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Growth-hormone loaded bioactive ceramics

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A 'bioactive' material, capable of active stimulation of osteogenesis, has been produced by adsorbing human growth hormone onto calcium phosphate ceramics. These materials can be used to deliver growth hormone at the bone–ceramic interface. The elution of the hormone occurs in two phases, with an initial rapid release followed by a slow continuous release for up to 25 days. Tricalcium phosphate was found to release growth hormone better than hydroxyapatite, probably due to the higher solubility of the ceramic. *In vivo* studies using a rabbit model were used to demonstrate osteointegration at the ceramic interface.

1. Introduction

A combination of porous materials with bioactive ceramic coatings is the favoured method of total joint replacement for the younger patient. This surgical technique relies on early bony ingrowth into the coating for early weight bearing. Pronounced enhancement of bone formation has been observed 2 and 4 weeks after implantation, with no differences observed after 12 weeks [1]. To stimulate new bone formation in the crucial post-operative period, we have used calcium phosphate ceramics as delivery agents for human growth hormone. Growth hormone is the only hormone known to stimulate longitudinal bone growth in a dose-dependent manner [2]. Whilst the 'somatomedin hypothesis' [3] implies that growth hormone acts on the skeleton by regulating the circulatory levels of somatomedin or insulin-like growth factor I (IGF-I), more recent evidence indicates that growth hormone may directly activate target cells to produce IGF-I; these findings have been observed *in vivo* in the rat growth plate [4] and *in vitro* in osteoblast cultures [5]. Growth hormone is now thought to stimulate bone cell differentiation and proliferation by both paracrine and autocrine responses.

We have previously reported the use of polymethylmethacrylate bone cement to release human growth hormone, and observed an increase in the formation of osteoid at the bone–cement interface [6]. In this work, we evaluate hydroxyapatite and tricalcium phosphate ceramics as possible delivery agents for growth hormone.

2. Materials and methods

Preparation of growth-hormone loaded calcium phosphate ceramic hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$ powder and tricalcium phosphate (TCP) ceramic $Ca_3(PO_4)_2$ obtained from BDH (British Drug Houses, Dagenham, Essex) were used to make calcium phosphate ceramic discs. The powders were compressed in a stainless steel die up to an applied pressure of 80 MN m^2. After the powder compact was pushed out, it was first sintered at 800 °C and then sintered at 1250 °C for HA and at 1150 °C for TCP. The discs were loaded with human growth hormone by placing discs of the ceramics in solutions of human growth hormone (12 IU ml^{-1}) for a period of 24 h at 4 °C.

2.1. Preparation of ^{125}I growth-hormone loaded calcium phosphate ceramics

Hydroxyapatite and tricalcium phosphate ceramic discs (diameter 3 mm, thickness 2 mm) were placed in a solution of ^{125}I-biosynthetic human growth hormone (10 μCi ml^{-1}) for two time periods: 24 h and 3 days.

2.2. Elution of growth hormone and protein

The release of growth hormone from the loaded material was investigated using an *in vitro* system. Discs of growth-hormone loaded material were placed in phosphate buffered saline (PBS) and continuously mixed on a rolamixer at 37 °C. At regular intervals the elution fluid was removed and replaced with fresh phosphate-buffered saline. The eluent was assayed for growth hormone by an in-house enzyme-linked immunoadsorbant assay, with a polyclonal guinea pig anti-hGH as the coating antibody, and a peroxidase labelled Fab′-fragment conjugate of guinea pig anti-hGH; the standard was biosynthetic 22k-hGH (provided by Novo-Nordisk, Denmark). Total protein was measured by a commercial dye-binding method (Biorad, Hemel Hempstead, UK) and a Cobas Bio analyser (Roche, Welwyn Garden City, UK).

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2.3. Measurement of the release of radioactive growth hormone

Single blocks of ^{125}I-loaded growth hormone TCP and HA ceramics were placed in 3 ml PBS and rolamixed continuously at 37 °C. At regular intervals, the PBS was removed (eluent) and replaced by fresh PBS. The eluent was counted for total ^{125}I counts, the protein was then precipitated with 40% trichloroacetic acid, and the precipitate and supernatant were counted.

2.4. *In vivo* studies

Hydroxyapatite (HA) and tricalcium phosphate (TCP) powders were compressed in a stainless steel die up to an applied pressure of 80 MN m^{-2}. After the powder compact was pushed out, first sintered at 800 °C, pins with diameter 2 mm and length 5 mm were manufactured and then sintered at 1250 °C for HA and at 1150 °C for TCP. HA and TCP ceramic pins were loaded with growth hormone (12 U ml^{-1}) for 24 h. The pins were allowed to dry and stored at 4 °C.

2.4.1. Rabbit model

Six mature Sandy Lop rabbits of least 3.5 kg were used in this study. Three growth hormone loaded pins were implanted into the lateral cortex of each femur, with three plain pins in the contralateral femur as a control. The rabbits were kept unrestrained for 1 month; after sacrifice the femora were dissected out and divided into three sections containing a pin surrounded by intact bone.

2.4.2. Histology and electron microscopy

The implants with surrounding tissue were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, dehydrated in graded series of alcohols, infiltrated in Spurr's resin and embedded after 21 days.

2.4.3. Light microscopy

Sections of 1 μm were cut and stained with methylene blue–azure II for 3 min and basic fuchsin for 30 sec, as described by Humphrey [7].

2.4.4. Electron microscopy

Sections of between 60 and 90 nm were cut, placed on 0.5% pioloform-supported copper grids, stained with uranyl acetate and lead citrate, and observed using a Philips CM12 electron microscope.

3. Results

3.1. Release of protein

The release of protein was monitored from both TCP and HA, a rapid release for the first 10 days followed by a slower release for the following 10 days (Fig. 1). There was 35% more protein released from the TCP ceramic than the HA after 20 days of elution.

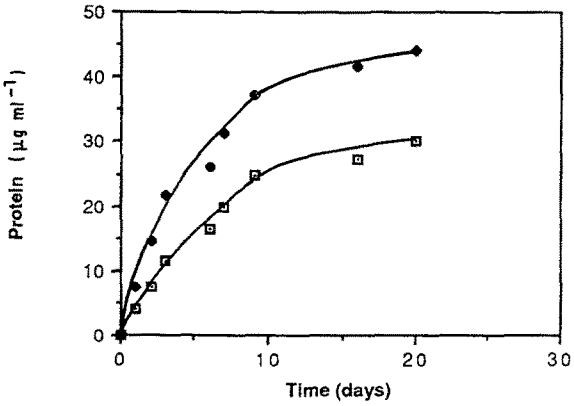

Figure 1 The *in vitro* release of growth hormone from loaded ceramics, as measured by the total protein (ceramics loaded with growth hormone for 24 h). ⊡, HA; ◆, TCP.

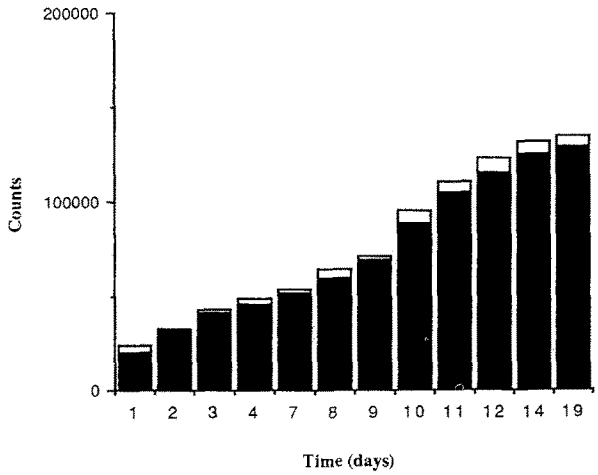

Figure 2 The *in vitro* release of ^{125}I growth hormone from hydroxyapatite ceramic (HA loaded with ^{125}I growth hormone for 3 days). ■, Mean; □, S.D.

3.2. Release of ^{125}I Growth hormone

The release of ^{125}I growth hormone was monitored from both TCP and HA ceramics. The quantity of hormone released was proportional to the length of time that the blocks were loaded with growth hormone. Tricalcium phosphate loaded with growth hormone for 3 days released 2.38 times more hormone than a similar block of TCP loaded for only 1 day.

The release of radioactive growth hormone from both TCP and HA demonstrated the reproducibility of the hormone release. Fig. 2 shows the mean counts for ^{125}I growth hormone released from four blocks of HA with time. There was little variation between the HA blocks, as demonstrated by the low standard deviations between the counts.

3.3. Release of human growth hormone

The elution of growth hormone from the ceramics occurs in two phases. The first phase is a very rapid release and occurs within the first 24 h of the elution; 91.6% of the total growth hormone is released from HA and 78.6% of the total growth hormone is released from the TCP.

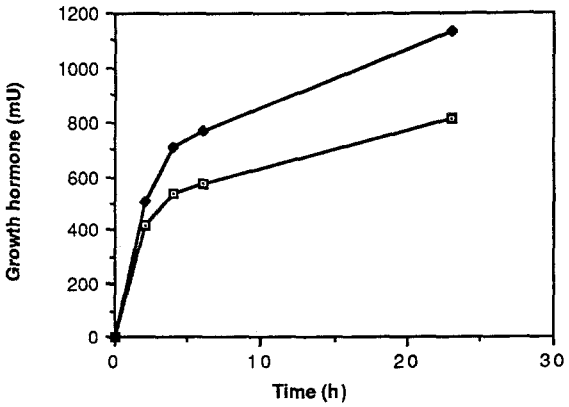

Figure 3 The release of growth hormone from calcium phosphate ceramics during the first 24 h of the elution period ceramics loaded with growth hormone for 24 h. ⊡, HA; ◆, TCP.

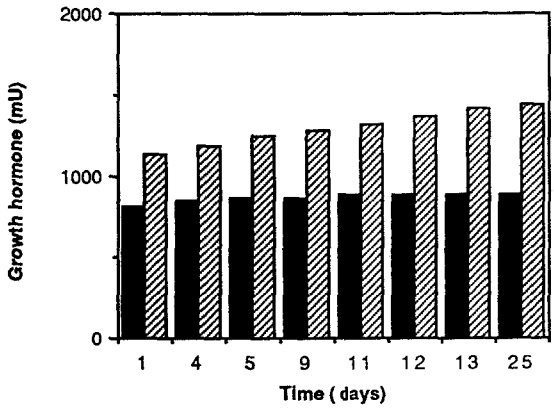

Figure 4 The release of growth hormone from growth-hormone loaded HA (■) and TCP (□) discs for up to 25 days (ceramics loaded for 24 h).

3.4. First phase of elution

During the first 24 h of elution, the most rapid release of growth hormone occurred from both HA and TCP: this has been called the first phase of elution (Fig. 3). The release of growth hormone was greater from the TCP than the HA ceramics, and this difference increased with time. There was no difference between the quantity of growth hormone released from HA and TCP 1 h after elution, but 16.6% more growth hormone was released from TCP than HA after 2 h and after 24 h this difference had increased to 25.1%.

3.5. Second phase of elution

During the second phase of the elution of growth hormone from the ceramics, there was a slow continuous release for up to 25 days. A greater release of growth hormone was observed from tricalcium phosphate than hydroxyapatite discs. 38.6% more growth hormone was released from the TCP than the HA samples after 25 days (Fig. 4).

3.6. *In vivo* results

3.6.1. Histology

The histology of the ceramic–bone interface revealed that there was close integration of bone with the ceramic; and collar of new bone had formed around the ceramic pins. The bone at the interface was composed of hard mineralized bone which, in some areas, was covered by an osteoid seam. More osteoid was visible at the growth-hormone loaded interface (Fig. 5) than at the plain ceramic interface (Fig. 6). There were areas in which the bone had not completely remodelled and marrow cells were in contact with the ceramic; an active osteoid seam was observed covering the mineralized bone (Fig. 7).

3.6.2. Electron microscopy

The ultrastructure of the plain ceramic interface revealed new collagen formation, hydroxyapatite crystals associated with the collagen and the formation of an advancing mineral front towards the ceramic. The

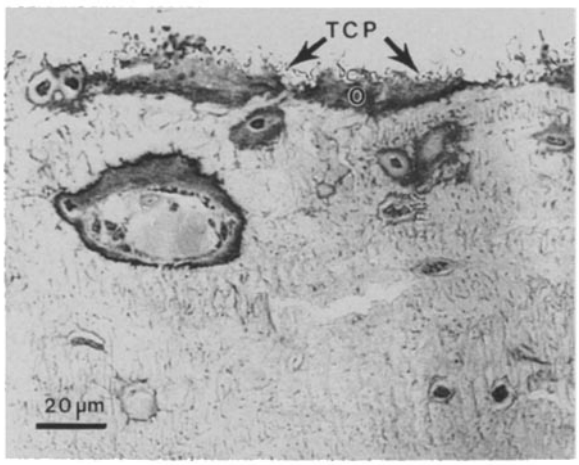

Figure 5 The histology of the interface between bone and growth hormone loaded ceramic. O, Osteoid seam; C, TCP ceramic (1 month after implantation in a rabbit model).

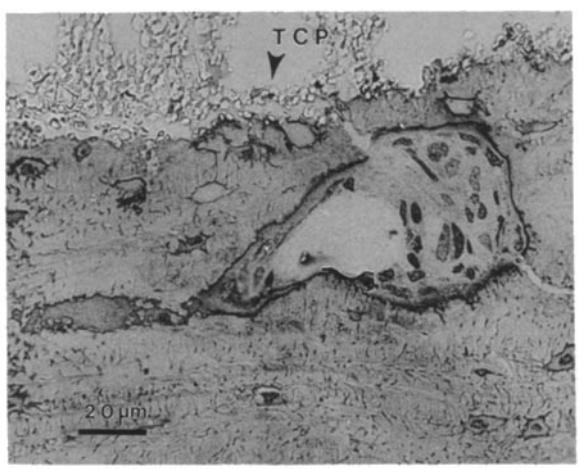

Figure 6 The histology of the interface between bone and plain TCP ceramic. C, ceramic (1 month after implantation in a rabbit model).

interface is that region between the TCP and the mineralizing collagen (Fig. 8), newly formed osteocytes could be observed along the interface as the osteoblasts become surrounded by mineral.

At the interface between bone and growth-hormone loaded ceramic, there was evidence of the formation of

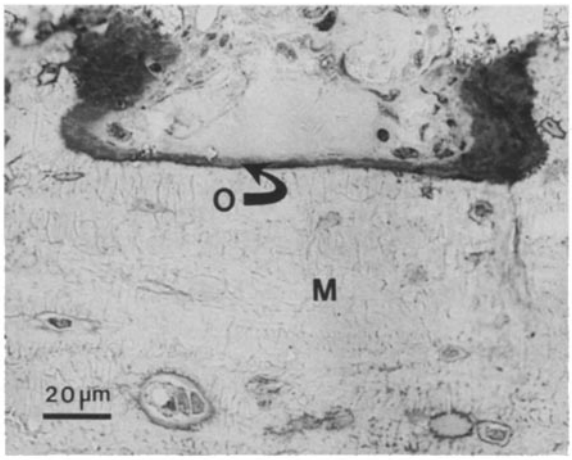

Figure 7 Histology of the interface between bone and growth-hormone loaded ceramic. Note the active osteoid seam at the interface.

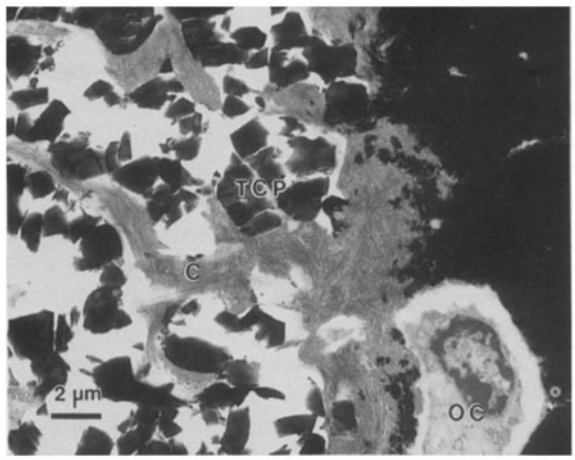

Figure 9 An electron micrograph of the interface between bone and the growth-hormone loaded ceramic. TCP, ceramic; OC, newly forming osteocyte; C, collagen. Note the finger-like processes of collagen between the particles of the ceramic.

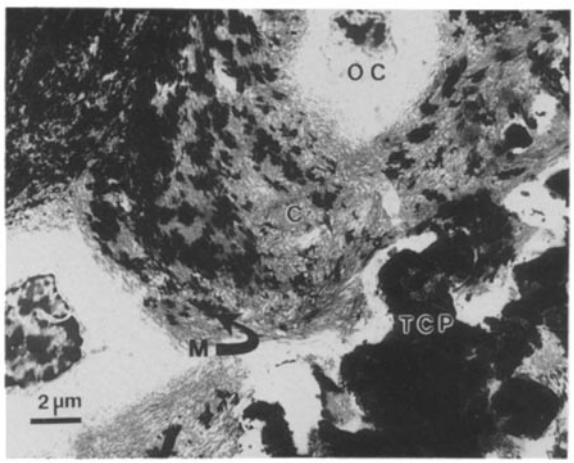

Figure 8 An electron micrograph of the plain ceramic interface – a newly formed advancing mineral front is observed. OC, osteocyte; C, collagen; M, mineral.

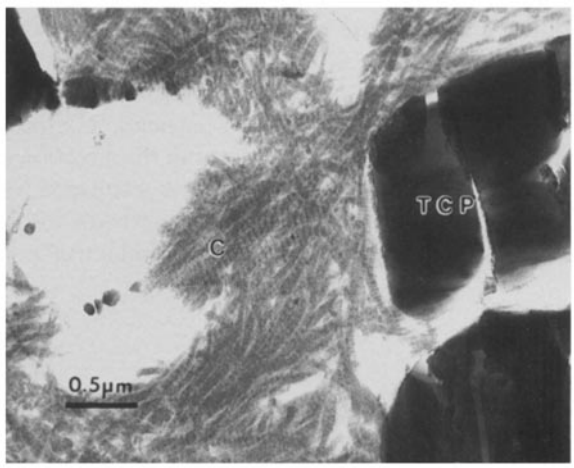

Figure 10 An electron micrograph of collagen fibrils surrounding growth-hormone loaded ceramic. C, collagen; TCP, ceramic.

new bone, i.e. an advancing mineral front and new osteocyte formation. There were marked differences in the appearance of the collagen at the growth-hormone loaded ceramic interface. The collagen was more dense with finger-like processes extending between the ceramic particles (Fig. 9). At higher magnification the collagen fibrils could be seen in direct contact with the ceramic (Fig. 10).

4. Discussion

The stimulation of bony ingrowth into the porous coatings of orthopaedic implants will considerably improve the mechanical fixation and long-term stability of the implant. The coating of metal implants with hydroxyapatite coatings has been described as a bioactive system which stimulated new bone formation [8]. This type of implant is favoured for the younger patient: early remodelling leads to early joint loading and faster patient recovery. In order to further improve bone remodelling at ceramic interfaces, we have incorporated human growth hormone in calcium phosphate ceramics.

Our studies show that calcium phosphate ceramics are good carriers for proteins. The proteins are adsorbed onto the ceramic and can subsequently be released into an elution fluid. There is obviously enormous potential for the use of ceramics as delivery agents of osteogenic proteins and peptide hormones or growth factors. In this work we have specifically studied the incorporation and release of human growth hormone. The elution of growth hormone from calcium phosphate ceramics has been described as two phases – a very rapid release during the first 24 h followed by a slow, continuous release. More growth hormone was released from the TCP than the HA. The quantity of growth hormone released was proportional to the time taken to load the ceramic, and provided that the loading of the ceramics was carefully controlled, very reproducible release rates were obtained. The quantity of protein released depended upon the type of ceramic used; tricalcium phosphate released more protein and hormone than hydroxyapatite, probably because the tricalcium

phosphate is more soluble than hydroxyapatite in the elution fluid.

In the *in vivo* system, growth hormone released from the ceramics should come into direct contact with the cells and tissue surrounding the implant. Calcium phosphate ceramics without the addition of growth hormone have been described as osteoconductive [1], and we confirm that an active remodelling process occurs at the ceramic interface after implantation in bone. However, the incorporation of growth hormone improves this remodelling process by actively stimulating osteogenesis and matrix production at the interface. Growth hormone is thought to increase tissue formation directly by promoting the differentiation of precursor cells, and indirectly by the stimulation of local production of IGF-I which promotes cell multiplication [9]. The stimulation of IGF-I production by growth hormone has been demonstrated using osteoblasts in culture [5, 10].

In a recent study, growth hormone was shown to increase osteoid formation at the growth-hormone loaded ceramic interface [11]. In this study we examined the ultrastructure of the ceramic–bone interface, and observed dense collagen along the original mineral with collagen fibres extended between, and in direct contact with, the ceramic particles. The presence of a mineralizing collagen front in the direction of the ceramic suggests that, with time, this collagen will completely mineralize and true bony ingrowth will be observed. The incorporation of osteoinductive proteins in calcium phosphate ceramics provides a truly bioactive biomaterial for use in orthopaedics.

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