Surface topography and HA filler volume effect on primary human osteoblasts *in vitro*

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HAPEX^{TM}, a bone analog material, with similar properties to cortical bone, has been studied *in vitro* with particular reference to the effect of surface topography. The stimulation of a favorable bone response by this composite depends on optimization of the hydroxyapatite (HA) content in relation to the material bioactivity without compromising the mechanical characteristics. In this study we have started to investigate the effects of surface topography on cell attachment and subsequent cellular behavior in relation to proliferation. Different volumes of HA (20% and 40%) were added to a high density polyethylene (HDPE) matrix to produce the test materials. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were used to examine cell morphology on HAPEX^{TM}, and the surface characteristics produced by different machining protocols. The measurement of cellular DNA and tritiated thymidine ([^{3}H] – TdR) incorporation has been used to asses cell proliferation upon the materials. The results show that the material surface topography has a large influence on cell proliferation and attachment, and with a controled material topography the 40% vol HA/HDPE composite gives the greater biological response compared to the 20% vol HA/HDPE composite.

1. Introduction

The study of the interaction between a biological system and a material is important for the classification of the material. In the study of bone substitution materials cell adhesion, proliferation, differentiation and subsequent mineralization observations are necessary to determine the ability of the material to perform in a specific manner.

Surface topography of materials, defined as the morphology at the surface, plays a significant role in cellular interaction. Topography can be subdivided into macro and micro topography; with macro topography dealing with the physical configuration of the implant (e.g. screw threads), and micro topography resulting from surface roughness or texture. Roughness is characterized by the presence of hills or pits of random size or distribution; possibly caused by the processing route during manufacture, or created by surface finishing. Texture represents the microconfiguration of a surface with grooves, ridges, pillars or pores [1].

To date, there is poor understanding of the material characteristic which influence tissue behavior at the cellular level. However, it is the initial interaction of bone cells that influence all subsequent responses relating to cell adhesion, spreading, proliferation and subsequent extra-cellular matrix synthesis and mineralization [2, 3]. Curtis and Wilkinson [4] state that topography appears to provide a set of very powerful signals for cells. It is only more recently that the idea of considering surface influence on cell phenotype has become of great importance when designing an ideal substrate for particular cells [4].

HAPEX^{TM}, a composite material incorporating bioactive hydroxyapatite (HA) particles reinforced with high density polyethylene (HDPE) matrix has been developed; the composite has optimal stiffness, toughness and bioactivity. HAPEX^{TM} offers the potential of a stable implant-tissue interface during physiological loading [5] and has established clinical uses for middle ear and orbital floor implants [6, 7]. The material is produced by a process of twin screw extrusion (Betol BTS40L) and compression molding, giving a homogeneous material of uniform composition [8].

A previous study showed that 20% vol HA/HDPE had a greater bioactivity than 40% vol HA/HDPE [9]; on closer examination it was found that variations in surface topography gave rise to different, and in some cases conflicting results. It is known that the topography of materials influences cellular orientation and migration

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[10], and that different surface characteristics may present cells with a variety of signals which may influence their behavior [4]. The variations in surface topography of the first batch production of HA/HDPE composites, supplied for the previous study [9], were due to a non-optimized machining process, which resulted in variable results.

The aim of this study was to examine the material topography, and determine its effect on biological response. For this purpose, a second batch of HA/HDPE composites was produced using a standard, reproducible machining process.

The *in vitro* cell culture model used primary human osteoblast-like (HOB) cells which are representative of the cell type in contact with the material *in vivo* [11]; the effect of surface topography and HA filler on the biological response and cell-material interaction were studied under controlled conditions.

The adhesion of HOB cells to the material surfaces was studied using immunohistochemistry. Focal adhesion contacts of the osteoblasts were visualized by vinculin staining.

2. Materials and methods

2.1. Materials

HA/HDPE at 20% and 40% volume HA was produced by incorporation of HA particles into HDPE through twin screw extrusion and compression molding [7]. The materials were sterilized by gamma irradiation at a dose of 2.5 Mrad (Swann Morton (Services) Ltd) using standard procedures for medical devices.

2.2. *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 $\mu g ml^{-1}$, 0.02 M L-glutamine, 0.01 M HEPES, 100 units ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. Thermanox (TMX, Nunc) was used as a negative cytotoxicity control and, as a positive cytotoxicity control, plastic disks conforming to BS5736 (Protex Limited, UK) were used. HOB cells were seeded onto the materials at a density of 7×10^5 cells ml^1 followed by incubation at 37 °C in humidified air with 5% CO_2 for a period of 1, 3, 7 and 14 days. The culture medium was changed at selected time intervals, with care to cause little disturbance to culture conditions.

2.3. Cell Proliferation

The growth and proliferation of the HOB cells on the materials were measured using $[^3H] - TdR$ 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 mg/ml, in saline sodium citrate buffer (pH 7.0). Fluorescence was measured on a Fluoroscan fluorimeter (Ascent, Life Science International, excitation wavelength of 335 nm, emission wavelength of 450 nm), and the sample DNA content calculated from the standard curve.

$[^3H]$-TdR was measured on days 1, 3, 7 and 14 on both the materials and the control TMX. The cells were incubated with 1 $\mu Ci ml^{-1}$ $[^3H]$-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 (Millipore, UK), 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 $[^3H]$-TdR incorporation measured by scintillation counting.

2.4. Cell Morphology

The materials were seeded with HOB cells at a density of 1×10^5 cells ml^{-1}. These were incubated at 37 °C in humidified air and 5% CO_2. The cells were fixed with 1% gluteraldehyde buffered in 0.1 M sodium cacodylate after 24 h and 48 h incubation periods. The cells were then fixed 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 35C SEM at an accelerating voltage of 10 keV.

2.5. Material Topography

Materials were examined pre- and post-gamma-irradiation, and also submerged in conditioned medium, for the time periods studies. All samples were processed as in section 2.4 before viewing by SEM.

2.6. Vinculin Expression

HOB cells were seeded onto the materials (5×10^6 cells ml^{-1}) and cultured for 48 h. At each point the cells were fixed in 4% formaldehyde/phosphate buffered saline (PBS). When fixed the samples were washed with PBS, and permeabilized using a permeabilizing buffer (10.3 g sucrose, 0.292 g NaCl, 0.06 g $MgCl_2$, 0.476 g Hepes buffer, 0.5 ml Triton X, in 100 ml water, pH 7.2) at 4 °C. The samples were then incubated at 37 °C for 5 min in 1% BSA/PBS, followed by the addition of anti-viculin primary antibody (hVIN-1 (Sigma)) for 1 h (37 °C). The samples were washed in PBS/Tween 20. A secondary FITC conjugated antibody (rabbit anti mouse, DAKO) was added for 1 h (37 °C). A further wash followed, and the samples were then viewed on a confocal laser scanning microscope (CLSM, Noran).

3. Results

SEM analysis of HOBs growing on the surface of the first batch of 20% and 40% vol HA/HDPE showed large differences in growth pattern depending upon the topography of the grooving caused by the turning of the material during machining (Fig. 1a and b).

Examination of the surface topography of the first batch of 20% and 40% vol HA/HDPE material by SEM revealed notable differences in the roughness and spacing (regularity of the surface grooves (Fig. 1c–f).

SEM surface analysis of the new batch of 20% and 40% vol HA/HDPE materials, used to obtain the biological data in this study, revealed a notably more consistent surface reflecting a controled machining process. The surface topography remained unchanged following γ-irradiation, and at all time points studied (Fig. 1g and h).

SEM morphological analysis on HOB cells cultured on the new second batch of materials showed normal HOB growth on the 20% vol HA/HDPE composite (Fig. 2), and the 40% vol HA/HDPE composite (Fig. 3).

The results of the [^{3}H]-TdR analysis (Fig. 4) show an increased initial cell proliferation on the 40% volume HA/HDPE composite compared to 20% volume HA/HDPE composite and TMX. The cellular proliferation was seen to decrease with time for all the materials.

CLSM imaging of vinculin expression within the HOB cells showed accumulation of vinculin at points of adhesion in cells on both test materials. The 40% vol. HA/HDPE sample (Fig. 5) appeared to have more adhesion sites when compared to the 20% vol HA/HDPE composite (Fig. 6).

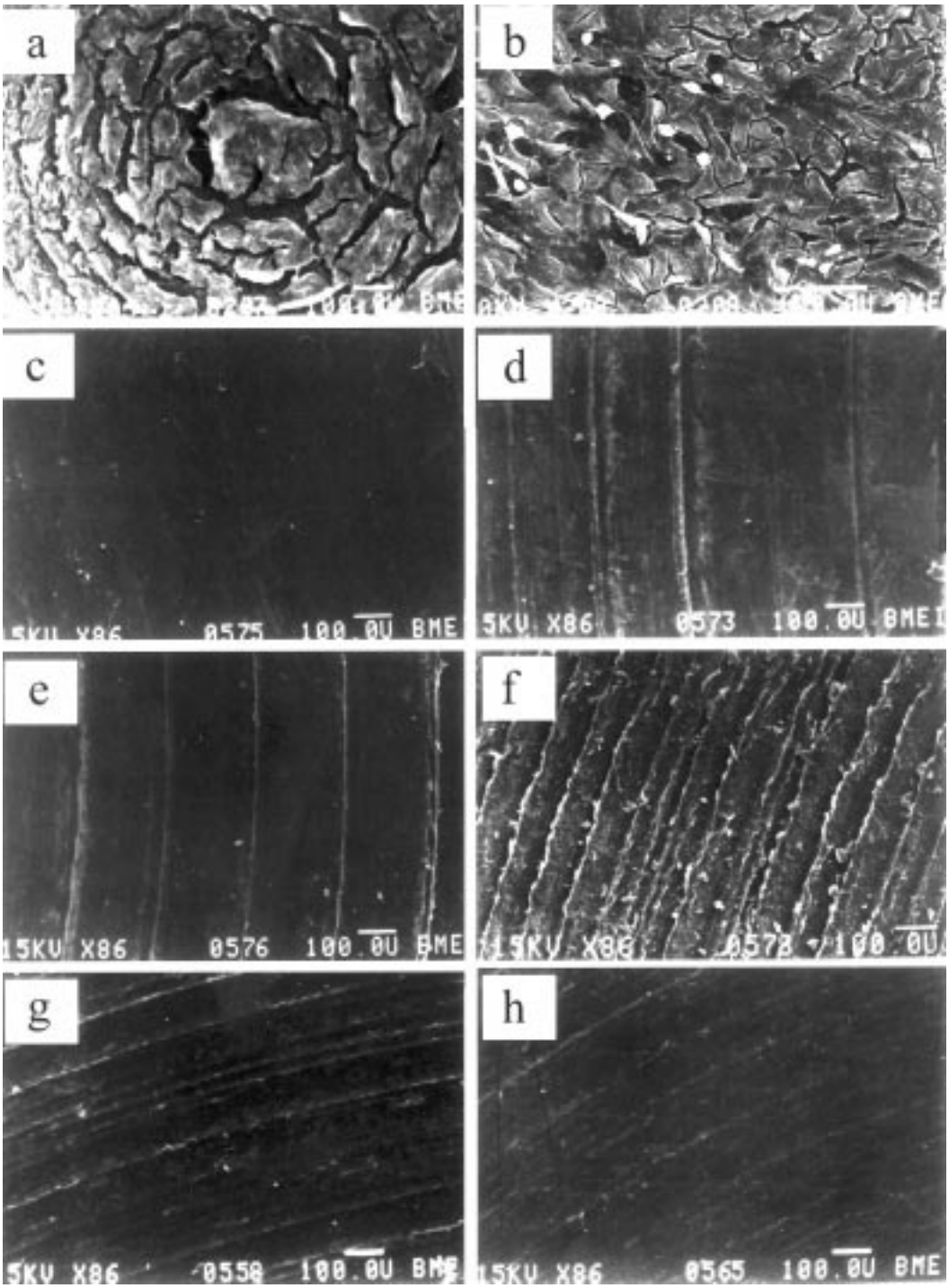

Figure 1 (a) SEM of HOB cells growing upon highly grooved HA/HDPE surface; (b) SEM of HOB cells growing upon smooth HA/HDPE surface; (c), (d), (e), and (f) SEMs of the differences in HA/HDPE surface roughness observed from the first production of the composites, ranging from smooth (c), to very rough (f); (g) SEM of typical material surface from the second production of HA/HDPE composites before γ-irradiation; (h) SEM of typical material surface from the second production of HA/HDPE composites after 14 days' submersion in cell culture complete medium.

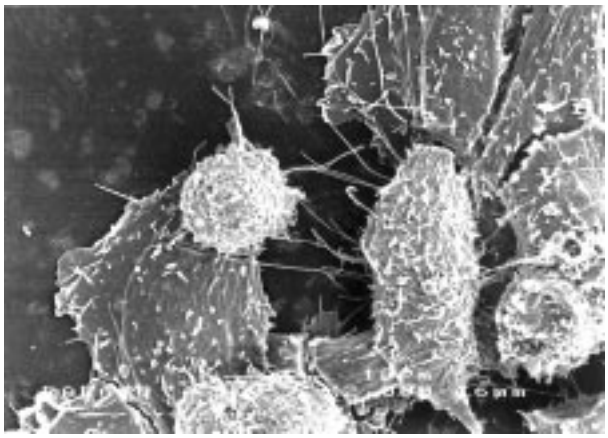

Figure 2 SEM of HOB cells cultured on 20% vol HA/HDPE. The cells showed normal osteoblast morphology and cellular processing.

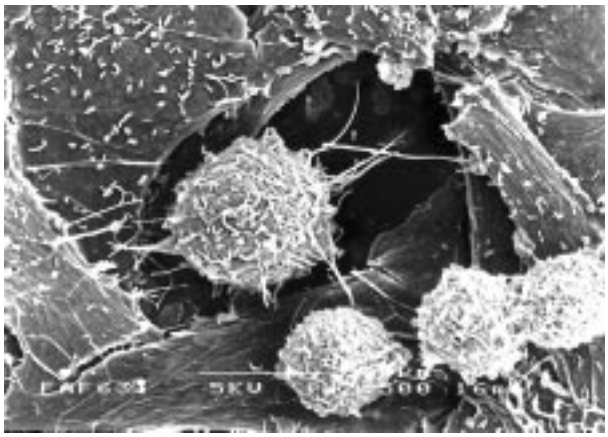

Figure 3 SEM of HOB cells cultured on 40% vol HA/HDPE. The cells showed normal osteoblast morphology and cellular processing.

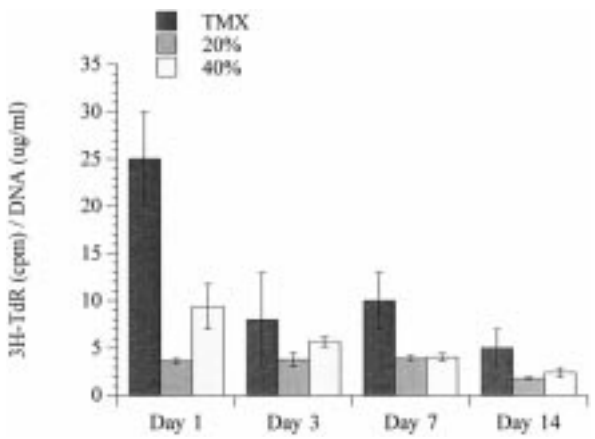

Figure 4 [^{3}H]-TdR incorporation (cpm) / DNA (μ/ml) for HOB cells on control TMX and the test materials, 20% and 40% vol HA/HDPE cultured over a 14 day period. The cell proliferation was seen to be highest on day 1 for all materials. The 40% vol HA/HDPE composite showed the highest initial rates of proliferation (results are the mean ± SD, $n = 3$).

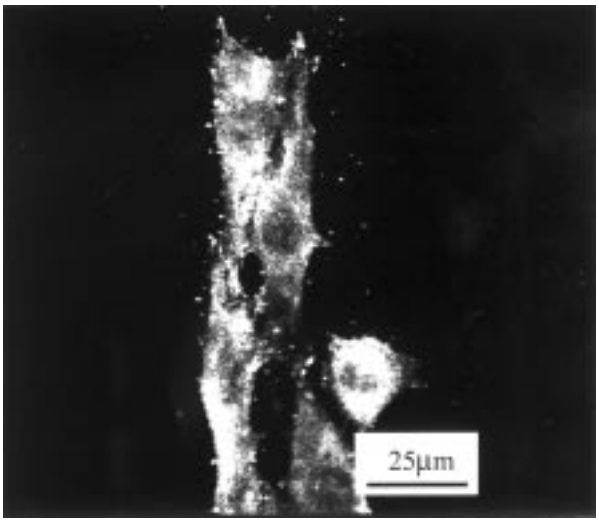

Figure 5 CLSM image of vinculin localization in HOB cell cultured upon 20% vol HA/HDPE composite after 48 h of culture (bar = 25 μm).

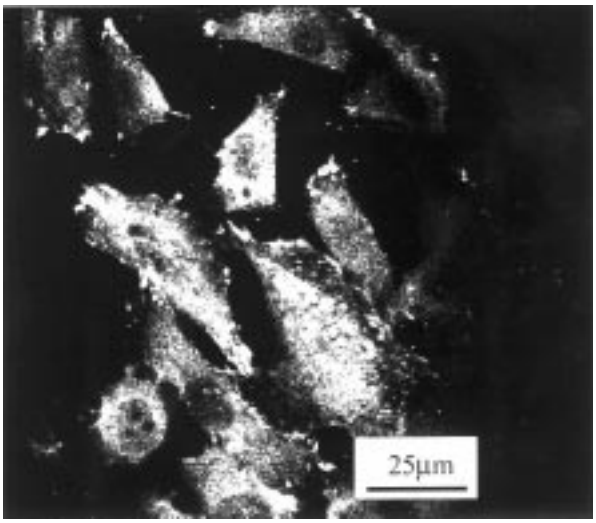

Figure 6 CLSM immage of viculin localization in HOB cell cultured upon 40% vol HA/HDPE composite after 48 h of culture (bar = 25 μm).

4. Discussion

These results show that the processing of the materials can have a notable effect on the biological response of the cells in contact. Materials of variable roughness can lead to differing results.

SEM investigations show very distinct differences in surface topography with both circular and longitudinal grooving. This had a significant effect on cell attachment, with cells perferentially lying along the groove planes of the surfaces (Fig. 1a), this observation could be due to contact guidance (as demonstrated using fibroblasts on microgrooved topographies [10]). The cells grown upon material surfaces with no visible grooving covered the entire surface, forming a confluent layer (Fig. 1b). The SEM observation of HOB cells following the groove patterns may be indicative of contact guidance, again demonstrating the effect of material topography upon the cells. Clark *et al*. showed cell alignment to be influenced by the depth of spacing of the grooves between 1–25 μm [12], if the tracks are too wide no cellular orientation is observed except at the edges [7]. It is thought that surface topography and chemistry may be interlinked, both

effecting cellular response [4]. Using techniques orientating a chemical cue (laminin), at right angles to a topographical cue, Britland *et al*. showed that at groove depths of 500 nm or less cells reacted mainly to the chemical cue, but at depths of 5 μm, the topographical cues orientated approximately 80% of the cells [13].

The results show that proliferation of osteoblasts upon the material is initially greater for the 40% volume HA/HDPE material, compared to the 20% volume HA/HDPE material. This suggests that the 40% composite supports a more rapid cell growth, and enhances cellular response.

The vinculin staining demonstrated the formation of more focal adhesion points on the 40% vol HA/HDPE composite. This is an important observation, as it is thought that regulation of cell growth and differentiation may be mediated by the molecules involved in focal contact formation, which is in turn determined by the nature of the substrate, i.e. the material surface [4]. The cell ''communicates'' with the material surface via transmembrane integrin proteins, which are able to generate specific signals to the cell cytoskeleton, resulting in new gene transcription and protein synthesis and this influences cell phenotype [15–17]. Cells with the ability to form tissue make close contact not only with other cells, but also with the substrate on which they are growing. Where the cell membrane makes such close contacts, a dense plaque of material is observed on its cytoplasmic surface. The adhesion plaques formed by the cells contain α-actinin and vinculin, which together bind F-actin and talin. Talin has been seen to bind to integrins, which have been observed to localize to focal contacts in fixed cells. Thus, the more adhesion plaques formed by the cell, the higher the levels of integrin signaling [15, 18, 19].

Studies in the area of topography have shown the implanted materials surface presented to the cells can be considered a foreign chemical species with reactive sites. the termination of polymer chains may also interact with reactive groups such as protein or carbohydrate molecules, ''biomolecules'', in serum. When a material is placed into a tissue, it is covered with a thin layer of extracellular fluid, and it is through this layer that the cells interact with the implant material [20]. This bonding may vary in strength from van der Waals attractions through to covalent bonding [21, 22]. The bonds may be strong enough to cause protein denaturation by the formation of multiple bonds, accompanied by the breaking of internal bonds within the protein. When cells are introduced they perceive the materials organic overlayer. Due to the dynamic nature of the cell membrane and the material the cells may respond by inducing an inflammatory response through to perceiving the material as tissue-like and no reaction being evoked. The nature of the cellular response will decide whether the implant is encapsulated in fibrous tissue, or direct bone growth occurs [23]. As a result of this, careful consideration is now being given to ensuring that material surfaces provide an ideal substrate, to optimize cell attachment and bone growth at the interface.

This work has looked at the initial reactions of the HOB cells to the materials under examination. Cell reaction to materials involves attachment, proliferation, differentiation and mineralization [2]. The results indicate that the 40% composite shows an enhanced bioactivity over the 20% composite, as indicated by a great number of cells adhering to the surface, and higher levels of proliferation. To follow on from this study observation of other cytoskeletal elements, and measurement of cell phenotype by alkaline phosphatase is required to fully characterize the cellular response to the materials.

5. Conclusion

When a cell is placed in contact with a material, it perceives the physical nature of the surface and its local environment. The results from this study have shown that the surface characteristics can significantly influence cell attachment. When considering a bioactive bone analog, the material selected, topography and material surface chemistry area all important factors for promoting bone growth upon the material surface. In the case of HA/HDPE, reproducibility of surface topography, and minimal batch to batch variations, allow the biological response to be attributed to content.

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