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EXTRACELLULAR MATRIX PRODUCTION BY OSTEOBLASTS ON BIOACTIVE SUBSTRATA *in vitro*

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Abstract

Some bone-substitute biomaterials have been classified as bioactive since they allow direct biological bonding to their surface *in vivo*. Using *in vitro* techniques, we have re-created the first stages of this biological bonding phenomenon and compared the initial, fibrillar, extracellular matrices produced by migrated primary osteoblast cell populations in contact with both dense and macroporous calcium phosphate substrata, apatite/bioactive glass composite (ABC) and 45S5 bioactive glass (BAG). The first formed fibrils in contact with these materials may be identified as collagen from their morphology as observed by scanning electron microscopy (SEM). However, the organization of this fibrillar material is significantly different on the five bioactive substrata examined. These *in vitro* findings may not only be related to both the surface morphology and surface chemistry of the substrata, but also correlated with their levels of *in vivo* bioactivity.

Key Words:- Osteoblast; extracellular matrix; collagen; *in vitro*; Calcitite™; Interpore 200™; Interpore 500™; apatite/bioactive glass composite; bioactive glass.

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Introduction

Bone-substitute biomaterials may be classified as either bone-bonding or non bone-bonding. The interface created, *in vivo*, between bone tissue and bone-bonding or "bioactive" substrata has been shown to comprise a direct biological bond with collagen fibrils interdigitating with the surface of the implant (Beckham *et al*, 1971). While this interfacial behaviour is of central importance to the clinical success of bioactive bone-substitute biomaterials, the mechanisms of these bonding phenomena are not understood (Ito *et al*, 1987). This is, in part, due to the fact that while *in vitro* models are necessary to deconvolute the complexities of established *in vivo* responses, no *in vitro* methods had, until recently, been developed to investigate these tissue/material interactions. However, we have shown using simple tissue culture techniques, that the migratory morphology of primary osteoblast populations on different calcium phosphate substrata can be compared (Davies *et al*, 1986), that such isolated cell populations can produce a mineralized extracellular matrix juxtaposed to the artificial substratum (Davies *et al*, 1987) and that the collagen bonding known to occur on 45S5 bioactive glass *in vivo* may be reproduced *in vitro* (Matsuda & Davies, 1987).

As the initial stage of collagen bonding is critical to the development of a bone-bond, we demonstrate here the ultrastructural characteristics of the fibrillar matrix formed, by primary bone cell cultures, at the created interface with four bioactive bone-substitute materials.

Materials and Methods

Calcitite™

Calcitite™ (Calcitec Inc., San Diego, CA) is a solid, dense non-resorbable form of hydroxyapatite ceramic. It was supplied as smooth rounded particles of 1-2 mm^3, in sealed plastic containers, which had been sterilized by ^{60}Co gamma-irradiation ready for clinical use.

Interpore 200™ and Interpore 500™

Both Interpore 200™ and Interpore 500™ (Interpore International, Irvine, CA) are macroporous forms of carbonate containing hydroxyapatite ceramic derived by hydrothermal exchange from the aragonite skeleton of a common sceractinian reef-building coral, genus: porities (Roy & Linnehan,

1974) but the mean pore sizes differ, i.e., 200 and 500 microns respectively.

The resultant material has been described as both non-biodegradable and exceptionally permeable to tissue ingrowth (Chiroff *et al*, 1975). Interpore 200™ was supplied as fracture particles (425-1000 microns) produced from a larger parent block and sterilized in a sealed glass bottle by ^{60}Co gamma-irradiation ready for clinical use. Interpore 500™ was supplied in block form which was fractured immediately prior to *in vitro* use and sterilized by immersion in 70% alcohol.

Apatite/Bioactive Glass Composite. (ABC)

This material was supplied by Nippon Kogaku k.k. (Tokyo, Japan). It was produced by first making the BAG and hydroxyapatite (HAp) components as described by Mochida *et al*, (1988). The BAG (of 45SF1/4 type) was melted at 1350°C in a platinum crucible and had a final composition of SiO_2(46.1%), Na_2O(24.4%), CaO(20.2%), CaF_2(6.7%) and P_2O_5(2.6%). After pouring into a mold the BAG was pulverized to obtain a particle size less than 75 microns. The HAp powder was prepared by reaction of $Ca(OH)_2$ and H_3PO_4 in aqueous solution.

The two powders were then mixed 1:1 by weight and re-pulverized to produce a mean particle size of 3 microns. After drying and pressing the mixture was heated at 960°C for 1 h. and subsequently annealed at 100°C/h. to room temperature. The final material was fractured to produce particles of approximately $1mm^3$ and irregular shape suitable for the *in vitro* methods employed. The particles were sterilized by dry heat at 160°C for 3.5 h. prior to use *in vitro*.

Bioactive Glass. (BAG)

The bioactive glass, used in these experiments, was of the 45S5 type and known to promote direct bone-bonding *in vivo* (Ito *et al,* 1987). This glass was also supplied by Nippon Kogaku k.k. (Tokyo, Japan) and was prepared by melting reagent grade Na_2CO_3, $CaCO_3$, H_3PO_4 and SiO_2 in a platinum crucible at 1200-1400°C for 2 h. before casting into steel molds as described by Fujui & Ogino (1984). The glass, having a final composition of SiO_2(46.1%), Na_2O(24.4%), CaO(26.9%) and P_2O_5(2.6 %), was fractured to produce particles of approximately $1mm^3$ and of irregular shape suitable for the *in vitro* methods employed. The particles were sterilized by dry heat at 160°C for 3.5 h. prior to use.

Tissue Culture Method

The tissue culture method is modified from that of Jones & Boyde (1977) who described the migration of osteoblasts over glass fragments. Neonate Albino Wistar rats (1-4 days old) were killed and the calvaria removed, using aseptic techniques, following reflection of the cranial skin. The endocranial and extracranial periostea were removed, while the tissue was bathed in phosphate buffered saline pH 7.2 (PBS) containing 10% foetal calf serum (FCS), to minimize fibroblastic contamination and expose the endocranial surface osteoblast population.

A central area of each parietal bone was then removed, devoid of sutural areas and transferred to Bigger's culture medium (Fitton Jackson modification - Flow Laboratories) containing 10% FCS, 20μl/ml 200mM glutamine, 10μl/ml Penicillin (5000iu/ml)/Streptomycin (5000μg/ml and 25μl/ml 1M Hepes; hereafter referred to as sFJm. The test bioactive bone-substitute material was then placed as an overlay on the resultant fragment of host bone, with an intact osteoblast population, and maintained in culture in a 5% CO_2 humidified atmosphere for 4 weeks in Linbro multiwell dishes containing 2 mls of sFJm. Hepes was used in conjunction with 5% CO_2 since the colour of the medium, and hence the pH was adversely affected using 5% CO_2 alone.

Preparation for Microscopy

Following the culture period the sFJm was replaced with 2.5% 0.1M cacodylate buffered glutaraldehyde (pH 7.2), dehydrated through a graded series of ethanols, critical point dried from carbon dioxide, using a Polaron E3000 and sputter coated with either gold or platinum prior to examination in an ISI 100A SEM (accelerating voltage 20kV) or a JEOL 1000 CXII STEM (accelerating voltage 20kV).

After initial examination, some samples were freeze fractured to allow observation of the created tissue/biomaterial interface by the following method. The sample was transferred to two changes of absolute ethanol before freezing in liquid nitrogen. A miniature stainless steel chisel was used to fracture the sample which was then returned to absolute alcohol before being critical point dried from carbon dioxide prior to re-coating with platinum as described above.

Results and Discussion

SEM Appearance of material surfaces

Figure 1 shows a montage of SEM photomicrographs of Calcitite™ (Figs 1a-d), Interpore 200™ (Figs 1e-h), Interpore 500™ (Figs 1i-l), ABC (Figs 1m-p) and BAG (Figs 1q-t), respectively. Both the varying degree of roughness and macroporosity of these materials could be compared at low magnifications.

Calcitite™ and BAG appeared smoother than the remaining three materials although the former had a pitted surface (Fig 1a) while only ABC demonstrated microporosity (Figs 1n,o). The appearance of Interpore 200™ and Interpore 500™ were significantly different with the latter being considerably rougher and exhibiting a surface with an apparent spheritic crystalline character (Fig 1j) while Interpore 200™ and Calcitite™ exhibited roughened surfaces with no apparent organization. Some areas of the Calcitite™ surface demonstrated fusion of ceramic grains (Fig 1d) but no evident microporosity.

At higher magnification, BAG exhibited a two-phase appearance with a homogeneous distribution of rounded micro-elevations (Figs 1s,t) and no evidence of microporosity. In contrast, ABC clearly possessed a microporous surface with fused ceramic grains which formed an irregular micro-trabecular structure (Figs 1o,p). However, an even greater surface area was exhibited by Interpore 500™ where the rounded surface elevations were formed from agglomerations of microcrystallites forming a microporous structure (Figs 1j,k). These microcrystallites possessed a plate-like morphology (Figs 1k,l) which was also evident on the surface of this material after being kept in culture medium for a period of four weeks (not shown). At high magnification the surfaces of the microtrabeculae of ABC were seen to be smooth (Fig 1p) and similar in surface morphology to Calcitite™ (Fig 1d). Of the five materials, Calcitite™ and Interpore 200™ were the most similar in surface morphology at high magnifications (Figs 1d,h).

SEM appearance after 4 weeks *in vitro*

After the 4 week culture period, cells had colonized all bioactive material fragments although the degree of coverage, in several series of experiments, was variable. The interface

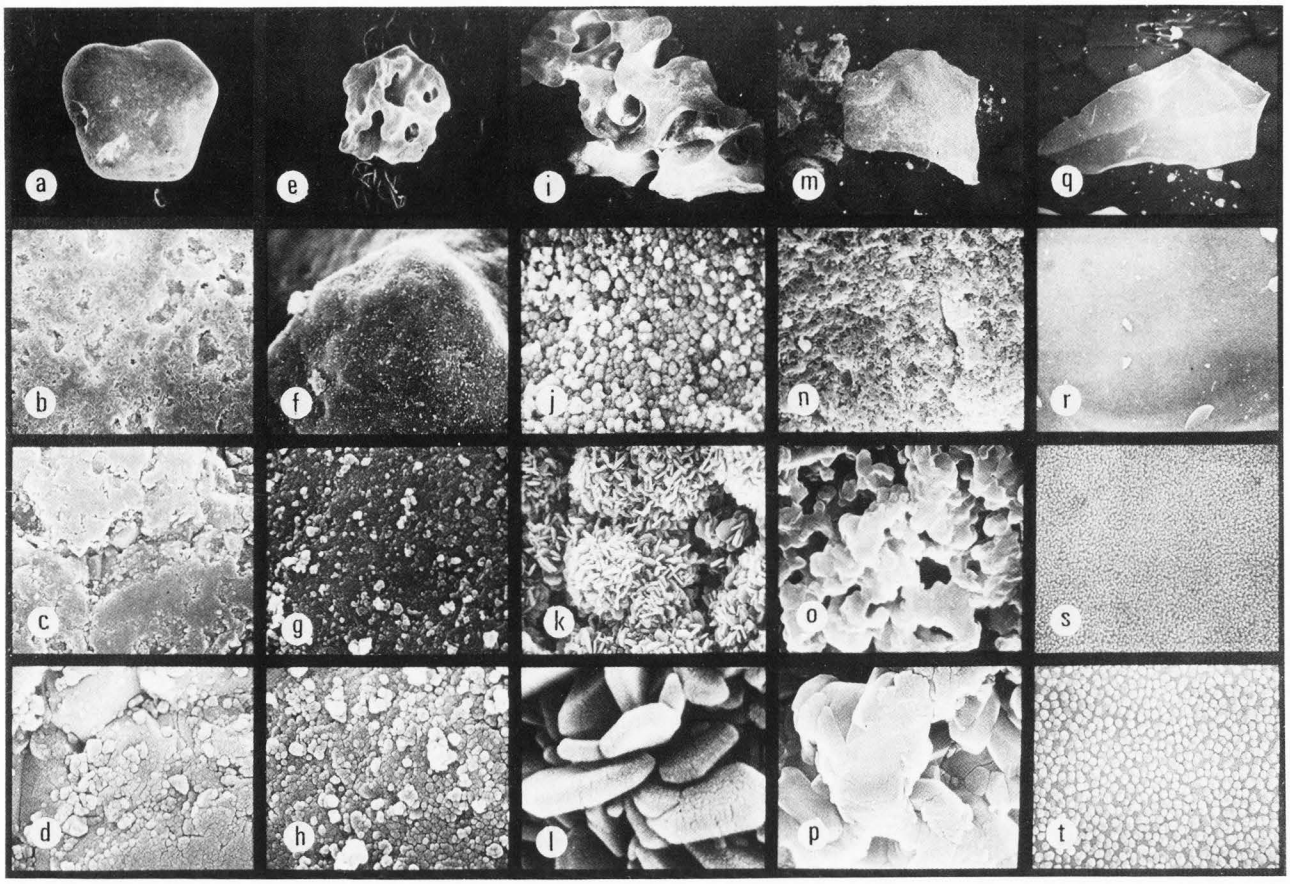

Figure 1. SEM micrographs of fragments of bioactive bone-substitute biomaterials. (a-d): Calcitite™. A dense calcium phosphate bioceramic. Field Widths = (a) 1.17mm; (b) 58.6 μm; (c) 5.86 μm and (d) 1.94 μm. (e-h): Interpore 200™. Both macroporosity and microporosity are lower in this material than Interpore 500™. Field Widths = as for a-d. (i-l): Interpore 500™. A macroporous calcium phosphate ceramic. j-l show the appearance of an internal pore surface. This is to be distinguished from the fracture surfaces created by fragmenting this sample from the parent material block. Note the fine plate-like micro-crystalline morphology at high magnification. Field Widths = (i-k) as for a-c, (l) 0.58 μm. (m-p): Apatite/bioactive glass composite, ABC. Microporosity is evident in this material. Field Widths as for a-d. (q-t): 45S5 Bioactive glass, BAG. A non-microporous, smooth surfaced material which exhibits a two-phase surface structure. Field Widths = as for a-d.

formed between the tissue elaborated by the migrated osteoblasts and the biomaterial could be examined in two ways.

First, shrinkage which occurred during the critical point drying procedure caused the tissue covering the biomaterial fragment to be fractured; thus exposing the interface. Second, the interface could be examined following the freeze-fracturing procedure described above.

Figure 2a shows an Interpore 500™ particle following the 4 week culture period and the shrinkage artifacts caused by the critical point drying procedure. Figure 2b shows the appearance of a sample of Interpore 500™ following freeze-fracturing. In both figures, the degree of tissue coverage after the 4 week culture period is evident. While the amount of tissue elaborated was variable, the degree of tissue reaction to Interpore 500™ was similar to that which we have previously observed on BAG by both light and electron microscopy (Davies *et al*, 1987; Matsuda & Davies, 1987).

Freeze-fracturing demonstrated the amount of ingrowth of cells into, and fabrication of, tissue in the pore structure of this material. This was greater than we have previously reported in Interpore 200™ where, after initial colonization of the pore structure by cells, continuing cell migration resulted in the pore openings being bridged by sheets of migrating cells which, in turn, reduced cell migration into, and tissue elaboration within, the pore structure (Rout *et al, 1987).*

We have already reported that this *in vitro* method is suitable for the examination of two created interfaces. First, that created between the host bone surface and the overlying test material and, second, that between the extracellular matrix (ECM) produced by the migrated cells and the dorsal overlay surface. Examination of the former interface demonstrated significant differences between the tissue appearance underlying bone-bonding and non bone-bonding materials. Bone-bonding interfaces were characterized by numerous cell processes which are torn on removal of the overlay material (Matsuda & Davies, 1987). We have seen a similar appearance, in the present work, beneath ABC, Interpore 200™ and Interpore 500™ (not shown). However, in this report we are con-

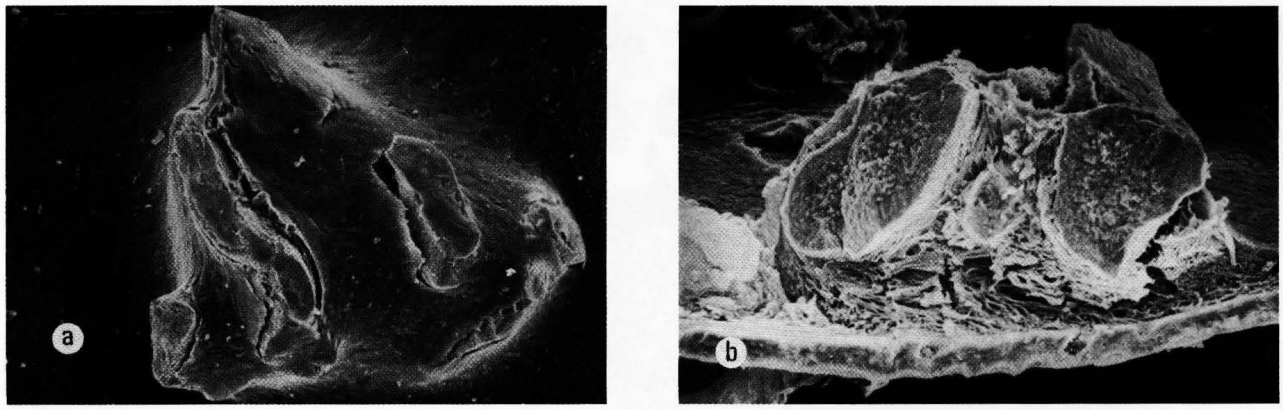

Figure 2. SEM photomicrographs of Interpore 500™. (a) Shows an overlay particle firmly anchored to the host bone by the elaborated tissue covering. The osteoblast multilayer on the parietal square is confluent over the overlay surface except where shrinkage artifacts have occurred during the critical point drying procedure. In these areas, the interface between tissue and artificial substratum may be seen. Field Width = 1.6mm. (b) A freeze-fractured sample showing the host bone surface below, the macroporous particle above and considerable tissue ingrowth into the single pore visible in this section. Field Width = 1.4mm.

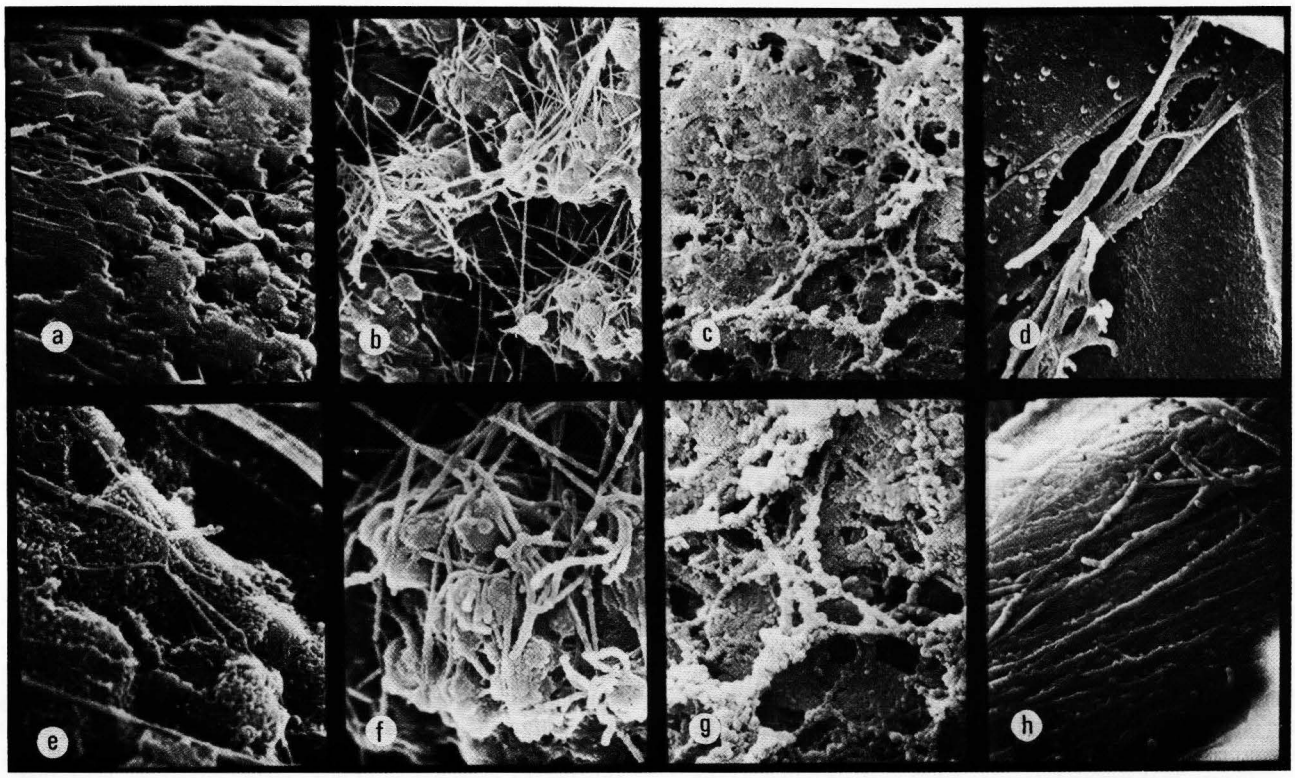

Figure 3. SEM photomicrographs of Calcitite™ (a,e); an Interpore 500™ surface fractured to create the fragment prior to the culture procedure (b,f); ABC (c,g) and BAG (d,h). (a): Note the pitted surface which may be compared with the appearance in Fig.1(b). Field Width = 8.3 μm. (e): The surface is covered with a micro-globular network to which randomly oriented collagen fibrils are attached. Note the banded morphology of the fibrils. Field Width = 1.66 μm. (b, f): Field Widths = 8.3 and 2.5 μm,respectively. Compare the appearance of these collagen fibril networks with those elaborated on the internal pore surface as shown in Fig.4. Note also the distinct banded morphology of the collagen fibrils (c): The micropore structure of this material, shown in Figs.1f-h, has been masked by the elaborated extracellular matrix. Field Width = 5 μm. (g): The fibrillar component of the ECM is coated and exhibits a nodular appearance. It is not possible to distinguish these fibrils as collagen. This appearance could be due to coating of collagen by non-collagenous proteins. Field Width = 2.5 μm. (d): The substratum has been coated reducing the surface roughness (above). Collagen fibre bundles from an overlying cell (bottom right) are attached to this surface coating. Field Width = 6.25 μm. (h): Ordered linear arrays of collagen fibrils with a discernible banded morphology. Field Width = 2.5 μm.

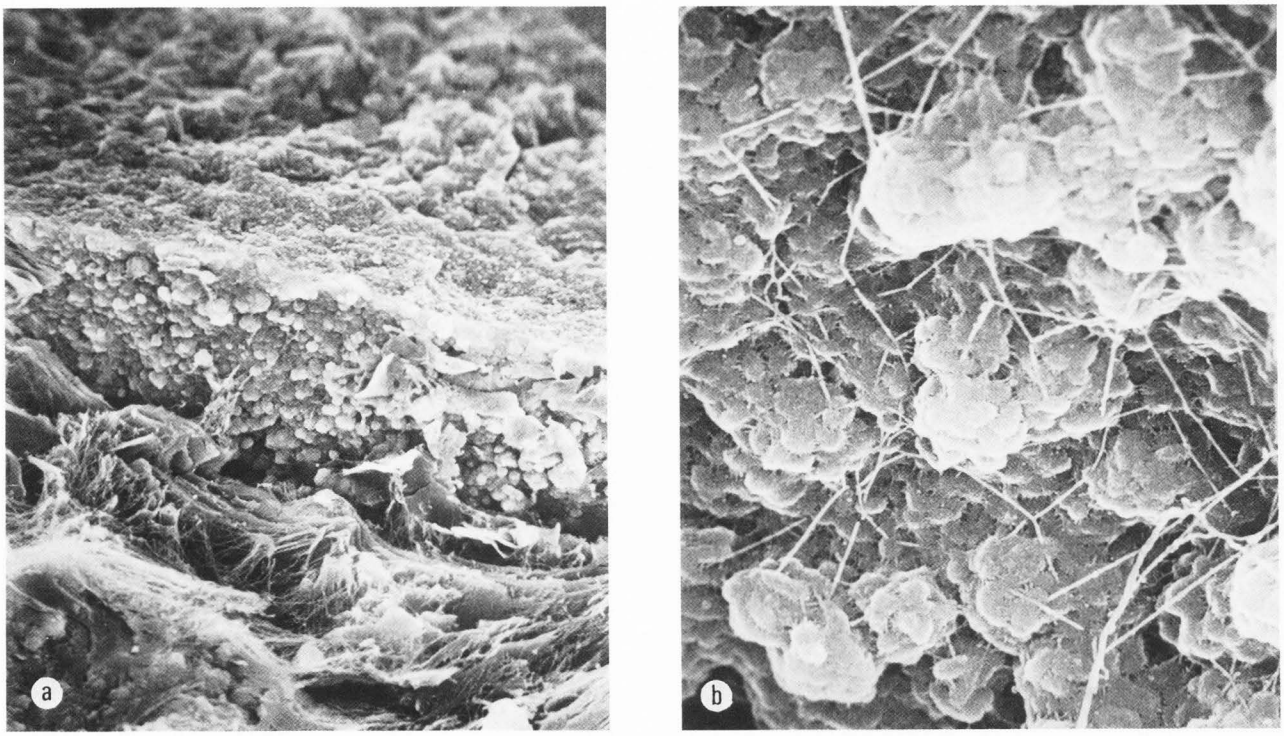

Figure 4. SEM photomicrographs of a freeze-fractured Interpore 500™ particle. (a) The interface revealed by fracturing shows the particle above with the fracture surface clearly delineated and the elaborated tissue within a pore below. The particle surface forming the interface is therefore an internal pore surface and may be compared to the morphology on a fracture surface created prior to culture (Figs. 3b,f). At this magnification the surface morphology is similar to that seen in the dry material (Fig.1). Field Width = 100 μm. (b) The internal pore surface at higher magnification. Note the interdigitation of the collagen fibrils with clusters of rounded bodies which themselves are connected by a finer filamentous network. Compare with Fig.1k. Field Width = 8.3 μm.

cerned with the initial stages of production of ECM on the dorsal overlay surface.

Elaboration of ECM at the interface

After 4 weeks culture all materials were found to have fibrillar material, elaborated by the colonizing cells, in intimate contact with the material surface. The ultrastructural appearance of all materials, except BAG, corresponded to the original dry appearance of the material surface. In the case of BAG, the dynamic surface chemistry of the glass, which results in the production of a calcium phosphate rich surface layer (Ogino & Hench, 1980), rapidly creates an interface with the macromolecular components of the culture medium before migrating cells reach the dorsal overlay surface.

Comparison of Figures 3(a-d) with the surface morphologies illustrated in Figure 1 demonstrates, except for BAG, the similarity between the original sample surface and that after 4 weeks *in vitro*. Only the appearance of Calcitite™ after the 4 week culture period (Fig.3a) could be directly compared to the dry material (Fig.1b) since the pitted surface structure was easily discernible. Nevertheless, at higher magnifications (compare Figs 1d and 3e which represent the same field width) it was evident that the surface was covered with a microglobular network to which the collagen fibrils were randomly attached (Fig. 3e). The surface of ABC could also be distinguished, although the microporosity had been masked by the elaboration of ECM (Fig.3c).

The fibril morphology on these four materials was significantly different. On Interpore 500™, a fine fibrillar arrangement tended to bind into globular accretions (Figs.3b,f). On ABC the fibrils appeared coated (Figs.3c,g) while on BAG the fibrils were organized into linear bundles of collagen fibres which were thicker than those found on the other substrata (Figs.3d,h).

It is clear from these SEM photomicrographs that ECM was elaborated on each of the five samples examined in the form of fine fibrillar material which was in intimate contact with the bioactive material surface. We assume these fibrils to be collagen both from the morphological evidence presented here (see the classical banded morphology in some of the fibrils in Figs.3 e,f which is also evident in Fig.3 h) and polarized light photomicrography and transmission electron microscopic (TEM) evidence that we have presented elsewhere (Davies *et al*, 1987; Matsuda & Davies, 1987).

It was difficult to compare the thicknesses of these layers of ECM elaborated on the five substrata due to both the variability from one culture batch to the next and the limitations of the SEM preparatory techniques (the fracturing technique caused considerable damage to the tissue layers on the dorsal overlay surfaces and we were unable to produce freeze-fractured specimens of Calcitite™ suitable for photomicrography). However, the "surface" appearances shown in Fig.3 were re-

producible and we are currently preparing batches of these samples for both light and transmission electron microscopy.

The interdigitation of collagen fibrils with the substrate surface, resulting in the elaboration of collagen fibre bundles, is one of the fundamental stages leading to the establishment of bone-bonding in these bioactive materials (Jarcho, 1981). Thus the comparison between the fibrillar structure of the collagen on the calcium phosphate ceramic samples and the fibre bundles forming after the same time period in culture on BAG is of direct relevance to the rapidity of bone-bonding associated with the latter *in vivo*. In fact, the BAG surface seemed to be coated in such a way as to smooth the micro-rough host material surface which is known to form on creation of the solid/liquid interface (Matsuda *et al*, 1987) and the collagen fibrils seemed to merge with this surface coating (Figs. 3d,h). This integration of collagen fibres with the crystallising surface calcium phosphate layer has also been shown to occur when bioactive glass is simply maintained *in vitro* in the presence of collagen fibres (Hench & Wilson, 1984).

It is clear from a comparison of Figs. 4(a) and 1(j) that the surface of Interpore 500™ after the 4 week culture period was similar, at low magnifications, to the dry material. However at higher magnification, Figs. 4(b) and 1(k), it was clear that the plate-like morphology of the microcrystallites comprising the rounded surface elevations seen in the dry material (Figs. 1k,l) were no longer visible and the fibril network, elaborated by the migrated cells, had been organized at two morphologically distinct levels. First, the collagen fibrils formed a random network interdigitating with globular masses. Second, these globular masses were apparently composed of smaller rounded structures which were clustered together by a fine filamentous network. These rounded structures obliterated the plate-like crystallites of the Interpore 500™ which supported them and thus the microstructure of the substratum could clearly be seen to be responsible for the primary organization of the ECM. We think it is possible that locally high Ca^{2+} and PO_4^- ion concentrations which would be created by partial dissolution of this high surface/volume ratio material could result in the Ca/P solubility product being exceeded resulting in re-precipitation of calcium phosphates on the fine fibrillar ECM. This would create a biological bond similar in nature to that seen in BAG and which we have demonstrated, at TEM level, using these *in vitro* techniques (Matsuda & Davies, 1987). However, we have so far failed to identify the composition of these globular masses using energy dispersive X-ray analysis due to difficulties in distinguishing the calcium and phosphorus emissions from those of the parent material.

Conclusions

Collagen containing extracellular matrix is produced by migrated osteoblasts on bioactive substrata *in vitro*. The morphological organization of this interdigitation of ECM and substratum surface differs considerably within the four material types examined. Furthermore, the degree of organized collagen bonding is greater on BAG than the other materials while Interpore 500™ exhibits a two-level interfacial organization governed by the plate-like microcrystallite morphology of its internal pore surface.

Acknowledgements

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Discussion with Reviewers

J. A. Gallagher: Some highly differentiated cells including osteoblasts exhibit a rapid loss of phenotype in culture. The authors have not taken any apparent steps to ensure that the cells which colonize these materials are osteoblasts or are capable of osteogenesis.
Authors: The loss of osteoblast phenotype in culture is a well known phenomenon and represents the major criticism of the majority of *in vitro* models used to assess osteoblast/biomaterial interactions. While it is not our purpose in this paper to prove recovery of osteoblast phenotype by the demonstration of the formation of a calcified extracellular matrix, we clearly reference, in the Introduction, previous work of ours where this was done. It is for this very reason that the model described is suitable for *in vitro* investigations of interfacial reactions on bone-substitute materials.

J. A. Gallagher: The value of this experimental approach is dependent on its capacity to reflect the interaction between tissue and implant *in vivo*. What data can the authors provide to support or validate this model?
Authors: See Beckham *et al* (1971), Jarcho (1981) and references cited in Hench & Wilson (1984) and Rout *et al* (1987). With specific regard to collagen interdigitation with the surface of bioactive glass, compare Figure 25 in Beckham *et al* (1971) with Figures 7a and 9 in Matsuda & Davies (1987).

J. A. Gallagher: Do the authors believe that this experimental model could distinguish between materials of differing bioactivities?
Authors: Yes, we demonstrate that this is the case in this paper. A comparison of the response of osteoblasts, using this model, to bioactive and non-bioactive glass is given in Davies *et al* (1987).

S. J. Jones: What changes occur at the bone surface below the onlay? In 4 weeks of culture, a "periosteum" has probably reformed, with the cells immediately in the bone (and, perhaps, bioactive overlay) being osteoblasts (and, perhaps, osteoclasts) whilst those in the multilayer above are "fibroblastic".
Authors: Much of our experimental evidence points to the assumption that at least two micro-environments are formed in this model system: one below the overlay, the other above. We refer to these as the ventral and dorsal surfaces of the overlay. We have seen no histological indication of a reforming periosteum on the ventral side. Although, the changes that occur depend upon the nature of the overlay material (Matsuda & Davies, 1987), we have observed the formation of tissue that microscopically resembles osteoid both on the bone surface and the ventral surface of the overlay itself.

S. J. Jones: What proportion of the cells were osteoblasts at the end of the culture period?
Authors: This model, which involves production of cell multilayers on the test material, is not amenable to cell counting. Enzymatic digestion followed by cell counting, which would be possible, would not enable a distinction to be made between osteoblasts or other cell types.

J. A. Gallagher: Do the authors have any information on the cellular responses evoked by these materials in explant cultures of tissues from non-osseous sites, for example skin? The responses observed may not be specific to bone cells but rather may be a general response expressed by matrix synthesizing cells. Perhaps the interaction of soft tissue fibroblasts with the substrates would serve as a useful control.
Authors: No. Bioactive glass, for example, is known to establish a soft-tissue bond with the tympanic membrane (Hench & Wilson, 1984). It may be of interest to examine the reactions of soft tissue fibroblasts to these materials, with other experimental end-points in mind, but these cells would not serve as a control population but simply a different cell population.

A. Maroudas: How do the differences in ECM structure relate to the comparative *in vivo* SEM studies, and the functional behaviour of these different implant materials?
Authors: We know of no comparative *in vivo* study which examines tissue reaction to these five materials. The *in vivo* functional behaviour of these materials is, separately, well documented (for example, Jarcho, 1981; Hench & Wilson, 1984 and references therein). It is difficult to gain information, from *in vivo* studies, of the relationship of fibrillar material directly at the interface with a bone-substitute due to the complexity of the *in vivo* environment, characterised by a heterogeneous cell population. Ito *et al* (1987) used several methods, including SEM, to compare the *in vivo* reaction of bone tissue to bioactive and silica glass. The materials were implanted in rabbit femora. Figure 5 is an SEM photomicrograph of a bioactive glass implant used in that study and may be compared to the present *in vitro* work in Figs.3(d) and(h).

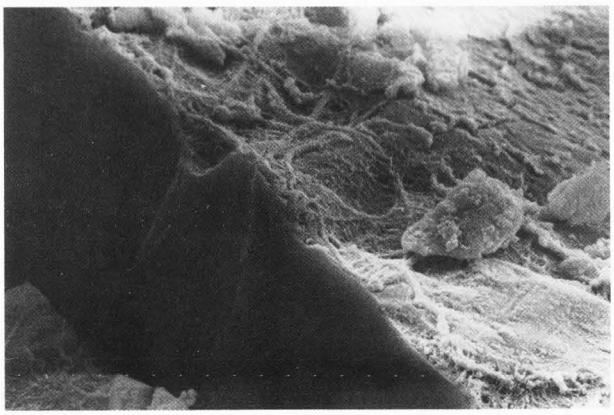

Figure 5. SEM photomicrograph of the fracture surface of a bioactive glass implant following an 8 week implantation in rabbit femur. The collagen fibre orientation on the glass surface may be compared with that demonstrated in Figs.3d & h. Field Width = 25 μm.

J. A. Gallagher: Have the authors considered the effects, on the bone substitution material, of incubation in tissue culture medium. It is conceivable that four weeks incubation in a protein-containing medium could alter the surface morphology, even in the absence of live tissue.

Authors: We have no evidence to suggest that proteins adsorbed to the surfaces of these substrata alter the morphology as examined at SEM level. Indeed we make specific reference to this with regard to the plate-like morphology of Interpore 500™.

U. Gross: What is the cause of the different appearance of fibrillar material on the 5 substrates?
Authors: We are unable to say, at this stage, what factors are specifically responsible for the wide variation in appearance of the fibrillar material but, as the fibrillar appearances on the five materials were reproducible, we assume the primary cause would be the physico-chemical nature of the material surface.

M. B. Engel: Do you think that surface charges on the substrates are important in determining the interaction between bone and the various biomaterials?
Authors: Yes, very important both as a general consideration for interfacial reactions at biomaterial surfaces (Davies, J.E. (1986) Exoemission for biomaterials research. Japan J. Appl. Phys. 24S24-4 43-47.) and in specifically affecting osteoblast behaviour. It is clear that both simple ionic and complex macromolecular species would be adsorbed on the material surface before cells colonize the material. However, we have shown that the surface charge of a material may dramatically affect cell behaviour even through the intermediary charge double layers and/or adsorbed molecules (Davies, J.E. *et al* 1986. The migration of osteoblasts over substrata of discrete surface charge. Biomaterials 7 231-233.).

U. Gross: Do you exclude that globular calcospheritic structures, made from apatite crystal precipitation from matrix vesicle calcification explain the globular structures demonstrated in Fig. 4b? Fibrils are entrapped in these globular structures. How do you explain this phenomenon?
Authors: We do not exclude the possibility of matrix vesicle calcification but we have not observed such structures in our TEM studies. Fibril entrapment is explained in the text.

M. B. Engel: Are non-collagenous macromolecules (proteins) involved in the adhesion of bone and substrate?
Authors: Non-collagenous macromolecules (proteins) are known to contribute to the collagen/apatite relationship in natural bone tissue and have aiso been implicated in the adhesion of collagen to bioactive substrata (Hench & Wilson, 1984).

U. Gross: What is the proof for the collagen nature of the fibrillar material?
J. A. Gallagher: Does the matrix that is laid down on these substrates have any specific characteristics of bone matrix in terms of collagen type or content of bone specific proteins? These questions could be addressed using immuno-probes or alternatively by solubilisation of the matrix followed by biochemical analysis.
S. J. Jones: Have the authors considered embedding the entire specimen and then doing block-face microscopy using TSM and SEM (BSE and CL) so that the interfaces are not disrupted? In conjunction with specific stains (eg., fluorescent antibodies to Type I, II and III collagens) the nature of the matrix and its possible mineralization could be investigated in the one specimen.

Authors: The typical morphology of collagen fibres is seen, for example, in Figs 3 e, f and h. We have also demonstrated collagen using transmission electron microscopy (Matsuda & Davies, 1987). We have not labelled either collagens or non-collagenous proteins to date although we feel that this is essential for future work, having now demonstrated that there are significant morphological differences to be explained.

Since we are particularly concerned with interfacial reactions, solubilisation of the matrix followed by biochemical analysis would not be a suitable method. Cells populate the overlays in multilayers, solubilisation would remove the spatial information we require.

We are currently preparing more specimens for block-face microscopy which we feel will add significant, but different, information. In the present work we have deliberately taken advantage of the exposure of the interface caused by critical point drying.

J. A. Gallagher: To what do the authors ascribe "the variability from one culture batch to the next"? Does this not bring into question the usefulness of this experimental model?
Authors: Of the many parameters which effect such variability, the most obvious are the irregular particle shapes and the differing contact geometry (with the host bone surface) that each particle will therefore exhibit. This presents a problem if one needs to compare the degree and rate of colonisation of different substrata. It does not bring into question the usefulness of the experimental technique in investigating the interfacial reactions described herein.