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Biomaterials 25 (2004) 2039–2046
Biomaterials
www.elsevier.com/locate/biomaterials

Nodule formation and mineralisation of human primary osteoblasts cultured on a porous bioactive glass scaffold

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Received 2 June 2003; accepted 25 July 2003

Abstract

The aim of this study was to analyse human osteoblast responses to a porous bioactive glass scaffold. It was hypothesised that osteoblasts would attach, proliferate and form mineralised nodules in response to culture on the bioactive glass. As dissolution products are a key feature of bioactive glasses, this was measured by inductively coupled plasma optical emission spectroscopy to determine effects of both the glass surface and ion release. Osteoblasts attached and proliferated on the foams as demonstrated by scanning electron microscopy. Nodule formation was also observed in the pores of the glass and also in conditioned medium containing dissolution products at certain concentrations and these nodules were shown to be mineralised by alizarin red staining. Undiluted dissolution products from the foams however caused significant apoptosis suggesting an ion concentration dependent response.

Keywords: Osteoblast; Bioactive glass; Ion release; Mineralisation; Apoptosis

1. Introduction

Surface reactive bioceramics were first developed by Hench and colleagues in the early 1970s [1]. Since then numerous studies have focused on bioactive glasses for bone tissue repair due to their ability to bond to bone [1]. Glasses of various compositions such as melt-derived 45S5 Bioglass® [2], and the sol–gel derived glasses of 58S [3], S70C30 [4] and 77S [5] composition have been investigated. It has been previously found that 45S5 Bioglass® causes osteoblast gene up regulation in response to leachable ions [6]. Tissue engineering is undergoing extensive research due to its excellent potential to repair diseased or damaged tissue via regeneration rather than replacement. Third generation biomaterials which are bioactive, resorbable and which stimulate specific and controlled cell responses at the molecular level are now being investigated [7]. Tissue engineering aims to regenerate tissue via cell seeded resorbable constructs which will be directed to regenerate the target tissue by the material itself, the cells seeded within it and also the body's own responses. Much research is aimed at bone tissue engineering due to its high capacity for self-repair and has focused on hydroxyapatite scaffolds due to the high hydroxyapatite content of bone [8] and calcium phosphate due to the high content of calcium and phosphate within bone [9]. Many porous polymer scaffolds are being investigated such as porous polycaprolactone [10] and composites of natural (to enhance biocompatibility) and synthetic components such as collagen and polycaprolactone [11]. Composites of hydroxyapatite and polymers such as polyethylene are also being investigated for bone repair and tissue engineering [12,13].

This study describes the initial responses of human osteoblasts to a third generation foamed and porous 58S bioactive glass [3], with a view to using the material for bone tissue engineering. We hypothesised that the scaffold would stimulate osteoblasts to attach, proliferate and form mineralised nodules in response to culture on the surface of the bioactive glass network itself and also in response to dissolution products from the material.

2. Methods

2.1. Glass synthesis

Colloidal solutions (sols) of 58S composition (60 mol% SiO_2, 36 mol% CaO, 4 mol% P_2O_5) were

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0142-9612/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2003.07.001

prepared by mixing (in order); distilled water, 2 N nitric acid, tetraethyl orthosilicate (TEOS), triethyl phosphate (TEP) and calcium nitrate [14]. Porous scaffolds were produced by foaming 50 ml aliquots of sol by vigorous agitation. An acidic catalyst (1.5 ml 0.5 wt% HF) was added as a gelling agent, to reduce the gelling time to 7 min and 1.5 ml of Teepol®, a detergent containing a low concentration mixture of anionic and non-ionic surfactants was added as a foaming agent. As the gelling point was approached the solution was cast into moulds. Samples were aged (60°C), dried (130°C) and thermally stabilized (600°C) according to established procedures [3].

Samples measured 25 mm diameter × 10 mm deep with a geometrical bulk density of 0.28–0.32 g/cm^3. Porosity and surface area values have been previously determined [3].

For cell culture studies, samples were used as above or cut to approximately 10 mm × 10 mm × 5 mm using a scalpel and placed into the wells of a 6-well plate containing 8 ml medium.

2.2. Inductively coupled plasma optical emission spectroscopy (ICP-OES)

Ionic concentrations of released silicon (Si), calcium (Ca) and phosphorous (P) after immersion of foams in culture medium (DMEM) for 24 h at 37°C in static conditions, to mimic cell culture conditions were determined using ICP-OES (3580B ICP Analyser, Applied Research Laboratories, IM35xx ICP Manager software, Micro-Active Australia Pty Ltd.). The samples were diluted 1:10 in a 2 N HNO_3 matrix before analysis. Three integrations were performed per sample and the mean and the standard deviation was calculated. The instrument detection limits for the elements of interest were 0.05 (Si), 0.10 (Ca) and 0.20 (P) ppm.

2.3. Osteoblast culture

Human osteoblast cells (HOBs) were isolated as described previously from femoral heads after total hip replacement surgery [15,16]. Bone fragments of approximately 3 mm × 3 mm were removed and washed several times in phosphate buffered saline (PBS) to remove blood cells and debris with a final wash in culture medium. Fragments were then placed in tissue culture flasks in complete Dulbecco's modified Eagles medium (DMEM) containing 10% foetal bovine serum (FBS) with 1% glutamine, 2% penicillin/streptomycin and 0.85 mM ascorbic acid. The fragments were incubated at 37°C in a humidified incubator with 5% CO_2. Medium was changed every 2 days and after approximately 4 weeks in culture, bone fragments were discarded and cells harvested using trypsin EDTA. Cells were seeded onto materials or Thermanox discs as positive controls, at a density of 80,000 cells/cm^2.

2.4. Culture in conditioned medium

Conditioned medium was made by incubating foam samples in DMEM for 24 h. Cells were then cultured in this conditioned medium neat, or diluted 1:1 or 1:4 with DMEM. Some samples were also buffered using 2 N HCl due to the increase in pH from released ions. Cells were harvested after 24 h culture and stained with 10 μg/ml propidium iodide in PBS for 1 min. Cells were mounted using Vectashield and viewed under an Olympus IX70 fluorescence microscope at 535 nm absorption and 617 nm emission wavelengths and percentage of viable or apoptotic cells counted. Conditioned media were also used supplemented with either dexamethasone, β-glycerophosphate or both to analyse their effects on mineralisation.

2.5. Scanning electron microscopy

Cells were cultured on foams or Thermanox discs and at required time-points fixed in 1.5% glutaraldehyde for 30 min at 4°C. Cells were then post-fixed in 1% osmium tetroxide for 1 h at 4°C. Cells were dehydrated through a series of increasing concentrations of ethanol and dried using hexamethyldisilazane (HMDS). Samples were sputter coated with gold and viewed using a Cambridge Stereoscan S360 scanning electron microscope operated at 10 kV.

2.6. Mineralisation

Ability of cells to produce mineralised matrix and nodules is important with regard to development of materials for bone regeneration. Whether mineralisation of matrix and nodules occurred was determined using alizarin red staining. Alizarin red is a common histochemical technique used to detect calcium deposits in mineralised tissues and cultures. Positive alizarin red staining for calcium has been demonstrated to represent calcium phosphate and osteoblast culture mineralisation by energy-dispersive X-ray analysis (EDX) [17,18].

Cells cultured in bioactive glass dissolution products were fixed in 70% ethanol and stained with 1% alizarin red for 2 min. Cells were then washed with distilled water and viewed under the light microscope.

For determination of mineralisation of cells cultured directly on the foamed scaffolds, nodules were trypsinised from the surface and then fixed and stained as above. This was done because calcium is present in the material making it difficult to visualise true positive staining and this was found to be an effective technique.

In cell free controls, no spontaneous nucleation of mineral or positive alizarin red staining was observed.

2.7. Statistical analysis

All experiments were done in triplicate and analysis of variance (ANOVA) was performed using the Tukey–Kramer multiple comparison test. In each case $n = 3 \pm$ standard deviation (SD).

3. Results

3.1. ICP

Table 1 shows ion release from 58S foam incubated in DMEM for 24 h, which was subsequently diluted in culture medium. Si release was found to be 230 μg/ml in the neat eluate, 120 μg/ml when diluted 1:1 in culture medium and 47 μg/ml when diluted 1:4 in culture medium. There was little change in Na, Ca and P concentrations in the conditioned medium dilutions.

3.2. pH

pH values were also measured after ion release. The neat eluate was found to have a pH of 8 as shown in Table 2 whereas the 1:1 dilution, 1:4 dilution and medium alone were 7.43, 7.3 and 7.2 respectively.

3.3. SEM

Pore structure of the 58S foam is shown in Fig. 1. Macropores were observed to be approximately 500 μm diameter with interconnecting pores ranging from 80 to 120 μm diameter. Cells were observed to attach and spread on Thermanox control and 58S foams as shown in Figs. 2A–F. Typical osteoblast attachment after 90 min culture is shown in Fig. 2A. Cells attach and spread in a comparable manner on the 58S foam as shown in Fig. 2B (low power showing cell colonisation of a single pore) and 2C (higher power micrograph showing cell spreading). By 24 h, cells were fully spread (Fig. 2D shows typical cells cultured on Thermanox control). Cells were observed to spread within the pores and also spread across smaller pores as shown in Fig. 2E. A higher power magnification of 2E is shown in 2F where an individual cell process (filopodia) is observed attaching to the rim of a pore.

Nodule formation after 10 days was also observed on the porous foams as shown in Fig. 3. No nodule formation was observed on the Thermanox controls at 10 days (Figs. 3A and B). Fig. 3C shows cell coverage over the surface of the foam where cells had proliferated to form a monolayer. Several nodules were observed within pores of the foams. Fig. 3D shows a low power micrograph of 2 nodules within a pore and Figs. 3E and F show higher power magnifications of individual nodules.

3.4. Apoptosis

Fig. 4 shows cells stained with propidium iodide to reveal nuclear morphology, after incubation in conditioned medium for 24 h. Cells are observed with condensed nuclear morphology, typical of apoptotic nuclear morphology (arrow heads). Some viable cells are also observed with uniform nuclear staining (arrows). The percentage of apoptotic cells was quantified by counting and is shown graphically in Fig. 5. Significant apoptosis of approximately 50% was observed when cultured in the neat eluate from the 58S foams. As shown in Table 2, the pH of this particular solution was 8.0. Therefore to determine whether the effects of the ions or pH or both were involved, a neutralised solution was run alongside the solution of pH 8. A 9% reduction in apoptosis was observed.

3.5. Mineralisation

To determine whether nodules formed in the foamed scaffolds were mineralised, nodules were trypsinised from the porous foam and stained with alizarin red to show staining of calcium deposits, as shown in Fig. 6. Nodules that formed in the 1:4 dilution of eluate:culture medium were also stained with alizarin red and positive staining is shown in Fig. 7 where a large nodule can be

Table 2
pH of conditioned DMEM from 58S foam measured at room temperature immediately after removal from 37°C incubation

Sample	pH
58S	8
1:1 58S:Med	7.43
1:4 58S:Med	7.3
Medium	7.2

Table 1
Ion concentrations in conditioned DMEM from 58S foam (24 h)

Sample	Si (μg/ml)	±	Ca (μg/ml)	±	P (μg/ml)	±
58S	230	44.7	37.674	6.416	52.096	18.79
1:1 58S:Med	120	17.7	6.689	1.491	56.141	4.149
1:4 58S:Med	47.7	7.845	0	2.198	67.631	14.091
Medium	0	5.315	4.866	3.036	65.701	4.848

seen, stained heavily with alizarin red. No nodule formation was observed in the neat dissolution products or the 1:1 ratio or on the Thermanox control substrates.

Results of mineralisation in dissolution products (conditioned medium) from 58S foams were compared to traditional mineralisation promoting culture conditions (culture medium supplemented with dexamethasone and beta-glycerophosphate). It was found that control cultures with no supplementation or dissolution products showed no alizarin red staining (Fig. 8A). Supplementation with dexamethasone alone (Fig. 8B) also showed negative alizarin red staining (some pink colour was observed but this was so faint that the authors determined this to be negative and probably due to the high cell concentration making nodular areas appear darker). Cultures supplemented with beta-glycerophosphate (Fig. 8C) or both dexamethasone and beta-glycerophosphate (Fig. 8D) showed positive staining. Cultures in 58S dissolution products with no supplementation (Fig. 8E), stained positively with alizarin red, with some areas of the micrograph appearing more densely stained than others.

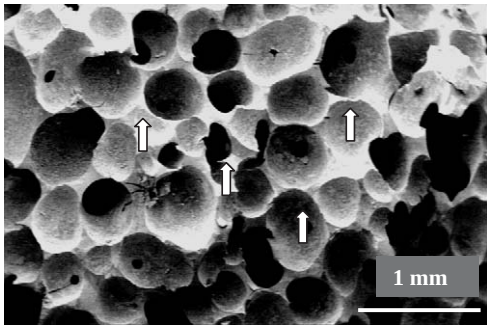

Fig. 1. Low power scanning electron micrograph showing porous structure of the foam and interconnecting porosity (arrows). Magnification × 28. Macropores are approximately 500 μm diameter and the interconnecting pores (arrows) are approximately 100 μm diameter.

4. Discussion

In this study, primary human osteoblasts were observed to attach, spread, proliferate and form mineralised nodules when cultured on 58S bioactive

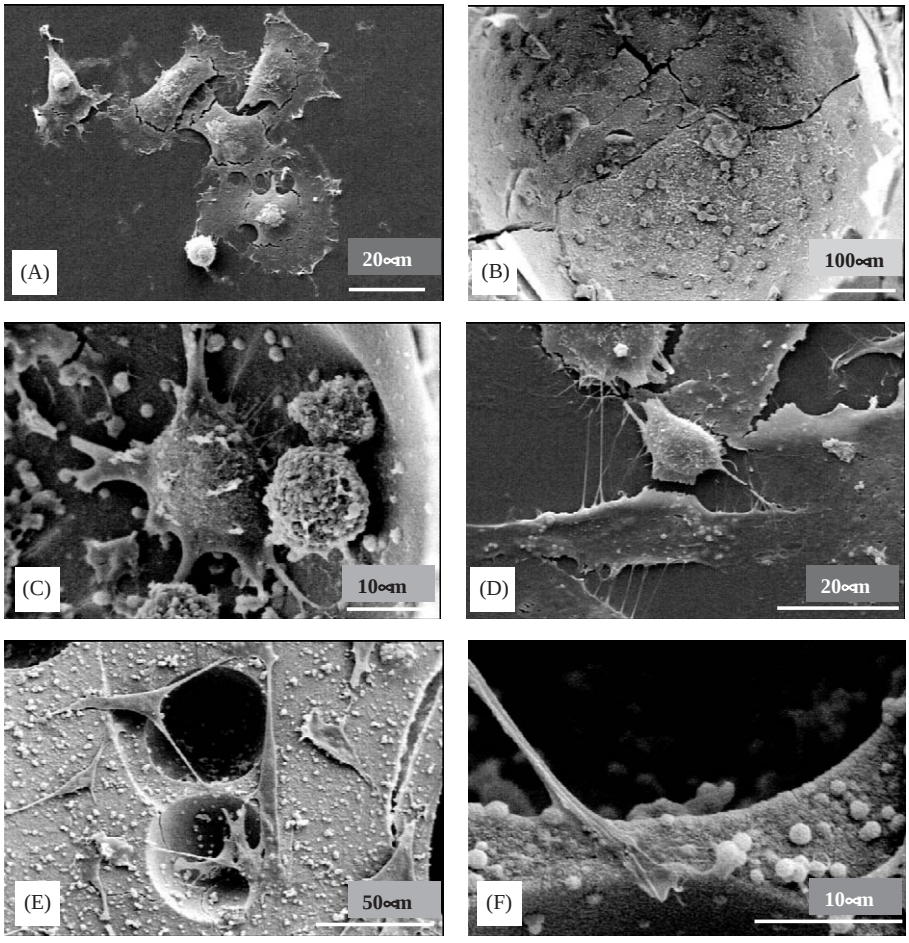

Fig. 2. Scanning electron micrographs of cells cultured on foamed sample (A: Thermanox control, 90 min, × 900; B: low power of cells within pore cultured for 90 min, × 220; C: higher power of cell spreading within pore cultured for 90 min; D: Thermanox control 24 h, × 1.7; E: low power of cells spreading across pores cultured for 24 h, × 650; and F: higher power of C showing specific site of cell attachment, × 3800).

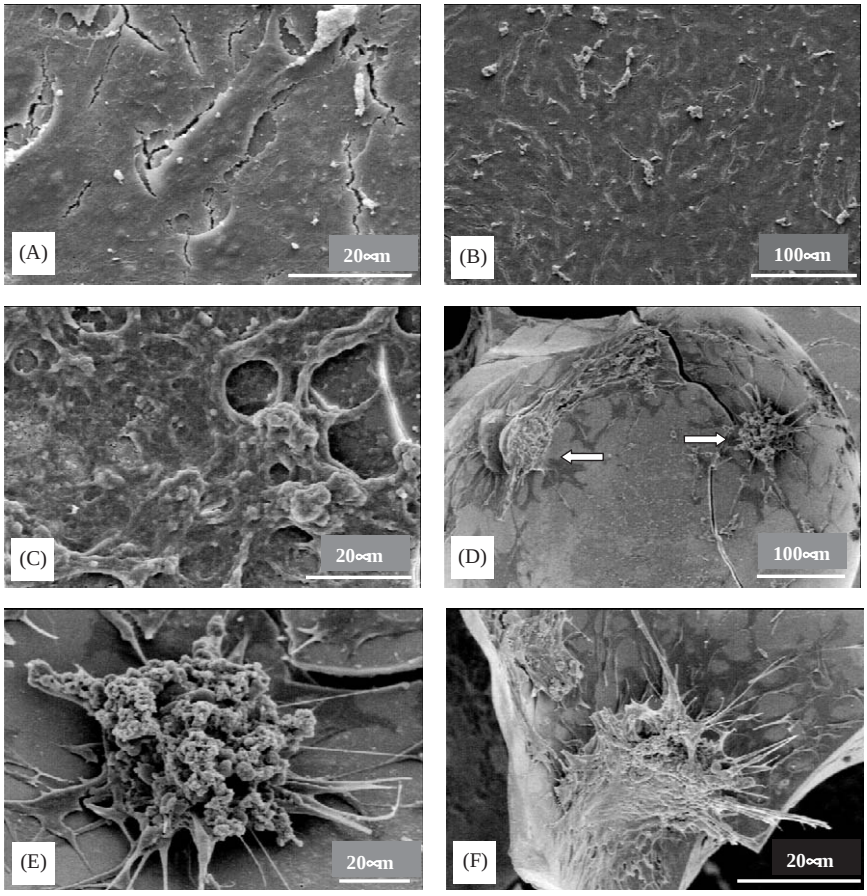

Fig. 3. Scanning electron micrographs of HOBs cultured for 10 days on Thermanox (A: ×1700, B: ×280) and foamed 58S (C: low power of cell coverage, ×1400; D: low power of nodules in pore, ×240; E, higher power of nodule, ×950; F: larger nodule, ×320).

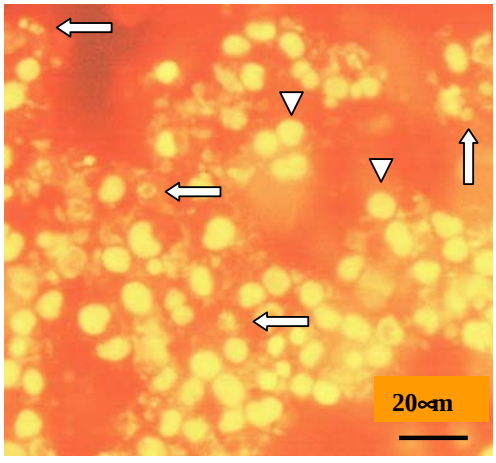

Fig. 4. Fluorescence micrograph of propidium iodide stained cells cultured in conditioned medium from 58S. Arrows show examples of apoptotic cells, arrow heads show examples of viable cells.

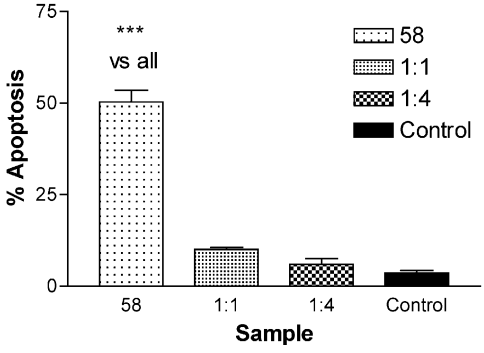

Fig. 5. Graph showing percentage apoptosis of osteoblasts cultured in conditioned medium. Approximately 50% of cells cultured in neat elute underwent apoptosis as demonstrated by propidium iodide staining of cell nuclei, which was significantly different to all other samples (one way ANOVA with Tukey–Kramer post-test) with $p = <0.001$ (***) where $n = 3 \pm$SD.

glass porous foams. Extensive nodule formation within pores of the scaffolds was observed by SEM [19] and compared to osteoblast cultures on Thermanox controls, where no nodules were observed in the time frame studied. As nodule formation can occur on Thermanox [19], it is more accurate to state that in this study, nodule formation was more rapid compared to control cultures. These nodules were also found to be mineralised as demonstrated by positive alizarin red staining, a technique commonly used to detect calcium deposition in osteoblast cultures. More importantly, mineralised

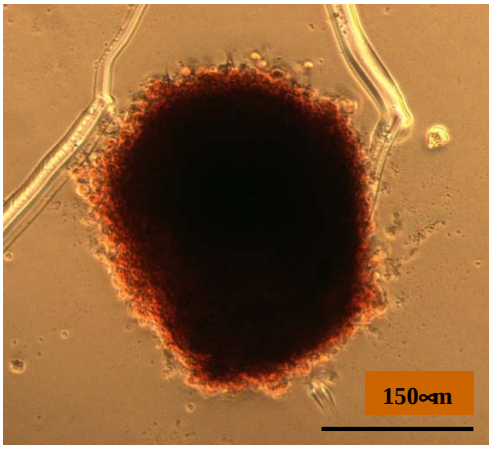

Fig. 6. Light micrograph of bone nodule trypsinised from 58S foam stained with alizarin red. Positive dark red staining shows presence of calcium deposits i.e. mineralisation.

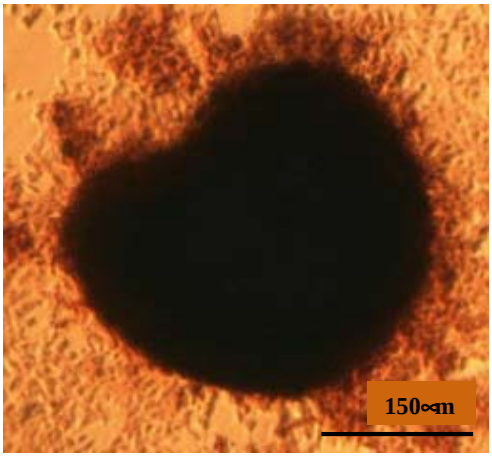

Fig. 7. Light micrograph of bone nodule cultured in 1:4 conditioned medium from 58S foam: normal medium, stained with alizarin red. Positive dark red staining shows presence of calcium deposits i.e. mineralisation.

cultures were observed as shown by alizarin red staining, in the absence of common mineralisation agents dexamethasone (a synthetic glucocorticoid) and beta-glycerophosphate, demonstrating the remarkable ability of 58S bioactive glass to cause osteoblast mineralisation. This is highly important with regard to developing materials for bone repair or bone tissue engineering/regeneration. Many materials are biocompatible with regard to certain cell types but often require addition of growth factors and other agents to improve the cell responses. Here we can show that without addition of any extra agents we obtain an improved cell response as shown by mineralised nodule formation. Control cultures on tissue culture plastic or Thermanox were only found to mineralise when supplemented with dexamethasone and beta-glycerophosphate. Many osteoblast cultures only mineralise in the presence of these agents and the effect of dexamethasone on mineralisation is contradictory and its action is unknown [20–24]. In our study, we demonstrated that mineralisation occurs without either agent, if cultured in the presence of 58S bioactive glass.

ICP results show that Si concentration is likely to be the key factor in mineralisation and nodule formation as there was little change in concentration of Ca and P in this study. Nodule formation was only demonstrated in a 1:4 ratio of dissolution products to DMEM, which was found to contain approximately 47 μg/ml, whereas the other concentrations of dissolution products were significantly higher. In fact, at these higher concentrations significant programmed cell death (apoptosis) was observed demonstrating a concentration dependent effect. In the neat dissolution product sample where Si concentration was 230 μg/ml, Ca concentration was 38 μg/ml and these are likely to be key in apoptosis as there was little change in the concentration of P.

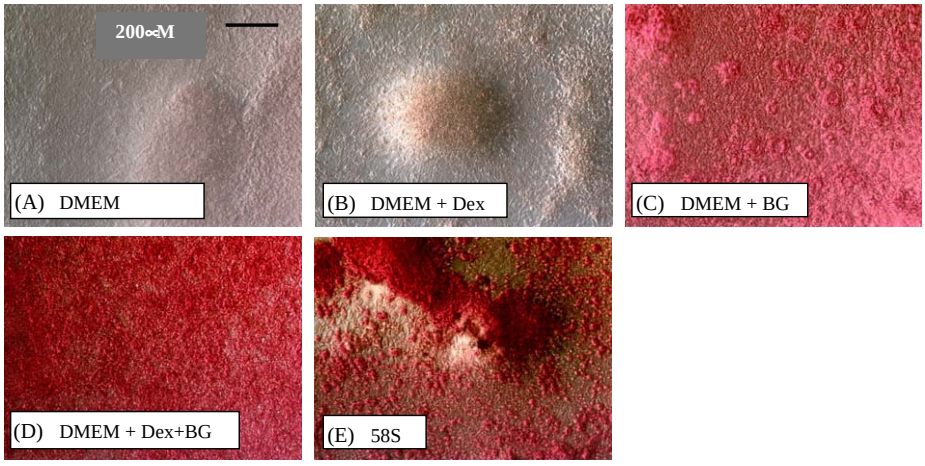

Fig. 8. HOBs cultured in dissolution products from 58S bioactive glass with or without mineralisation agents dexamethasone and beta-glycerophosphate. A: DMEM control (i.e. no supplementation); B: DMEM with 10^{-7} M dexamethasone, C: DMEM with 10 mM beta-glycerophosphate, E: 58S dissolution products with no supplementation. Positive alizarin red staining was observed in micrographs C, D and E. Bar = 200 μm on all images.

Previous work by Anderson [19] and Xynos [25] also demonstrated that certain concentrations of Si cause nodule formation and apoptosis. Calcium has been demonstrated to have an effect on apoptosis induction [26]; however the levels in our study are very low and therefore Si concentration may be more important in apoptosis induction.

Cells including osteoblasts are sensitive to changes in pH and are generally cultured in medium maintained at pH 7.2–7.4. Changes in extracellular pH are likely to cause apoptosis or necrosis as the potential across the cell membrane may be altered and ion exchange systems may be inhibited such as Ca^{2+} and $Na+/H+$. This may result in a build up of intracellular $H+$ and denaturation of proteins [27,28]. The effects of pH ranges from 7.0 to 7.8 in human osteoblasts have been investigated [29]. Activity was shown to increase as the pH increased although their data suggest that a pH of 7.2 may be best for these cells.

Careful consideration is therefore required when designing osteoinductive biomaterials with inclusion of Si. Certain dissolution product concentrations have a highly beneficial effect, which has also been demonstrated by Xynos et al. [25] and Peaker et al. [30] but higher concentrations appear to cause programmed cell death, as shown here. The authors have demonstrated similar effects of Si release from other bioactive glasses [31]. Further work is currently underway with regard to 58S processing to obtain the optimal Si release rates. Detailed analyses of effects of ion release on osteoblasts are also underway including gene expression studies during mineralisation.

5. Conclusions

Foamed 58S bioactive glass scaffolds support the attachment, proliferation and mineralised nodule formation of human primary osteoblasts. The concentration of ionic dissolution products is key to the survival, apoptosis and mineralisation of these cells. High concentrations of ionic products (Si and Ca in particular) cause apoptosis or programmed cell death of the osteoblasts when cultured in conditioned medium and prevent mineralised nodule formation. Culture in lower concentrations promotes mineralised nodule formation which was also observed when osteoblasts were cultured directly on the foamed scaffolds. Importantly, such mineralisation occurred in the absence of common mineralisation agents dexamethasone and beta-glycerophosphate demonstrating the remarkable ability of bioactive glass to induce mineralisation. This scaffold therefore has potential in bone tissue regeneration.

Acknowledgements

The authors would like to thank Tim Ryder and Margaret Mobberley, Electron Microscopy Unit, Department of Histopathology, Charing Cross Hospital, London for assistance with the scanning electron microscopy and Priya Saravanapavan, Department of Materials, Imperial College, London for operating the ICP. This study was funded by the MRC and Lloyds Tercentary Foundation.

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