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Evaluation of the osteoblast response to a silica gel *in vitro*

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Many bioactive glasses and glass ceramics contain silica, yet the effect of silica on the osteoblast is not well understood. The osteoblast cell response to a silica surface, without the interference of the other ions present in glasses and glass ceramics has been investigated. A silica sol–gel was prepared which gave a molar ratio of 1:4:4 tetraethyl orthosilicate (TEOS): ethanol : acidified water 0.2 M HCl) and spin cast on to thermanox discs. The gel was characterized in terms of bioactivity and release of silicic acid. Primary human osteoblasts (HOBs) were seeded on the surface of upright or inverted silica discs. Cell activity (alamar blue reduction), number (DNA content) and differentiation (alkaline phosphatase activity, nodule formation and mineralization) were measured. There was no apparant difference in cell number, activity or alkaline phosphatase activity between silica discs and controls. Nodules formed much earlier on the silica surfaces and these eventually mineralized. Nodule formation was reproducibly enhanced on the silica surface and less markedly on the inverted discs. It is likely that both the surface characteristics of the silica gel and silicic acid release from the disc affect osteoblast behaviour. © *1998 Kluwer Academic Publishers*

1. Introduction

Some silica containing glasses and glass ceramics are bioactive and are used as biomaterials [1, 2]. Silica is the main component of these materials but little is known about the role of silica in bone bonding. Bone bonding ability is lost when the silica content exceeds 60 mol% [2]. It has been proposed that a prerequisite to bone bonding is the formation of a biologically active carbonate-containing hydroxyapatite layer on a material surface under physiological conditions [1, 2]. This intermediate apatite layer has compositional and structural characteristics common to those of bone apatite. It is thought that hydrated silica, which is formed on the implant surface in the body, may have a role in the nucleation of the apatite layer [3]. It is difficult to study this phenomenon because apatite inducers (such as CaO, Na_2O and P_2O_5) dissolve from the materials. This problem has been partly overcome by the use of a pure silica gel prepared by hydrolysis and polycondensation of tetraethoxysilane (TEOS) in aqueous solution containing poly(ethylene glycol) to study the mechanism of apatite formation [4]. Much of the work to date has used an *in vitro* approach, using simulated body fluid (SBF) to ascertain the conditions under which a silica gel will form a surface apatite layer [5–8]. Other studies have used animal models to elucidate further the interaction of silica gels with bone [3, 4]. Few studies have examined the behaviour of bone cells on silica-containing surfaces [9–11]. The process by which osteoblasts respond to silica surfaces is not well understood.

The aim of this work was to assess the osteoblast response to a silica surface without the interference of the other ions present in glasses and glass ceramics. We have investigated the properties of a silica gel deposited on to thermanox overslips in terms of silica release and bioactivity. The behaviour of human osteoblasts on the surface was evaluated using markers of osteoblast differentiation and mineralization.

2. Materials and methods

2.1. Preparation and characterization of silica-coated discs

A silica sol–gel was prepared using 10 ml tetraethyl orthosilicate (Sigma) mixed for 1 h with 10.23 ml dried ethanol and 3.165 ml 0.2 M HCl giving a final molar ratio of 4:4:1 TEOS:ethanol:acidified water. This was spin cast on to 12 mm diameter Thermanox® discs (Nunc) and allowed to dry so that a thin, even film was formed.

The bioactivity of the silica-coated discs was assessed using a simulated body fluid (SBF). SBF was prepared as described previously [5, 6] using the reagents NaCl, $NaHCO_3$, KCl, K_2HPO_4, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ and Na_2SO_4 added in that order to give an ionic concentration almost equal to that of human plasma. The pH of the solution was brought to 7.4 using HCl and Tris buffer. Silica-coated discs ($n = 4$) were incubated in SBF at 37 °C for 11 d. The surfaces were air dried and viewed using a Philips XL30 FEG environmental scanning electron microscope (ESEM) operated in auxillary mode at 10 kV with energy dispersive X-ray (EDX) microanalysis.

Silica-coated discs and Thermanox® discs ($n = 3$) were immersed in SBF for 2, 4 and 11 d. The amount of monomeric and dimeric orthosilicic acid released was determined using the molybdenum blue assay. The method was based on that of Mullen and Reilly [12] and modified according to Perry and Keeling-Tucker [13]. Briefly, silica standards were prepared from 1000 ppm solution of SiO_2 (as sodium metasilicate; BDH) in a solution containing 6% acidified ammonium molybdate solution. Test samples were prepared in the same way. After 10 min, 30% of a reducing solution containing metol was added and the absorbance at 810 nm was read after 2–48 h.

2.2. Cell culture

Silica gels were placed in 24-well tissue culture plates (Falcon) with the silica surface uppermost. Primary human osteoblasts (HOBs) were obtained from femoral head trabecular bone [14] and cultured in phenol red free Dulbeccos modified eagles medium supplemented with 10% foetal calf serum and 150 $\mu g l^{-1}$ *L* ascorbic acid at 37 °C; 5% CO_2. These were seeded on the silica surfaces at a density of 8×10^5 cells ml^{-1} and maintained in culture for up to 31 d. Uncoated Thermanox® discs were used as controls. Cultures were refed every 2–3 d. Some silica discs were placed in an inverted position to rule out topographical effects.

Cell activity on silica-coated discs and Thermanox® controls was measured over 31 d using the alamar blue assay (Serotec). This assay quantifies metabolic activity by utilizing a redox indicator which changes to a fluorescent product in response to the chemical reduction of the medium by growing cells. The medium was removed from the cultures and a 5% solution of alamar blue in Hanks' balanced salt solution (HBSS) was added to each well. These were incubated at 37 °C; 5% CO_2 for 20 min. The fluorescence was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Cell number was determined at 2, 4 and 7 d using bisbenzimidazole (Hoechst 33258, Sigma) as described by Rago *et al.* [15]. DNA standards were prepared from 20 $\mu g\,ml^{-1}$ stock solutions of purified calf thymus DNA (Sigma) to give a DNA content of 10, 6, 5, 4, 3, 2, 1, 0.5 and 0.25 $\mu g\,ml^{-1}$ per well. Aliquots from cell lysates were placed in 96-well tissue culture plates. A 1 in 50 dilution of the dye in TNE buffer (10 mM Tris, 2 M NaCl and 1 mM EDTA; pH 7.4) was prepared from a 1 $mg\,ml^{-1}$ H_2O stock solution and an equal volume of this was added to the test solutions and to the standards in the plate. Fluorescence was measured immediately at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. A standard curve was prepared and DNA values were calculated.

Alkaline phosphatase (AP) activity was measured at 2, 4 and 7 d using 4-nitrophenylphosphate as the substrate. The absorbance was read at 620 nm.

Mineralization was observed by staining with alizarin red S according to the method of Ohgushi *et al.* [16] to identify calcium-containing deposits. The medium was removed from 14 d cultures and they were rinsed in Mosconas minus phosphate buffer (0.137 M NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$ and 11 mM dextrose; pH 7.4). Alizarin red S (0.25 g alizarin red S in 50 ml 0.1 M barbital buffer, pH 9) was added to the wells for 4 min, after which the discs were removed to a fresh plate containing buffer, and photographed immediately.

Mature cultures (14 d) were examined by tetracycline labeling counterstained with propidium iodide (PI). Tetracycline is incorporated into calcium-containing deposits as they form and can therefore be used as a marker of mineralization. Tetracycline (9 $\mu g\,ml^{-1}$) supplemented growth medium was added to each well and incubated for 24 h (JE Davies, oral communication). Discs were then fixed in a 50% ethanol solution containing 1% acetic acid and stained with 0.01 $mg\,ml^{-1}$ propidium iodide for 30 s. They were then rinsed in phosphate buffered saline, mounted, and viewed usig a Leica TCS confocal laser scanning microscope (CLSM).

The amount of silicic acid present in tissue culture medium from blank silica-coated discs and silica-coated discs seeded with HOBs was quantified to indicate any uptake of silica by osteoblasts. Four replicates were used for each type of disc. The medium was collected at 2 and 4 d and the silicic acid content of the medium was determined using the molybdenum blue assay.

3. Results

3.1. Characterization of silica gel

ESEM of the silica-coated disc surface revealed that it was flat and featureless even at high magnifications. Bioactivity was determined using ESEM with EDX to detect the presence of an apatite layer on the surface of the silica gel after an 11 d immersion in SBF at 37 °C. Calcium phosphate deposition was observed over much of the surface of the silica gel. The calcium : phosphorous (Ca : P) ratio varied from 1.2 to 1.6 showing that apatite had formed over at least some of the gel surface.

The amount of silicic acid (in the monomeric and dimeric forms) released into SBF from the silica gels was measured using the molybdenum blue assay. The silica gels had leached ~ 3 p.p.m. ml^{-1} silicic acid into SBF in 3 d. The release of silicic acid from the gels over time was measured in SBF. The amount of silica

released over 11 d was equivalent to ~ 1 p.p.m. ml^{-1} per day.

3.2. Osteoblast response to silica gel coatings

3.2.1. Morphology

HOB cells cultured on silica-coated surfaces and Thermanox® controls were confluent by 48 h. In some areas the cells were clearly oriented along discontinuities in the silica coating. Cultures had formed multilayers on all surfaces by 7 d. Cell clustering was seen in control cultures at 14 d and nodules began to form between 18 and 24 d. Nodules were formed earlier on the silica-coated discs than on the controls (Fig. 1). This was independent of the timescale of nodule formation on the controls. Osteoblasts grown on silica-coated surfaces produced mature nodules between 4 and 10 d when control samples were at the multilayer stage. Nodules were larger and more numerous on the silica-coated surfaces at later timepoints up to 31 d. The shape of the nodules formed on the silica-coated surface was sometimes elongated in comparison to the controls. This was attributed to the earlier alignment of cells along defects in the gel caused by contact with aqueous medium. HOBs were also grown on inverted silica-coated discs. These cells had access to the silica released from the gels but were not influenced by the surface topography nor surface chemistry. Nodule formation was earlier than control cultures on these discs (8–14 d) but was not as advanced as on the upright discs.

3.2.2. Cell biology

The response of HOBs to growth on a silica gel surface was determined biochemically using parameters such as cell activity (alamar blue assay), cell number (DNA content) and differentiation (AP activity). Cell activity was comparable on both surfaces with no significant difference between thermanox and silica-coated discs over a period of 31 d. DNA content and AP activity were also similar for experimental and control samples for 2, 4 and 7 d.

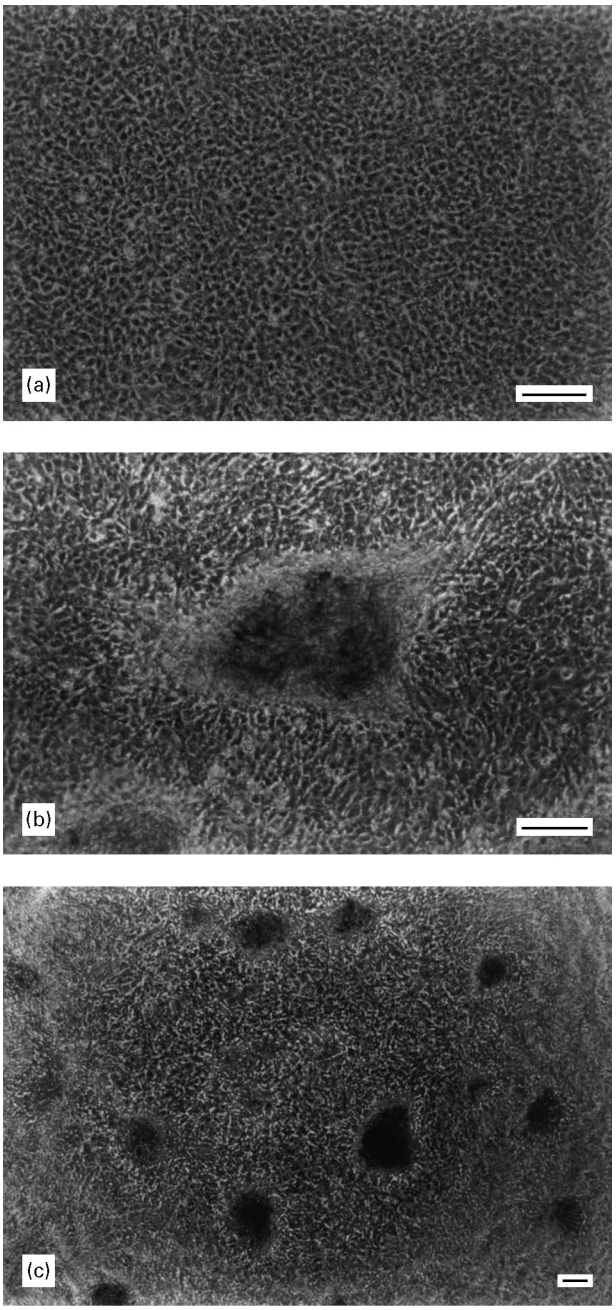

Figure 1 Osteoblast growth on silica coated discs. (a) HOBs grown on Thermanox for 10 d. (b) 10 d HOB culture on a silica-coated disc showing a mature nodule. (c) Distribution of nodules on silica-coated discs after 10 d in culture.

3.2.3. Mineralization

Cultures were labeled with tetracycline at 14 d when cells on silica gel surfaces had formed mature nodules and control cultures were beginning to form cell clusters. Fig. 2 shows minimal teracycline incorporation by control cultures compared with striking positively labeled nodules formed on silica-coated surfaces. Nodules on silica-coated surfaces also stained positively with alizarin red S at 14 d (not shown).

3.2.4. Silicic acid uptake by osteoblasts

Silicic acid release into tissue culture medium was similar to that in SBF at 2 and 4 d. The amount of silicic acid in the medium was significantly lower ($p < 0.01$) when osteoblasts were cultured on the silica-coated surface and was close to the control level (Fig. 3).

4. Discussion

In this study the behavior of osteoblasts grown on silica surface was investigated. HOBs were used because they have been shown to express many markers of osteoblast differentiation and to form mineralized nodules without the addition of mineral promotors [14].

HOBs were grown on a thin film of silica gel in order to establish the effect of silica on osteoblast growth and differentiation. Silica gels have been used in the past to eliminate the effects of the other ions present in glasses and glass ceramics, but these have

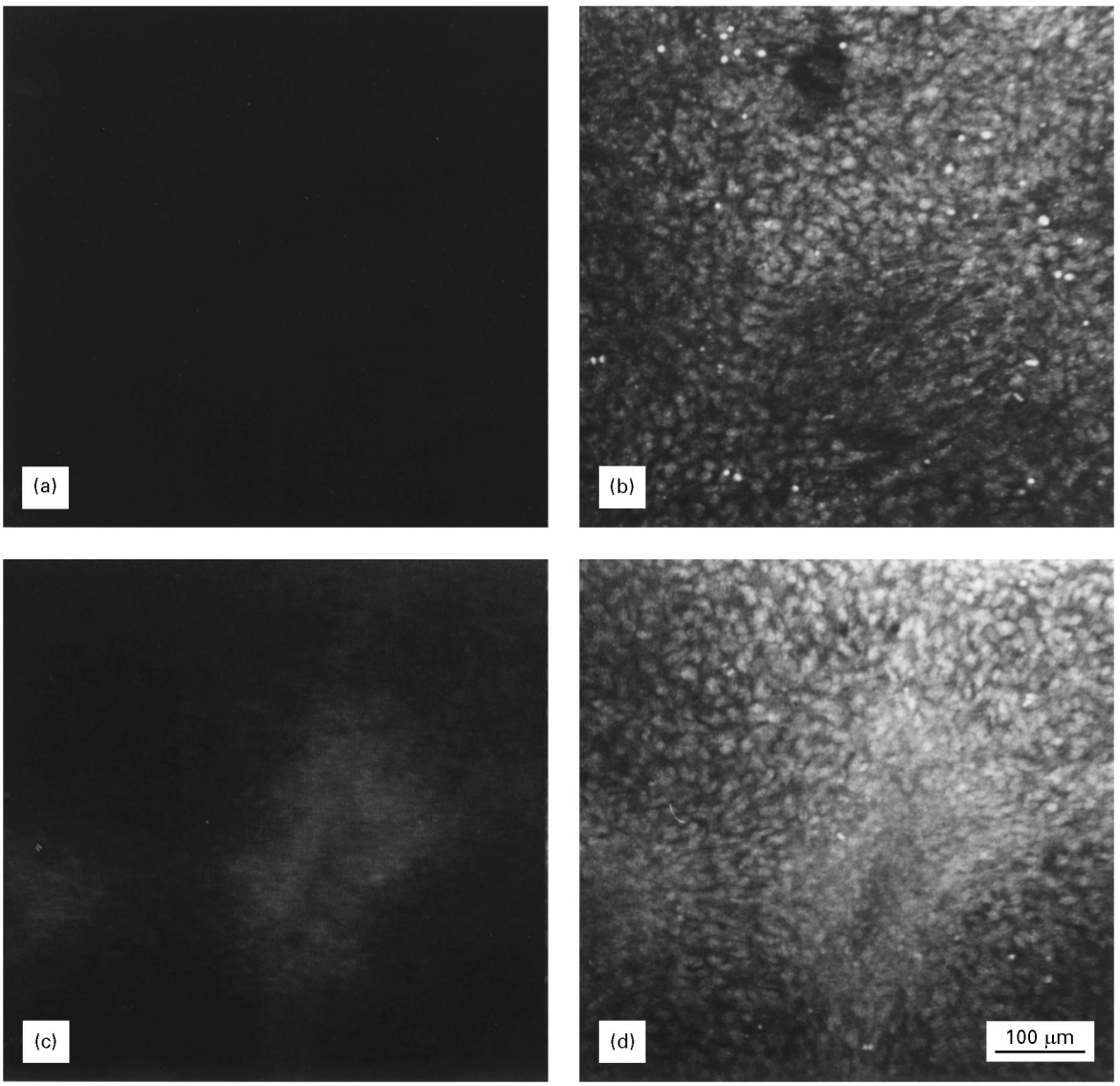

Figure 2 CLSM images of mineralization of nodules on (a, b) Thermanox and (c, d) silica-coated discs. (a, c) Tetracycline incorporation in calcium-containing deposits representing mineralized areas. Nuclei of cells in the same areas are stained with PI to show the localization of mineral. (b) PI stained nuclei of a cell multilayer on Thermanox. (d) PI stained nuclei of a nodule on the silica coated surface.

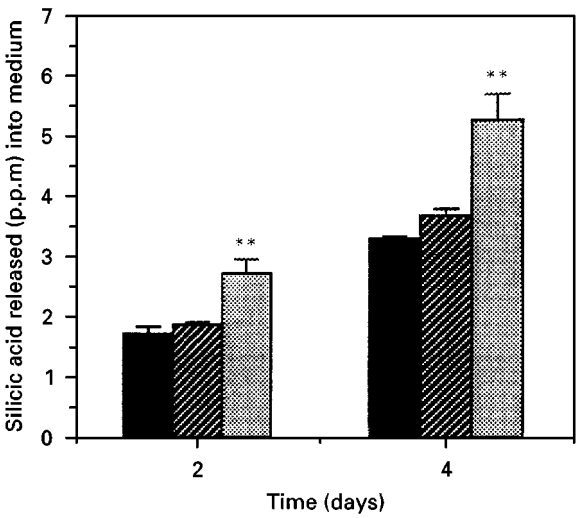

Figure 3 Silicic acid content of the medium from wells containing silica-coated discs (SCD) with and without cells as determined by the molybdenum blue assay. The amount of silicic acid released from the silica-coated discs is significantly reduced ($P < 0.01$) when cells are cultured on the surface. ■ Medium from thermanox, ▨ Medium from Cells on SCD, ▩ Medium from SCD

concentrated on the ability of the gels to form an apatite layer on their surface when soaked in SBF for an extended period of time. Few studies have investigated the cell response to growth on a silica surface. The gel used in this work was shown to produce a CaP layer when immersed in SBF for 11 d. EDX analysis of this layer showed the Ca:P ratio to be in the range 1.2–1.6 which approaches that of hydroxyapatite. The Ca:P ratio of the mineral portion of bone is ~ 1.5. It was concluded that the gel surface was bioactive.

The response of osteoblasts to the silica gel surface was striking. HOBs formed nodules on silica surfaces as early as 4 d. Nodule formation was always earlier on silica-coated surfaces than on controls, and nodules were larger and more numerous. The use of the methods outlined above clearly show that the nodules had mineralized. In some cases, osteoblasts aligned along defects in the silica gel and nodules later formed in these areas. It is clear that in these cases early nodule formation may have been due to the physical topography of the surface rather than the silica present on the surface. However, this was not always the

case and cells grown on discs which remained more stable in solution also produced nodules reproducibly earlier than on controls. HOBs were also grown on inverted silica discs to address the problem of topography. In this way cells had access to silica that was released from the discs but the surface available to the cells was the same as that exposed to the controls (Thermanox®). In these cultures, nodules formed earlier than on controls but not as early as the upright silica discs. This demonstrates that topography alone was not responsible for the early mineralization of HOBs on silica surfaces. It is still unclear whether surface chemistry of the gel affects the osteoblast response but it is thought that the surface of the gel is composed of silanol (OH) groups [17] which are reported to be favorable sites for apatite nucleation [8]. This has yet to be confirmed.

The gel used in the present work was shown to release silicic acid into SBF and tissue culture medium in the monomeric and dimeric forms. Silica is thought to be metabolized in the body as monomeric silicic acid [18]. When cells were cultured on the silica gel-coated surfaces, the amount of silicic acid in the medium was comparable to the control level. This preliminary work implies that osteoblasts either prevent the release of silicic acid from the gels or that they absorb the silicic acid released. Such a relationship between osteoblasts and silicic acid has never been previously demonstrated. The apparent ability of osteoblasts to absorb silicic acid coupled with the early nodule formation on both upright and inverted silica discs shows that osteoblast mineralization is enhanced on a silica surface *in vitro*.

It is interesting to note that despite reliably early nodule formation on silica-coated discs, cell activity, number and AP activity were not affected. *In vitro* studies of the osteoblast response to bioactive glasses and glass ceramics have shown increased AP activity on the silica containing surfaces [9, 11]. To our knowledge the osteoblast response to silica gels has not been documented. Silica has been linked with collagen formation [18] and may affect the expression of osteocalcin and it is possible that silica affects these and/or other proteins involved in the mineralization process.

5. Conclusion

A bioactive silica gel-coated disc was used to investigate the osteoblast response to a silica surface. Nodule formation was reproducibly enhanced on the silica surface and less markedly so on the inverted discs. It is likely that both the surface characteristics of the silica gel and silicic acid released from the disc affect osteoblast behavior. These two possibilities are under investigation.

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