In vitro evaluation of a new polymethylmethacrylate cement reinforced with hydroxyapatite

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The nature of the orthopedic implant surface affects the interaction between cells and subsequent bone formation. The bone/cement interface in cement-held prostheses is considered to be the main cause of fracture leading to implant revision. It is thought that the introduction of a bioactive phase, such as hydroxyapatite (HA), to cement may permit a stronger implant by encouraging direct bone apposition rather than encapsulation of the implant by fibrous tissue. Thus, a poly(methylmethacrylate) (PMMA) cement incorporating 17.5% HA by weight has been investigated. In this study, in order to analyze the interaction at the cellular level, the *in vitro* biological response of the HA/PMMA to a similar PMMA without HA incorporation has been studied. Primary human osteoblast-like cells (HOB) were used as they are a model of the cell type the cements might encounter *in vivo.* Cell proliferation and growth were assessed by measurement of total cellular DNA and tritiated thymidine ([^{3}H]-TdR) incorporation. Alkaline phosphatase (ALP) production was measured as an indicator of HOB phenotype upon the cements. The results showed that HA/PMMA was a better substrate for HOB cells, resulting in increased proliferation and ALP activity. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) showed that HOB cells cultured on the HA-filled PMMA preferentially anchored to HA particles exposed at the cement surface, with a close intimacy observed between HA and HOB cells.

1. Introduction

Poly(methymethacrylate) (PMMA), a self-curing cement, was developed in the early 1960s by Charnley and Smith following their observation that friction wedging and mechanical screwing led to loosening of the joint [1]. PMMA is the current standard for cement held prostheses, providing immediate structural support. PMMA cements have been described as inert materials, with fibroblastic cells observed at the cement/bone interface [1, 2]. The bone/cement interface is often considered to be the weak link in cement-held prostheses, providing a barrier to direct fracture healing. As there is no obvious chemical bonding of cement to bone, mechanical retention occurs as a result of interdigitation [1]. Mechanical characteristics have been a problem (historically) with PMMA cements; polymers produced by mixing the cement phases are usually brittle, and have a poor fatigue life. Fracture of cements has been reported to lead to aseptic loosening and tissue necrosis [3]. When considering implant materials there are a variety of factors to consider; these include, biocompatability, material surface conditions, the state of the host bed, surgical technique, and the loading conditions after implant insertion. The addition of small amounts of particulate materials, such as hydroxyapatite (HA), offers the possibility of strengthening, without affecting stress distribution and causing flow problems. HA addition does, however, increase modulus and decrease fracture energy. Although the addition of HA to PMMA improves the cement flexural properties, only a relatively small volume may be added before mechanical deterioration is observed, due to poor tensile and shear strength [4]. It is known that HA can lend bioactivity to composite materials [5]; however the extent of this activity is dependent on the volume of HA incorporated in the PMMA cements. The objective of the study was to investigate a potentially bioactive cement capable of strengthening the mechanical retention of the implant by allowing direct bone apposition. In order to examine this, we used an *in vitro* tissue culture model to evaluate the biological response, both on conventional PMMA and PMMA/HA. *In vitro* systems allow the study of tissue–

material interactions without the complexities associated with *in vivo* models [5]. Furthermore, primary human osteoblast-like (HOB) cells were used as they are representative of the cell type in contact with the material *in vivo* [6]. Assessment of proliferation and measurement of cell phenotype activity was performed to access the bifunctionality of the materials bioactivity. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed in order to examine cellular behavior on the materials.

2. Materials and methods

Discs of 1.2 cm diameter PMMA cements, with and without the addition of 17.5 wt % HA were prepared by addition of the monomer to the polymer and stirred, until fully wetted, under controlled temperature conditions ($22 \pm 2\,^{\circ}C$). The prepared discs were sterilized by gamma irradiation at a dose of 2.5 Mrad (Swann Morton, UK) using standard procedures for medical devices.

2.1. *In vitro* cell culture

HOB cells were cultured on the materials and control Thermanox (TMX, Life Technologies) at 5×10^6 cells ml^{-1} for 1, 3, 7 and 14 days under conditions described in a previous study [7].

2.2. Cell growth, proliferation and differentiation

Cell growth and proliferation were assessed using total DNA and [^{3}H]-TdR incorporation, cell phenotype was quantified by biochemical measurement of ALP. These methods have been described in a previous study [7].

2.3. Cell morphology

2.3.1. SEM

The materials were seeded with HOB cells at a density of 1.5×10^4 cells ml^{-1}. These were incubated at 37 °C in humidified air and 5% CO_2. The cells were fixed with 1.5% gluteraldehyde buffered in 0.1 M sodium cacodylate after a 48 h incubation period. The cells were stained in 1% osmium tetroxide and 1% tannic acid, then dehydrated through a series of alcohol concentrations (20, 30, 40, 50, 60 and 70%), stained in 0.5% uranyl acetate, then dehydrated further (90, 96 and 100% alcohol). The final dehydration was in hexamethyldisilazane, followed by air drying. Once dry, the samples were sputter-coated before examination under a Jeol SEM at an accelerating voltage of 15 keV.

2.3.2. TEM

The materials were seeded with HOB cells at a density of 5×10^6 cells ml^{-1}. These samples were incubated at 37 °C in humidified air and 5% CO_2. The cells were fixed with 1.5% gluteraldehyde buffered in 0.1 M sodium cacodylate after a 21 days in culture. The cells were stained in 1% osmium tetroxide, dehydrated in 70% alcohol, stained in 0.5% uranyl acetate, followed by further dehydration (90, 96 and 100% alcohol). Once dehydrated the samples were resin embedded and polymerized at 70 °C for 18 h. Ultra-thin sections were cut and viewed by a Phillips CM12 TEM.

3. Results

An increase in total cellular DNA (with time) was observed on both materials over the 14 day culture period. Initial high levels of [^{3}H]-TdR incorporation (expressed per μg of DNA) were observed on the TMX control, and both cement samples. Proliferation was seen to peak at day 3, with the HA filled PMMA cement giving higher incorporation values at all time points. Levels of proliferation were seen to be negligible on both materials after day 7 (Fig. 1). When the ALP results were normalized for DNA (Fig. 2), the ALP activity was seen to be similar in PMMA and PMMA/HA. An increase in ALP activity was observed on PMMA/HA on day 3.

SEM of HOB cells growing on the unfilled PMMA samples showed cells with normal osteoblast morphology, flattened with small processes attaching to the surrounding material (Fig. 3). SEM of HOB cells growing on the PMMA/HA cement again showed a normal osteoblast morphology, however, cell processes could be seen attaching preferentially to HA particles exposed at the cement surface, rather than the cement polymer (Fig. 4).

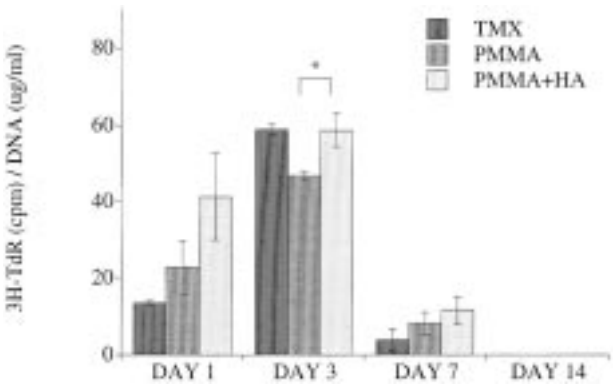

Figure 1 [^{3}H]-TdR (c.p.m.) incorporation/DNA (μg) for HOB cells on control TMX and the test materials PMMA and 17.5 wt % HA/PMMA cultured over a 14 day period (results are the mean $\pm$ SD, $n = 3$, $^* = t$-test $p < 0.05$).

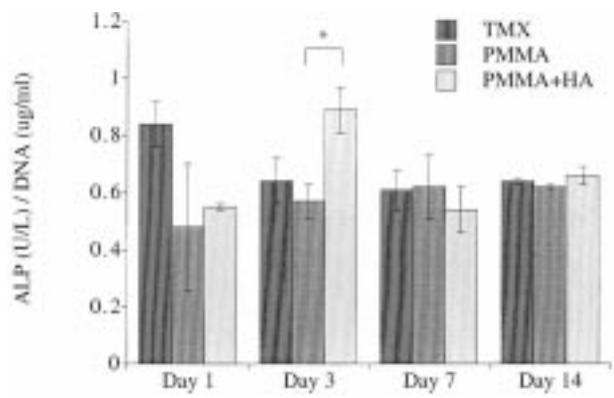

Figure 2 ALP (U/L)/DNA (μg) for HOB cells on control TMX and the test materials PMMA and 17.5 wt % HA/PMMA cultured over a 14 day period (results are the mean $\pm$ SD, $n = 3$, $^* = t$-test $p < 0.05$).

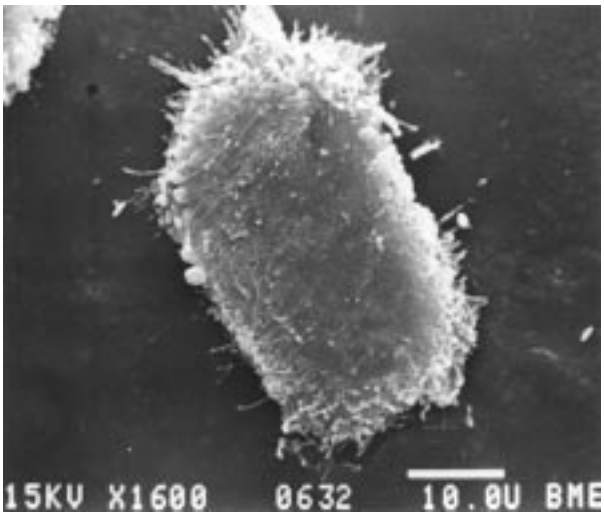

Figure 3 SEM of HOB cell cultured on unfilled PMMA. The cell has normal osteoblast morphology, with a flattened appearance, and small processes attaching to the surrounding material.

TEM showed a high level of intimate contact between HOB cell layers and exposed HA crystals on the cement surface (Fig. 5). Enlargement of the cells in contact with the HA crystals showed particles within the cell layer, close to the site of cell / HA contact (Fig. 6).

4. Discussion

Both the unfilled and PMMA/HA cements were able to support the proliferation and differentiation of HOB cells upon the material surface. The biochemical results for [^{3}H]-TdR incorporation and ALP production show quantitatively that the 17.5 wt % filled PMMA offers higher levels of HOB cell proliferation and phenotype expression. An initial high level of proliferation was observed, which was followed by a decrease in proliferation and a subsequent increase in ALP production. This result is indicative of normal osteoblast growth and phenotype activity [8].

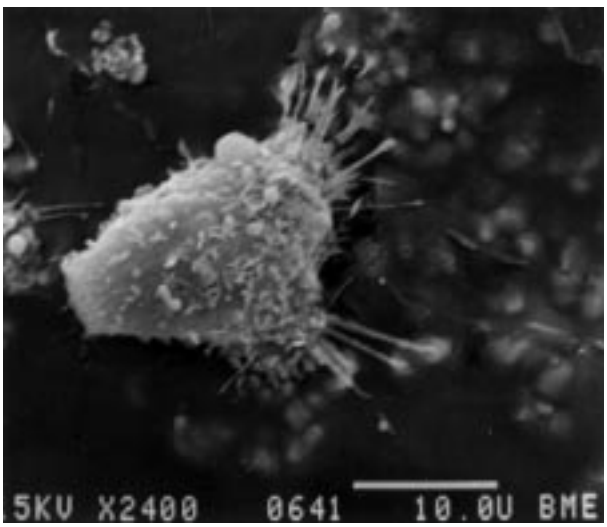

Figure 4 SEM of HOB cell cultured on HA filled PMMA. The cell has normal osteoblast morphology. The cellular processes can be seen to extend, and preferentially attach, to HA crystals exposed at the cement surface.

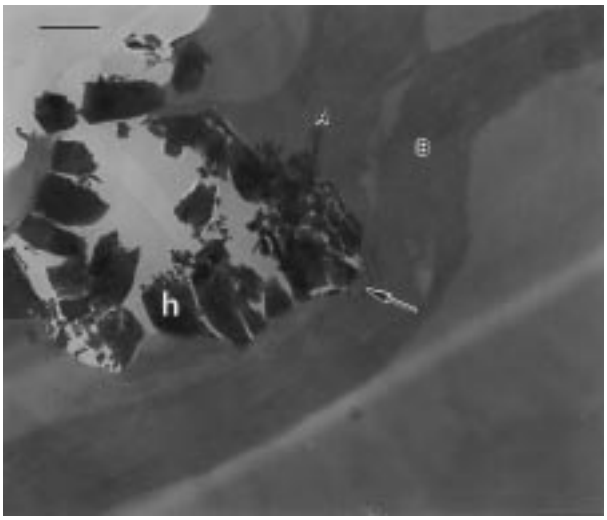

Figure 5 TEM of confluent HOB cell layers (A and B) growing in close intimacy with a HA crystal (h) exposed at the cement surface, after 21 days in culture. Crystalline deposits could be observed in cells with close contact to the HA (arrow). (bar = 1 μm).

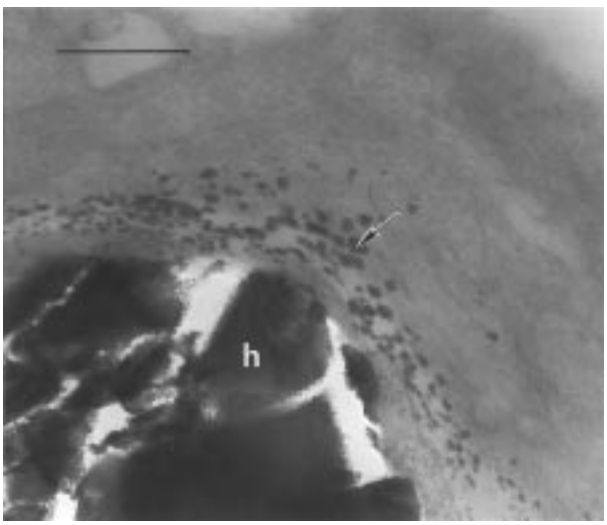

Figure 6 High magnification TEM of crystalline deposits (arrow) observed in HOB cell layer, after 21 days in culture. (bar = 250 nm).

SEM also showed that both materials were able to support normal osteoblast cell growth, but clearly demonstrated HA's bioactive properties with the preferential anchoring of HOB cells to exposed HA particles, rather than the cement polymer.

TEM observation of particles within the HOB cell layers juxtaposed to the HA particles suggests the cells are either entrapping the HA particles, or are producing crystalline particles in response to the presence of HA. This interesting observation requires further investigation by electron probe microanalysis.

5. Conclusion

It has been shown, both histologically and biochemically, that the addition of a low amount of HA to PMMA (17.5 wt %, approximately 6 vol %), offers increased bioactivity, and preferential cell anchorage. It is to be noted, however, that it may be necessary for greater HA incorporation before direct bone apposition is observed

in vivo. Research with the highly bioactive material HAPEX^{TM}, showed incorporation of HA volumes greater than 20% were required for the transition from fibrous encapsulation to direct bone growth [9]. Alternative cements are being developed, for example poly(ethylmethacrylate)/*n*-butylmethacrylate cement, which can incorporate a greater percentage volume of HA than PMMA; which may be the solution to transforming bone cement into a truly bioactive material [10].

Acknowledgments

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References

1. E. P. LAUTENSCHLAGER, S. I. STUPP and J. C. KELLER, *Func. Behav. Orthop. Biomater.* **2** (1984) 87.
2. S. DOWNES, M. V. KAYSER, G. BLUNN and S. Y. ALI, *Cells Mater.* **1** (1991) 171.
3. T. A. GRUEN, *Clin. Orthop. Rel. Res.* **141** (1979) 17.
4. A. SOGAL and S. F. HULBERT, *Bioceramics* **5** (1992) 213.
5. J. HUANG, L. DI SILVIO, M. WANG, K. E. TANNER and W. BONFIELD, *J. Mater. Sci.*: *Mater. Med.* **8** (1997) 1.
6. L. DI SILVIO, PhD Thesis, University of London, 1995.
7. L. DI SILVIO, M. J. DALBY and W. BONFIELD, *J. Mater. Sci.*: *Mater. Med.* **9** (1998) 845.
8. G. STEIN and J. LIAN in ''Endocrine reviews'' edited by M. Node (Academic Press, London, 1993) p. 424.
9. W. BONFIELD, *Biol. Biomech. Perf. Biomater.* (1986) 299.
10. L. L. HENCH, *J. Amer. Ceram. Soc.* **74** (1991) 1487.

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