Fig. 2). The bone was aligned longitudinally, parallel to the canal in the chamber, in a fibrovascular stroma. Bone was more prominent in the sections containing TGF β 1, but qualitatively, the bone in these sections resembled that in sections without TGF β 1. By six weeks, the thin longitud-

Fig. 2a

Fig. 2b

Four-week harvest. Figure 2a – Photomicrograph from a chamber with PE particles. The harvested tissue is composed of more mature, longitudinally orientated trabecular woven bone in a fibrovascular stroma. Figure 2b – Photomicrograph from a chamber with PE particles and TGF β . The harvested tissue contains thicker, longitudinally orientated bone trabeculae in a fibrovascular stroma (haematoxylin and eosin $\times 66$).

Fig. 3a

Fig. 3b

Six-week harvest. Figure 3a – Photomicrograph from a chamber with PE particles. The harvested tissue is composed of thicker, more mature bone trabeculae and a fatty fibrovascular interstitium. Figure 3b – Photomicrograph from a chamber with PE particles and TGF β . The harvested tissue contains thick, more mature bone trabeculae in a cellular fibrovascular stroma (haematoxylin and eosin $\times 66$).

Fig. 4a

Fig. 4b

Four-week harvest. Figure 4a – Photomicrograph from a chamber with PE particles. Vitronectin-receptor-positive cells are seen in the interstitium and adjacent to longitudinally orientated trabecular bone. Figure 4b – Photomicrograph from a chamber with PE particles and TGF β . More trabecular bone is seen. There is noticeably more brown staining for the vitronectin receptor (osteoclast-like cells) both in the interstitium and adjacent to bone (immunostaining and monoclonal antibody CD51 for the vitronectin receptor, counterstaining with Toluidine Blue $\times 66$).

Table II. Mean ($\pm$ SEM) percentage of bone in the BHC with PE particles with/without TGFβ

Time (wk)	PE particles alone	PE particles + TGFβ	95% CI for difference	p value
2	16.9 $\pm$ 2.7	7.8 $\pm$ 1.3	-14.0 to -4.3	0.002
4	22.5 $\pm$ 2.0	31.2 $\pm$ 3.4	1.0 to 16.4	0.03
6	28.8 $\pm$ 3.5	38.2 $\pm$ 3.9	-4.6 to 23.3	0.16

Table III. Mean ($\pm$ SEM) percentage of bone in the BHC without carrier or particles

Time	Right	Left	95% CI for difference
Control after two-week harvest	23.4 $\pm$ 1.4	24.2 $\pm$ 2.5	-7.1 to 5.5
Control after four-week harvest	20.3 $\pm$ 1.7	19.7 $\pm$ 1.8	-3.4 to 5.5
Control after six-week harvest	22.6 $\pm$ 2.8	25.4 $\pm$ 1.6	-10.4 to 4.7

inal trabecular woven bone strips had matured into thicker plates of bone (Fig. 3) and a marrow cavity was forming in the centre of some of the specimens. Bone was even more conspicuous in the six-week sections and more bone also appeared to be present in sections from chambers with TGFβ1 treatment compared with those without. Vitronectin-receptor-positive cells were still abundant at four and six weeks, with more of these cells in the interstitial tissue rather than abutting bone (Fig. 4). Staining with tartrate-resistant acid phosphatase paralleled the results for the vitronectin receptor.

Control sections which had neither particles nor TGFβ1 were composed of longitudinally orientated woven bone trabeculae in a fibrovascular stroma.

Histomorphometric findings. At two weeks, the mean ($\pm$ SEM) bone area as a percentage of the total area was less in chambers containing TGFβ1 than in those with particles alone (7.8 $\pm$ 1.3% v 16.9 $\pm$ 2.7% respectively; 95% confidence interval (CI) for difference, -14.0 to -4.30; p = 0.002, Table II). At four weeks, this finding had reversed; the percentage area of bone was greater in chambers containing TGFβ1, compared with those with particles alone (31.2 $\pm$ 3.4% v 22.5 $\pm$ 2.0%, respectively; 95% CI for difference 1.0 to 16.4; p = 0.03). There were no statistical differences at six weeks, despite a higher mean value with treatment with TGFβ1 (38.2 $\pm$ 3.9% v 28.8 $\pm$ 3.5%; 95% CI for difference -4.6 to 23.3; p = 0.16). There were no significant differences between the two control sides which had neither particles nor TGFβ1 at any of the time periods (Table III).

The number of vitronectin-receptor-positive cells was greater in the treatment group with particles and TGFβ1 compared with that with particles alone at two weeks with total cells (p = 0.0005) and those in the interstitium (p = 0.0001) and at four weeks with total cells (p = 0.04) (Table IV). Trends were found for increased numbers of vitronectin-receptor-positive cells in the TGFβ1 group at four weeks for cells adjacent to bone (p = 0.1) and in the interstitium (p = 0.06), and at six weeks for the total number of cells (p = 0.07) and those adjacent to bone (p = 0.07).

There were no differences in the numbers of vitronectin-receptor-positive cells after the first and third three-week

Table IV. Mean ($\pm$ SEM) number of cells positive for the vitronectin receptor in the entire section from the BHC with particles with/without TGFβ

Time (wk)	PE particles alone			PE particles $\pm$ TGFβ		
	Total VR $\pm$ *	VR $\pm$ adj†	VR $\pm$ away‡	Total VR $\pm$	VR $\pm$ adj	VR $\pm$ away
2	207.5 $\pm$ 43.6	66.8 $\pm$ 15.4	140.7 $\pm$ 29.2	510.4 $\pm$ 51.5 (172.4 to 433.4)§	52.8 $\pm$ 11.2 (-61.6 to 33.6)	457.6 $\pm$ 48.7 (206.7 to 427.1)
4	353.1 $\pm$ 28.6	134.3 $\pm$ 11.1	218.8 $\pm$ 21.4	491.8 $\pm$ 48.8 (-5.8 to 271.6)	183.6 $\pm$ 21.6 (-11.5 to 110.1)	308.2 $\pm$ 33.5 (-5.4 to 184.2)
6	309.4 $\pm$ 44.6	113.5 $\pm$ 17.5	195.9 $\pm$ 30.4	445.3 $\pm$ 40.2 (-13.5 to 285.3)	183.9 $\pm$ 24.2 (-6.2 to 147.0)	261.4 $\pm$ 34.1 (-51.1 to 182.1)

* the total number of vitronectin-receptor-positive cells in the entire section

† the number of vitronectin-receptor-positive cells which are immediately adjacent to bone in the entire section

‡ the number of vitronectin-receptor-positive cells which are not immediately adjacent to bone in the entire section

§ 95% CI of difference

Table V. Mean ($\pm$ SEM) number of cells positive for the vitronectin receptor in the entire section from the BHC in the control harvests

Time in situ	Right			Left					
	Total VR+†	VR+adj‡	VR+away§	Total VR+	95% CI for difference	VR+adj	95% CI for difference	VR+away	95% CI for difference
Control 1*	327.5 $\pm$ 29.9	90.4 $\pm$ 9.9	237.1 $\pm$ 22.5	309.3 $\pm$ 31.4	-47.3 to 83.7	89.6 $\pm$ 12.4	-28.5 to 30.1	219.7 $\pm$ 22.9	-26.6 to 61.4
2	558.0 $\pm$ 84.4	161.3 $\pm$ 29.5	396.7 $\pm$ 58.2	314.1 $\pm$ 41.6	113.6 to 374.2	110.2 $\pm$ 15.3	10.1 to 92.1	203.9 $\pm$ 27.6	98.0 to 374.2
3	496.6 $\pm$ 62.6	161.1 $\pm$ 18.7	335.5 $\pm$ 47.2	507.7 $\pm$ 73.3	-272.4 to 250.2	159.3 $\pm$ 30.7	-77.4 to 81.0	348.4 $\pm$ 50.6	-207.4 to 181.6

* control 1 = three-week harvest with no particles or carrier which follows the two-week harvest with particles; control 2 = three-week harvest with no particles or carrier which follows the four-week harvest with particles; and control 3 = three-week harvest with no particles or carrier which follows the six-week harvest with particles

† the total number of vitronectin-receptor-positive cells in the entire section

‡ the number of vitronectin-receptor-positive cells which are immediately adjacent to bone in the entire section

§ the number of vitronectin-receptor-positive cells which are not immediately adjacent to bone in the entire section

control periods in which both chambers received neither particles nor TGF β 1 (Table V). After the second control harvest, the chamber which had previously received particles and TGF β 1 still had more total vitronectin-positive cells ($p = 0.002$), both adjacent to bone ($p = 0.02$) and in the interstitium ($p = 0.001$).

Discussion

Osseointegration of cementless implants is especially challenging in revision hip surgery because of the damaged bone bed and the frequent presence of residual particulate debris and granuloma. Localised application of growth factors may be a possible solution to the difficulty in obtaining a long-lasting stable bone-prosthesis interface at revision. TGF β is an anti-inflammatory, immunosuppressing cytokine, important in the repair and remodelling of mesenchymal tissue. It induces osteogenesis and chondrogenesis by mesenchymal cells, stimulates osteoblasts to proliferate and synthesise collagen and extracellular matrix, and inhibits the function of osteoclasts.¹⁻³ Previous studies using the BHC and the rabbit tibial model have shown a suppression of net bone formation in the presence of polyethylene particles⁹⁻¹² as a result of a decrease in the formative pathways of bone and an increase in bone resorption.⁹⁻¹¹ We hypothesised that exogenous TGF β would enhance tissue differentiation into bone and reverse in part the suppressive effects of polyethylene particles on the net formation of bone in the BHC.

After two weeks, treatment with TGF β 1 was associated with a decrease in the net formation of bone and an increase in the number of vitronectin-receptor-positive cells in total and in the interstitium, compared with treatment with polyethylene particles alone. As mentioned previously, TGF β 1 is a pleotropic growth factor which can modulate cellular events in a time- and concentration-dependent manner.¹⁻³ Its effects on proliferation and differentiation may be stimulatory or inhibitory, depending on a variety of conditions including the cell type, growth conditions, the state of cell differentiation, the presence of other growth factors, the local concentration of TGF β , etc.¹⁻³ Removal of the contents of the BHC after two weeks in the presence of TGF β may reflect the residual effects of high local concentrations of this growth factor and/or an evolving biological environment of mesenchymal cells and capillaries temporarily conducive to bone resorption rather than formation. This may also be the case in other studies in which higher doses of TGF β did not result in increased bone ingrowth or formation in the short term.^{4-6,8}

After four weeks, TGF β 1 was shown to enhance tissue differentiation into bone in the presence of polyethylene particles. This effect was less pronounced, but was still present at six weeks. The increase in the net formation of bone occurred despite an increase in the number of vitronectin-receptor-positive cells, i.e., osteoclast-like cells. It

would appear that after four weeks, and to a less degree at six weeks, the effects of TGF β 1 on bone formation may predominate over those on bone degradation. They may be due to numerous factors in relation to the local biological environment as outlined above. The data at two, four and six weeks suggest an early window, at between two and six weeks, in which TGF β 1 may favourably affect bone ingrowth in the present model. These effects are probably not due to a decreasing local load of polyethylene particles. Similar studies at three and six weeks with comparable volumes of polyethylene particles showed a persistent suppression of net formation of bone.¹¹

It is recognised that the rabbit BHC containing polyethylene particles is not a weight-bearing joint arthroplasty and therefore direct application of this experiment to the clinical situation in man is speculative. We did attempt, however, to use parameters which would parallel the human condition as closely as possible, using a simplified model of bone ingrowth. Thus, the particles were sub-micron in size and composed of medical grade ultra-high-molecular-weight polyethylene, comparable with those from retrieved human tissues. When corrected for particle size, this concentration corresponded to the same order of magnitude in volume as that used in previous studies in rabbits, using larger high-density-polyethylene particles.^{9,10} Other doses of TGF β 1, different particle types and concentration and different harvest periods may have yielded different results. Despite this, the addition of TGF β 1 was found to have a significant, mitigating effect on the adverse consequences of polyethylene particles on bone ingrowth in the BHC. Thus, growth factors such as TGF β may be a useful adjunct in obtaining osseointegration and bone ingrowth, especially in the situation of residual particulate debris.

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