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In vitro response of osteoblasts to hydroxyapatite-reinforced polyethylene composites

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A primary human cell culture model was used to investigate a range of hydroxyapaptite (HA)-reinforced high-density polyethylene (HDPE) composites (HAPEX™). These materials are being developed as potential bone-substitute materials. When designing and optimizing a second-generation biomaterial, it is important to achieve a balance between mechanical and biological properties without compromising either. Biochemical and histological parameters have been used to compare the biological response of 20% and 40% volume HA in HDPE. Cellular DNA and incorporation of tritiated thymidine was measured to assess cell proliferation. Alkaline phosphatase (ALP) production was used as a marker of osteoblast phenotype expression. In this preliminary study, osteoblasts cultured on the 20% HAPEX™ showed a greater increase in the rate of proliferation and osteoblast expression as indicated by an increase in ALP activity compared to the 40% HAPEX™ over the time period studied. Osteoblast-like cells showed a flattened morphology on both composites and in some cases a greater covering was observed on the 20% HAPEX™. These results indicate that the composites may not be identical in terms of bioactivity and that further research on surface topography and physico-chemical properties is required to assess fully the biological response of these composites. © *1998 Kluwer Academic Publishers*

1. Introduction

A composite material incorporating bioactive hydroxyapatite (HA) particles reinforced with high-density polyethylene (HDPE) matrix has been developed (HAPEX™), with optimal stiffness, toughness and bioactivity. HAPEX™ offers the potential of a stable implant–tissue interface during physiological loading [1] and has established clinical uses for middle ear and orbital floor implants [2, 3]. The material is produced by a process of twin-screw extrusion (Betol BTS40L) and compression molding, giving a homogeneous material of uniform composition [4]. This method has been shown to produce better mechanical characteristics in the finished material than two-roll milling [5].

HA and HDPE complement each other well for use as bone substitute material. HA has similar mechanical and biological properties to bone mineral, and HDPE has comparable mechanical properties to collagen (bone matrix). Increasing the HA volume in the composite has been shown to increase Young's modulus and tensile strength, whilst reducing strain to fracture.

In vivo bioactivity studies of HA/HDPE have shown cancellous and cortical bone growth adjacent to the implanted ceramic surface in rabbit femoral condyles [6]. Bone apposition, opposed to fibrous encapsulation, has been observed *in vivo* for HA/HDPE composites with a percentage volume of HA greater than 20. This transition from fibrous encapsulation to bone apposition represents a ten-fold increase in interfacial shear strength, and hence it can be concluded that an HA of between 20 and 40% in a HDPE matrix gives a bioactive, fracture-tough implant, whose modulus matches that of bone.

In vitro systems using osteoblast cells allow the study of tissue–material interactions without the complexities associated with *in vivo* models. Previous studies have been performed to examine the mechanical and biological properties of HAPEX™ (40% volume HA in HDPE). The mechanical properties of

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HAPEX™ were investigated following immersion in simulated biological fluid. In addition, the biological response of osteoblasts to the material was assessed. No significant water absorption, or decrease in tensile strength, Young's modulus or fracture strain were noted, even after 3 mon period at 70 °C for the material tested. Furthermore, the study showed that cell proliferation was greatest on day 1 with an increase in cell differentiation with time, with a peak at day 14. Morphologically, the cells appeared flattened with cell filapodia frequently seen attached to the surface HA particles [7].

Studies have been performed to investigate the mechanical properties of HA/HDPE composites with different formulations [8]. Although *in vivo* investigations have shown that they encourage bone formation, osteoblasts undergo a progressive phenotype differentiation.

In vitro studies allow a more direct study of the biological response and cell–material interaction under controlled conditions. The aim of this study was to investigate whether altering the volume of HA from 20% to 40% in the HA/HDPE composites had any significant effect on the bioactivity. Primary human osteoblast-like (HOB) cells were used for this study as they are representative of the cell type in contact with the material *in vivo* [9].

Total DNA, tritiated thymidine incorporation ([^{3}H]-TdR), alkaline phosphatase (ALP) quantification have been used to assess cell proliferation and differentiation.

2. Materials and methods

HA/HDPE at 20% and 40% volume HA was produced by incorporation of HA particles into HDPE through twin-screw extrusion and compression molding [4]. The materials were sterilized by gamma irradiation at a dose of 2.5 Mrad (Isotron, UK) using standard procedures for medical devices.

2.1. *In vitro* cell culture

HOB cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids, *L*-ascorbic acid (150 g ml^{-1}), 0.02 M *L*-glutamine, 0.01 M HEPES, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Thermanox (TMX, Nunc) was used as a control. HOB cells were seeded on to the materials at a density of 7×10^6 cells ml^{-1}. The culture dishes were incubated at 37 °C in humidified air with 5% CO_2 for 1, 3, 7, and 10 d. The culture medium was changed at selected time intervals, with care to cause little disturbance to culture conditions.

2.2. Cell proliferation

The growth and proliferation of the HOB cells on the materials were measured using [^{3}H]-TdR incorporation and total DNA content. For the total DNA content, the cells were lysed at each time point using a freeze/thaw cycle ($3 \times (-70$ °C for 20 min/37 °C for 20 min)). Hoechst 33285 (DNA specific fluorescent dye) was reacted with lysates and DNA standards of concentrations 0, 0.31, 0.62, 1.25, 2.5, 5, 10, and 20 μg ml^{-1}, in saline sodium citrate buffer (pH 7.0). Fluorescence was measured on a Fluoroscan fluorimeter (Ascent, Life Science International. Excitation wavelength of 355 nm, emission wavelength of 450 nm), and the sample DNA content calculated from the standard curve.

[^{3}H]-TdR was measured on days 1 and 7 on both the materials and the control TMX. The cells were incubated with 1 μCi ml^{-1} [^{3}H]-TdR (Amersham International, UK) for 24 h before lysis (freeze/thaw). Tri-chloroacetic acid (TCA) precipitation of the lysates was used to measure the thymidine incorporation. The precipitate was filtered on to a membrane using a Millipore filtration system (Milli-pore Multiscreen), and any unbound radionucleotide was washed away by filtering 10% TCA through the membrane. The precipitate was dissolved in 0.01 M KOH solution, and the [^{3}H]-TdR incorporation measured by scintillation counting.

2.3. Cell differentiation

Osteoblastic phenotype was determined biochemically by measuring ALP production from the HOB cells. The cells were lysed on days 3, 7, and 10 (freeze/thaw). ALP activity was determined using a COBAS-BIO (Roche, UK) centrifugal analyzer. *P*-nitrophenol phosphate in a diethanolamine buffer (Merck, UK) was used as a substrate for ALP. The reaction product, *p*-nitrophenol is yellow at alkaline pH (9.8), and can be quantified at a wavelength of 405 nm.

2.4. Cell morphology

The materials were seeded with HOB cells at a density of 1.5×10^5 cells ml^{-1}. These were incubated at 37 °C in humidified air and 5% CO_2. The cells were fixed with 2.5% 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%, 70%), stained in 0.5% uranyl acetate, followed by further dehydration (90%, 96%, 100% alcohol). The final dehydration was in hexamethyl-disilazane, followed by air drying. Once dry, the samples were sputter coated before examination under a Jeol SEM at an accelerating voltage of 15 keV [7].

3. Results

An increase with time in total cellular DNA was observed throughout the 10 d period for the TMX control. Both the 20% and 40% HA/HDPE showed an increase in growth up to day 7, but then showed no further increase. A high rate of [^{3}H]-TdR incorporation (expressed per μg DNA) was observed on day 1 for the control TMX. The 20% HA/HDPE showed a higher proliferation rate than 40% on both day

1 and day 7, but in both cases this was lower than the control TMX (Fig. 1). By day 3, as proliferation began to fall, an increase in differentiation of the cells was observed on all test materials. An increase in ALP production with time was seen on the TMX control, with a similar trend observed for the 20% HA/HDPE. Interestingly, there was little change in the ALP production on the 40% HA/HDPE from day 3 to 7, with a fall observed on day 10 (Fig. 2).

SEM revealed a good cell covering on both materials, with the cells retaining their flattened, polygonal morphology. Generally, a greater number of cells was observed on the 20% compared to the 40% HA/HDPE. A dense layer of cells was observed on both materials; however, after the 48 h culture period the cell layer was seen to "break" in some areas, exposing the material surface (Figs 3 and 4). This may be due to the thickness of the cell layer or it could be a processing artefact.

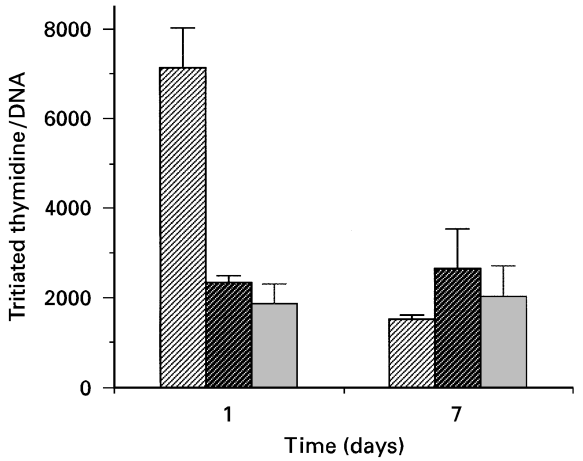

Figure 1 [^{3}H]-TdR (c.p.m.) incorporation/DNA (μg) for HOB cells on control TMX and the test materials 20% and 40% HAPEX™ cultured over a 7 d period. The cell proliferation rate was highest on day 1 for the control TMX with a fall observed by day 7. Both 20% and 40% HAPEX™ showed similar proliferation rates for days 1 and 7 (results are the mean ± S.D.). ▨ TMX, ▩ 20% HA, □ 40% HA.

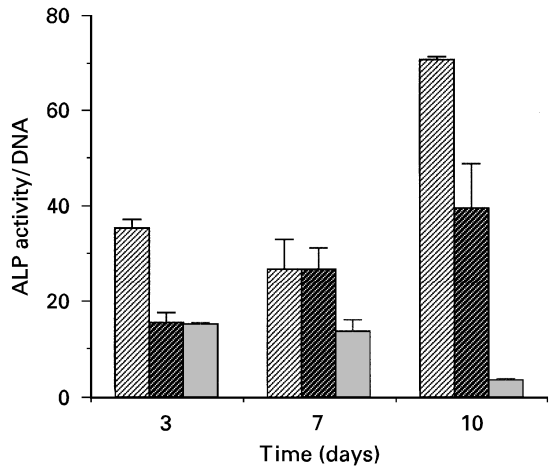

Figure 2 ALP activity of HOB cells cultured on control TMX and the test materials 20% and 40% HAPEX™. An increase in ALP activity was seen on day 3 in the control TMX compared to the test materials, this continued to increase with time. A similar trend, but lower activity was observed for the 20% HAPEX™ compared to the control. In contrast, cells on 40% HAPEX™ showed a fall in ALP activity by day 10 (results are the mean ± S.D.). ▨ TMX, ▩ 20% HA, □ 40% HA.

4. Discussion

The results from this study have shown that both 20% and 40% HAPEX™ have retained their bioactivity. Morphological observations have shown that HOB cells were able to adhere and proliferate on the materials. Biochemical analysis showed that both material surfaces were a suitable template for cell proliferation and the maintenance of osteoblast phenotype. The primary human cell model used was selected to represent the "appropriate response" of the cell the material will contact *in vivo*, thus giving results in relation to the materials final purpose and use as a bone analoge. When evaluating biological responses it is necessary to select the appropriate cell type, as the biocompatibility of a material is determined by its

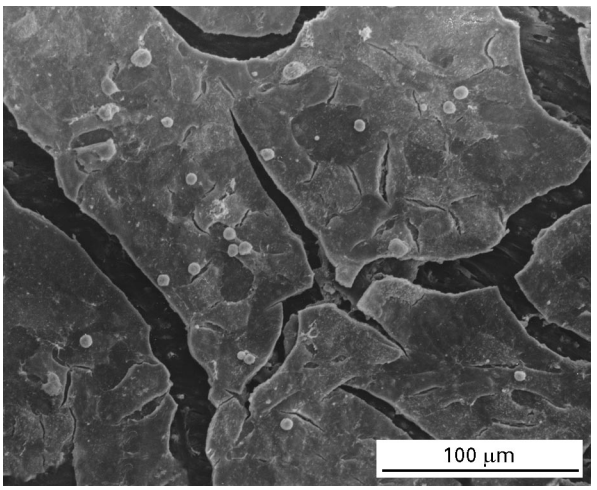

Figure 3 Scanning electron microscopical image showing HOB cells on the surface of 20% HAPEX™ after 48 h culture. A dense cell layer was observed covering the material. In some areas the material surface was visible.

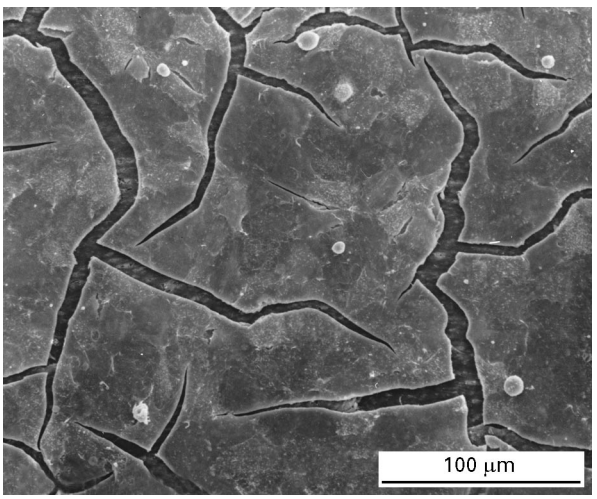

Figure 4 Scanning electron microscopical image showing HOB cells on the surface of 40% HAPEX™ after 48 h culture. A similar dense cell covering to the 20% HAPEX™ was observed.

ability to elicit a specific host response in a specific application.

The 20% volume HA/HDPE composite showed a higher level of biological activity, producing a suitable template for rapid cell proliferation. The fall in proliferation was followed by a subsequent increase in cell differentiation with a rise in the production of ALP with time. Although cells on the 40% volume HA/HDPE composite, seemed to have a similar proliferation trend, ALP production was much lower.

These initial *in vitro* findings have confirmed that this bioactive composite provides a favorable surface for cell attachment and proliferation at both 20 and 40 vol % HA. What is surprising is that the lower volume percentage of HA appears more bioactive. Given that, in each case, the chemistry, particle size and distribution, and morphology of the HA are identical, then the effect of particle spacing merits further study.

5. Conclusion

Initial observations indicate that the 20% HA/HDPE material appears to offer increased bioactivity over the 40% HA/HDPE material. This finding could be important in the development of an optimal HA/HDPE composite when taking into consideration biological and mechanical properties.

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