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AN ELECTRON MICROSCOPICAL STUDY OF THE INTERACTION OF BONE WITH GROWTH HORMONE LOADED BONE CEMENT

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Abstract

Growth hormone was incorporated into Polymethylmethacrylate (PMMA) with a view to improving the quality of the bone-cement interface. Growth hormone was released from the PMMA and delivered to the cells at the bone-cement interface. The *in vivo* response to the growth hormone loaded cement was compared to the plain cement, using light microscopy, transmission electron microscopy and scanning electron microscopy, in a rabbit model. The results indicate that growth hormone released at the bone-cement interface stimulated osteogenesis and the reorganization of the tissue components. An advancing mineral front was observed in the direction of the bone cement with new bone formed in direct apposition to cement. This was compared to the interface with plain PMMA cement, which showed little organization of the tissue components, and spaces between the bone and the cement containing areas of fibrous tissue.

Key words: Bone-cement, Polymethylmethacrylate, growth hormone, osteogenesis, bone, interface, osteoid, transmission electron microscopy, scanning electron microscopy.

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Introduction

Loosening of the implanted prosthesis remains the mode of long term failure of total hip arthroplasty (15, 17, 19). In the past decade the rate of aseptic loosening has been reduced by improved cementing techniques in order to achieve better interlock at the bone-cement interface (6, 13) and improved prosthesis design to prevent both fracture of the prosthesis and bone resorption (1, 8). However, there has been little improvement in the formulation of PMMA bone cement for the past twenty years.

In order to elucidate the mechanism of aseptic loosening it is necessary to examine the bone-cement interface after implantation of the cement in the medullary canal. According to Willert *et al.* (20) the anchorage of cement to bone is never more stable than immediately after surgery. This is followed by three phases in the histological reaction of bone to acrylic cement: degeneration, reparation and formation of a fibrous connective tissue membrane. The formation of this fibrous tissue at the bone-cement interface can compromise the stability of the prosthesis (7). Histological evaluation of the tissues surrounding loosened components has revealed a soft tissue membrane with macrophages with high acid phosphatase activity (11) and the presence of a synovial-like membrane associated with bone lysis (5). Fluorescent labelling demonstrates that reduced remodelling or necrosis occurs adjacent to cement (14). Histological examination of the stable cemented interface has revealed a spectrum of tissue reactions ranging from direct bone contact with cement, to a fibrous membrane measuring 1.5 mm in thickness. Necrotic bone resulting from surgical trauma had largely been resorbed and replaced by viable bone (10). Malcolm reported good bone cement integration with no fibrous layer in 60 out of 78 patients who had long term prosthetic implants that were clinically stable (12).

In this work we investigate whether growth hormone released at the bone-cement interface has an effect on the remodelling and new bone formation at this interface. Previous studies have shown that PMMA bone cement can be used to release growth hormone *in vitro*; an initial rapid release is followed by a slower release for

up to 40 days. Aliquots of released hormone were tested for bioactivity using the MTT-ESTA on Nb2 cells. All aliquots were found to contain bioactive growth hormone. Light microscopy and histomorphometric analysis of the bone-cement interface revealed more osteoid present, after use of growth hormone-loaded cement, one month after surgery (2). We now examine the ultrastructure of the bone-cement interface using both growth hormone-loaded and plain bone cement, in a rabbit model.

Materials and Methods

Preparation of growth hormone loaded bone cement

All the reagents, mixing bowl and spatula were kept at room temperature for one hour before mixing. Using sterile technique with sterile conditions, 10 g of polymethylmethacrylate powder (CMW Densply) were added to a plastic mixing bowl: 12 IU (2.5 mg) growth hormone (Novo Nordisk, Denmark), as a lyophilized powder, was then added and mixed thoroughly for one minute using a stainless steel spatula. The ampoule of the methacrylate monomer component of the cement was opened and 5 ml added to the powder in a well ventilated area. The cement was mixed for a further minute until in a "dough" state. It was then inserted into a sterile plastic syringe for insertion into the rabbit.

Animal Model

Three adult Sandy Lop rabbits weighing at least 3.5 kg were used in the experiments. Access to the knee was gained through a medial parapatellar capsulotomy and, using a 3 mm diameter drill bit, the medullary cavity of the femur was reamed to a depth of 2 cm starting at the intercondylar notch. One femur was filled with growth hormone loaded cement, with plain cement in the contralateral femur as a control. One month after surgery the rabbits were sacrificed and the femora removed.

Processing and Embedding

The adherent tissue was removed from the bone and the undecalcified femora cut into two halves longitudinally starting at the intercondylar notch. The tissue was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for two hours. A small area (3 mm x 6 mm) was sectioned out and further fixed for 24 hours in fresh fixative at 4°C. Secondary fixation was in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for one hour. The block was washed in sodium cacodylate buffer, dehydrated in a graded series of alcohols (70%, 90%, 100%), and impregnated with 1:1 (by volume) alcohol and Spurrs' resin for 6 hours, two hours of which were by vacuum impregnation at 150 mbar using an Anglia Scientific Vacuum embedding chamber. This was followed by 4 changes of 12 hours each, with Spurrs' resin, alternating every 6 hours with vacuum impregnation. The block was finally embedded and cured at 70°C for 18 hours.

Light Microscopy

For light microscopy 1 μm sections were cut using a diamond knife, floated onto a drop of water on a glass slide and dried on a hotplate. Sections were then stained with methylene blue-azure II and basic fuchsin (Humphrey's stain). Similar areas from both the growth hormone loaded and the plain cement interface were selected for ultrastructural studies.

Transmission Electron Microscopy

Selected areas were further trimmed down and sectioned with a diamond knife between 60-90 nm, then picked up onto 0.5% pioloform support films on copper grids. The sections were then stained with 2% uranyl acetate and lead citrate (Reynolds) for ten minutes each. Observation was made using a Philips CM 12 transmission electron microscope with an EDAX PV 9800 microanalysis system.

Scanning Electron Microscopy

Whole resin blocks were taken, the surfaces polished using a diamond knife and then plasma etched. The specimens were sputter coated with 20 nm gold and viewed with a JEOL JSM 35C scanning electron microscope.

Results

Light Microscopy

Fig. 1 shows the interface between bone and growth hormone loaded cement. A layer of active cuboidal shaped osteoblasts, a wide osteoid seam and new mineral formation were observed along the interface. Numerous osteocytes with visible nuclei were observed in the mineralized bone. Polarized light was used to visualize a distinct area of new bone formation. This was compared to the interface between bone and plain cement (Fig. 2). The bone at the interface between plain cement appeared to be much less "active"; some cells were observed along the interface but there was less evidence of new osteoid formation.

Transmission Electron Microscopy

Ultrastructural studies of the tissues present at the bone-cement interface revealed marked differences in the response to growth hormone loaded cement, as compared to plain cement. At the interface with growth hormone loaded cement there was a layer of active osteoblasts and newly formed collagen (Fig. 3a). There were also many newly formed osteocytes along the advancing mineral front and bone matrix components in direct apposition to the cement (Fig. 3b). It was difficult to determine the exact bone-cement interface because the cells and matrix had been laid down in the outer 30 μm of the cement. Bone cement is opaque at the TEM level and it was necessary to use X-ray microanalysis to localize the zirconium dioxide which is present in the cement as a marker. Deposition of hydroxyapatite along the collagen fibres and the formation of a new mineral front were observed along the cement interface (Fig. 4). The hydroxyapatite

crystals in Fig. 4 gave Ca:P ratios of 1.667:1 as compared to a known hydroxyapatite standard with X-ray microanalysis (EDAX).

At the boundary between bone and plain cement there were a few inactive cells, debris containing bone dust and marrow components remained at the interface (Fig. 5).

Scanning Electron Microscopy

Scanning electron microscopy was used to examine the entire bone-cement interfaces after use of both growth hormone-loaded cement and the plain cement. At the growth hormone-loaded cement interface direct contact was observed between the connective tissue and cement. An osteoid seam lined the mineralized bone with new osteocytes along the surface (Fig. 6). In some areas, cells lining the cement interface had extended processes in the cement between the polymer beads (Fig. 7). Conversely, at the plain cement interface, there was a gap between the bone and cement along most of the interface (Fig. 8). In some areas, an acellular tissue composed of fibres with a "mesh-like" structure was observed in the spaces between the bone and the cement; this has been called a fibrous-type tissue (Fig. 9). We believe that the examination of the intact bone-cement interface (without dissolving at the cement) has enabled us to examine this fibrous tissue.

Discussion

Polymethylmethacrylate (PMMA) bone cement has been widely used in Orthopaedic surgery to fix metal prostheses to bone. Bone necrosis occurs at the bone-cement interface due to the trauma of the surgery, loss of vascularization and leakage of cytotoxic monomer at the interface (20). The bone cement shrinks after polymerization, leaving a small gap between the bone and the cement (3). Thus the implant is in an unstable environment and remodelling of the bone at the interface is essential for long term stability. Various tissue responses to PMMA bone cement have been reported, ranging from direct bone contact (10) to the formation of a dense fibrous tissue capsule around the implant (9). A previous study (2) has shown that growth hormone loaded cement can be used to stimulate the early production of osteoid at the bone-cement interface, the difference becoming less significant at two and four months after surgery.

Stimulation of new bone formation in the crucial early post-operative period may improve the strength of the bond between bone and cement. PMMA can be used as a delivery agent for human growth hormone which may directly act on target cells at the bone-cement interface. Whilst the exact mechanism by which growth hormone stimulates bone growth remains unclear, there is evidence that growth hormone can exert a direct effect on osteoblast cells in culture, via the local synthesis of insulin-like growth factor-I (4, 18). In our study, we have examined the cellular and tissue response to growth hormone loaded cement, one month after surgery. Growth hormone released at the interface appears to have stimulated osteogenesis as shown by the increased number of active osteoblasts and the increased collagenous matrix, along the bone cement. Osteoblasts lining the interface showed organelle-rich cytoplasm of protein synthesis with numerous lamellae of rough endoplasmic reticulum and Golgi bodies. There was a marked increase in the synthesis of collagen at the interface and cytoplasmic processes from the cells radiating into the collagenous matrix. Spinelli (16) reported an advancing mineral front in the direction of the cement in a stable implant. We have demonstrated the formation of hydroxyapatite crystals associated with the collagen fibrils. This has lead to the mineralization of the matrix and the formation of an advancing mineral front towards the bone cement. Numerous osteocytes have been formed, as the osteoblasts at the interface have become surrounded by mineral and incorporated in the new bone.

Growth hormone at the interface has stimulated osteogenesis and the advancement of the mineral front, thus improving the stability of the implant. Moreover, cells have been observed extending into the polymer matrix, indicating that growth hormone loaded PMMA is osteoconductive.

Conclusions

The addition of growth hormone to Poly(methyl)-methacrylate bone cement stimulates osteogenesis at the bone-cement interface. Such improvement in the quality of the bone and increase in the strength of the bone to cement bond may improve the long term stability of the implant.

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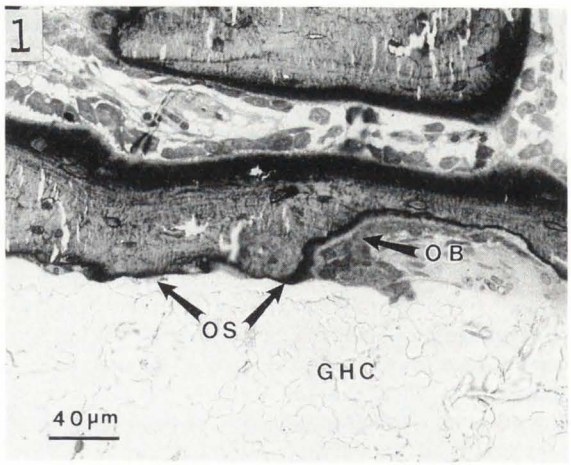
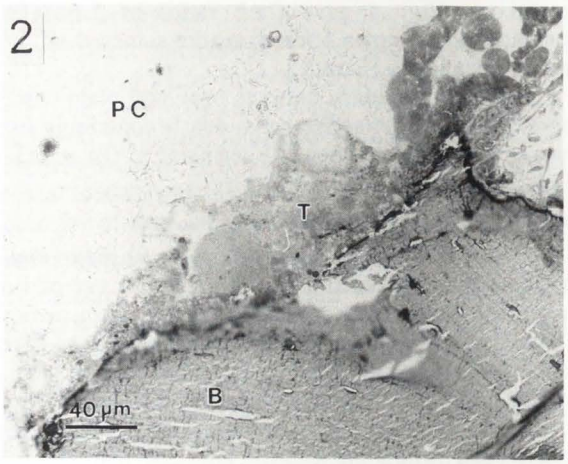
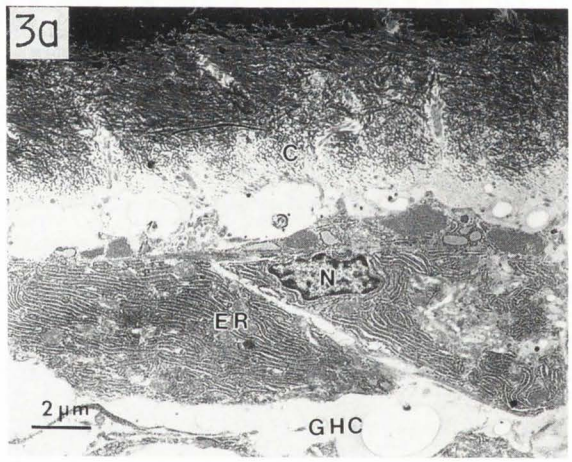






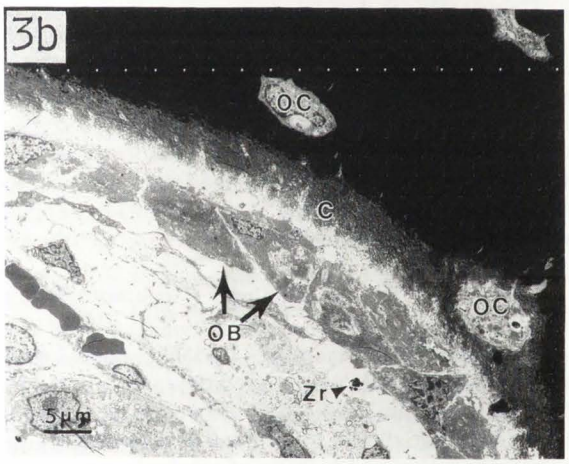


Figure 1. Interface between bone and growth hormone loaded cement (GHC) (stained with Humphrey's stain). Note the healthy osteoblasts (OB) and the osteoid seam (OS) lining the interface.

Figure 2. Interface between bone (B) and plain cement (PC) (stained with Humphrey's stain). Note the amorphous tissue (T) containing bone dust, lipid cells and marrow cells.

Figure 3. a. Ultrastructure of the osteoblasts lining the growth hormone loaded cement (GHC) interface (stained with Uranyl acetate and Lead citrate). Note the rough endoplasmic reticulum (ER), nucleus (N) and the processes from the cells into the collagenous matrix (C).

Figure 3. b. Electron micrograph of the interface between bone and growth hormone loaded cement, zirconium marker (Zr) (stained with uranyl acetate and lead citrate). Note the active osteoblasts (OB), collagen (C) and formation of osteocytes (OC) by the advancement of the mineral front.

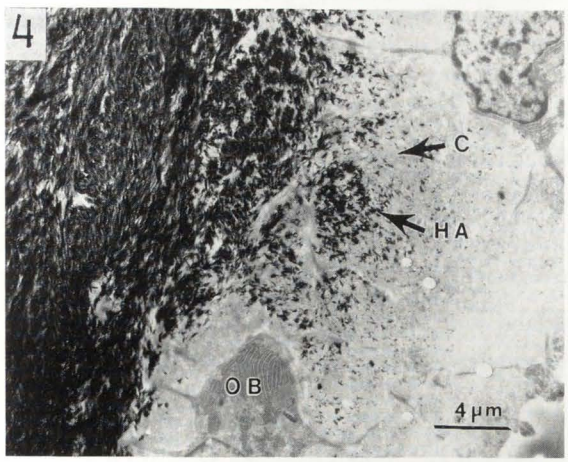
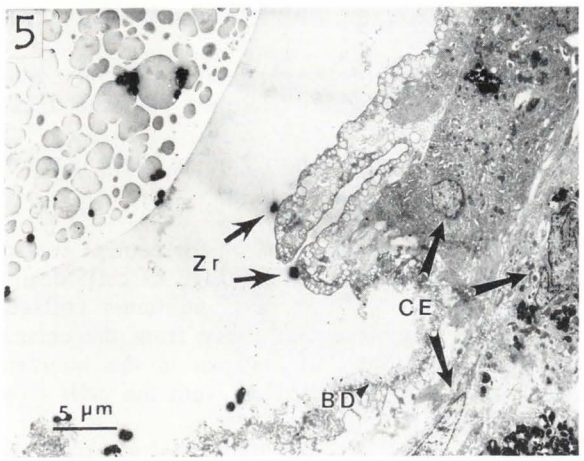
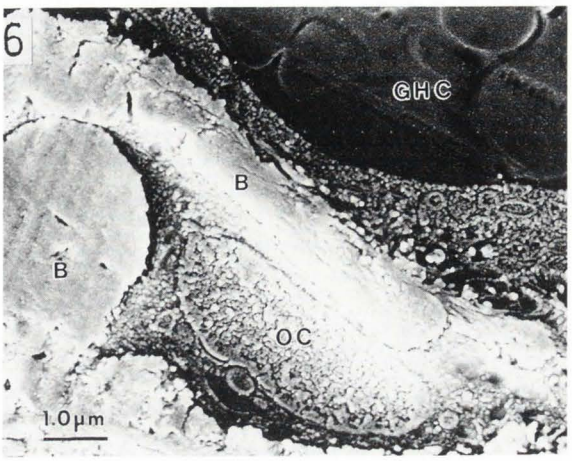


Figure 4. Electron micrograph of the mineralization at the growth hormone loaded cement interface (unstained). New sites of hydroxyapatite (HA) were observed associated with collagen fibres (C), advancing between osteoblasts (OB). These hydroxyapatite crystals gave Ca:P ratios 1.667:1 with the EDAX.





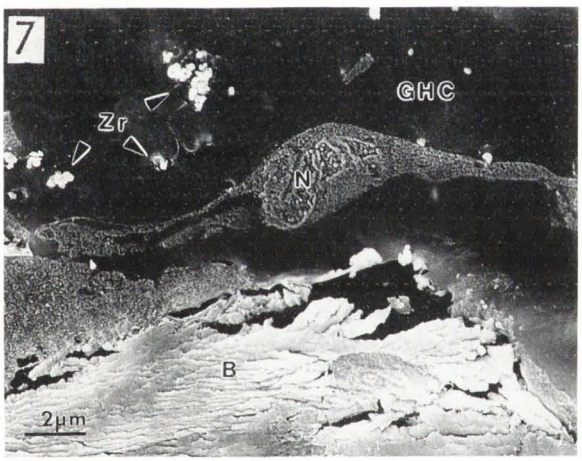


Figure 5. Electron micrograph of the plain cement interface, Zirconium markers (Zr) (stained with Uranyl acetate and Lead citrate.). Note the mixed tissue reaction with several inactive cells (CE) and bone dust (BD) along the interface.

Figure 6. Scanning electron micrograph of growth hormone loaded cement (GHC) interface (plasma-etched and sputter-coated with gold). Newly formed osteocyte (OC) surrounded by the advancing mineral front (B).

Figure 7. Scanning electron micrograph showing a cell with nucleus (N) within the pores of the growth hormone loaded cement (GHC) (plasma-etched and sputter-coated with gold). Note the bone (B) at the interface with the cement containing Zirconium dioxide markers (Zr).

Figure 8. Scanning electron micrograph of the plain cement interface (PC) showing the gap (G) between the bone (S) and the cement (plasma-etched, and sputter-coated with gold). Note numerous cells (CE) along the interface but no osteocyte formation was observed.

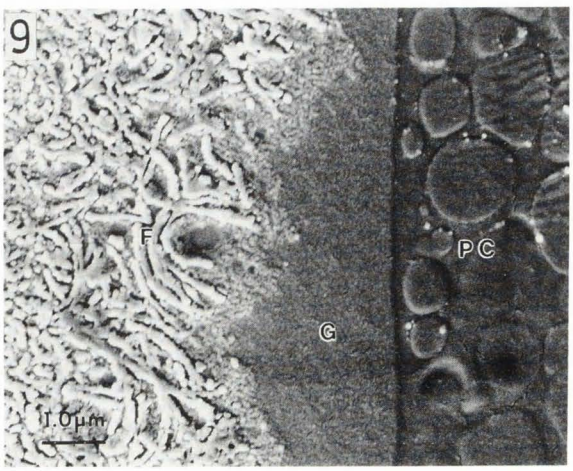


Figure 9. Scanning electron micrograph of the interface with the plain cement (PC) showing a gap (G) and fibrous-type tissue (F) (plasma-etched and sputter-coated with gold).

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Discussion with Reviewers

B. Lowenberg: What is the nature of the granular zone seen in Figure 9 between the "fibrous-type tissue" F and the "gap G"?
Authors: It is obviously the edge of the "fibrous-type"; it has not been characterized.

M.W. Lundy: Is the gap seen in the cement control group artifact, produced by shrinkage of cells during inadequate fixation? If there is minimum collagen between cells, they may pull away from the cement surface. The presence of collagen in the interface between bone and GFC could prevent the cells from shrinking away from the cement.
Authors: The fact that tight interfaces are observed between bone and cement indeed suggests that the gaps observed in the plain cement are not artifact.