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Bioceramics composition modulate resorption of human osteoclasts

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Biomaterials used in bone regeneration are designed to be gradually resorbed by the osteoclast and replaced by new bone formed through osteoblastic activity. The aim of the present study is to analyze the role of osteoclasts in the resorption process. The attachment of human osteoclasts and the appearance of their resorption lacunae, when cultured on either the resorbable crystalline, calcium orthophosphate materials or on the long-term stable bioceramic material was investigated. The resorbable materials contain $Ca_{10}[K,Na](PO_4)_7$ (AW-Si) and $Ca_2KNa(PO_4)_2$ (GB14, GB9 & D9/25) as their main crystal phases, however they differ in their total solubility. These differences result from small variations in the composition. The long-term stable material consist of about 30% fluorapatite beside calcium zirconium phosphate ($Ca_5(PO_4)3F + CaZr_4(PO_4)_6$) and shows a very small solubility. AW-Si has an alkali containing crystalline phase, $Ca_{10}[K,Na](PO_4)$. While GB14, GB9 and D9/25 contain the crystalline phase $Ca_2KNa(PO_4)_2$ with small additions of crystalline and amorphous diphosphates and/or magnesium potassium phosphate (GB14). D9/25 and AW-Si is less soluble compared to GB14, and GB9 among the resorbable materials. Resorbable and long-term stable materials vary in their chemical compositions, solubility, and surface morphology. Osteoclasts modified the surface in their attempts to resorb the materials irrespective of the differences in their physical and chemical properties. The depth and morphology of the resorption imprints were different depending on the type of material. These changes in the surface structure created by osteoclasts are likely to affect the way osteoblasts interact with the materials and how bone is subsequently formed.

1. Introduction

Bone remodeling plays an important role in bone function and maintaining the mechanical integrity of the skeleton. Bone is formed by a series of complex events involving mineralization of extracellular matrix proteins regulated by particular cells (osteoblasts and osteoclasts) with specific functions of maintaining bone integrity [1]. New bone formation is required for the repair of bone defects, which is impaired in large defects. Limitation in the amount of the donor bone available, coupled with donor site morbidities led to the use of bone graft substitutes. Bioactive calcium phosphate ceramics and bioactive glasses are candidate biomaterials, which qualify as bone substitutes for maxillofacial and orthopaedic applications. These materials can provide an appropriate open cell porous structure, which enables the osteoblasts (OBs) to deposit bone [2]. Depending on their application, bone substitute materials need to be either highly resorbable as in the case of alveolar ridge augmentation, or long-term stable implants for use as coatings where more stable materials are desirable [3, 4]. All of these materials are biocompatible [2, 5, 6] and osteoconductive [2, 5]. However, they differ considerably in the rate of resorption, which may play a critical role in determining the quality of the bone formed.

Novel resorbable crystalline, calcium orthophosphate materials with stable crystalline phases of $Ca_2KNa(PO_4)_2$ that exhibit higher degree of solubility and biodegradability than the currently available materials have been developed [7]. Previously, we reported that these novel materials support osteoblastic cellular proliferation and differentiation to a great extent [4, 8]. Ideally, biomaterial surfaces

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should be conducive to cell interactions that balance osteoclastic and osteoblastic activity. To date, much of the research has been focused on studying the interaction of novel materials with osteoblasts and little information is available regarding the behavior of osteoclasts (OCs) on these materials [4, 8–10]. The complex signaling between the OCs and OBs occurs through the regulation of cell surface molecules and the stimulation of secretion of osteoclastogenic cytokines [11]. OCs are the multinucleated cells derived from hematopoietic stem cells and these cells are predominantly responsible for resorbing the bone mineral by acid secretion [12–14]. The Receptor activator of nuclear factor kB ligand (RANKL) is the necessary molecule to induce the differentiation of OCs [15], which is physiologically provided by OBs or bone marrow stromal cells. The discovery of the RANKL has made it possible to generate the OCs *in-vitro* without the support of osteoblastic stromal cells. Apart from RANKL other important factors such as $1,25(OH)_2$ vitamin D3, interleukins (IL)1- are involved in up-regulating OCs formation [16]. The present study analyses the appearance of the resorption lacunae and the attachment of human OCs cultured *in-vitro* on different types of novel resorbable and the long-term stable bioceramic surfaces, which vary in their chemical compositions, solubility, and surface morphology.

2. Materials and methods

2.1. Material preparation

Novel resorbable ceramic discs of 10 mm in diameter were produced by a melting process and subsequently sintered. Materials were either rapidly resorbable, as in the case of AW-Si, GB9, GB14 and D9/25, or long-term stable containing about 30% fluorapatite beside calcium zirconium phosphate, denoted as FA7Z. AW-Si has an alkali containing crystalline phase, $Ca_{10}[K,Na](PO_4)_7$. While GB14, GB9 and D9/25 contain the phase $Ca_2KNa(PO_4)_2$ with small additions of crystalline and amorphous diphosphates and/or magnesium potassium phosphate (GB14). Detailed description for the preparation of these materials has previously been described [7]. The discs were sterilized for cell culture, using ethylene oxide. Dentine, cut from a sperm whale tooth, was used as the positive control.

2.2. Human osteoclast cell culture

Peripheral blood mononuclear cells were isolated from the human buffy coats acquired from the Australian Red Cross Blood Service as described previously [17]. The cells were purified over the Ficoll-Paque (Nycomed, Norway) and centrifuged (1500 g, 30 min, room temperature, without brake). The cell layer on top of the Ficoll-Paque was collected, resuspended in 10 ml of warm Hanks balanced salt solution (HBSS) and diluted in 40 ml of HBSS and centrifuged at 1500 g for 15 min. Cells were counted using a hemocytometer and seeded at a concentration of 2×10^6 cells/ml onto the bioceramic discs and dentine (Gift from Australian Customs) placed in individual wells of 15 mm diameter 24 well tissue culture trays. Cells were left to adhere to the material at 37 °C, 5% CO_2 for 24 hrs and non-adherent cells were removed and replaced with fresh complete medium. Media was changed every 3 days and the monocytes were allowed to differentiate into functional OCs over a period of 17 days cultured in complete α-MEM consisting of α-Eagle Minimum Essential Medium (α-MEM) in 10% FCS, 5 μg/ml Penicillin, 50 U/ml of streptomycin (Sigma-Aldrich) and 1% L-Glutamine (Invitrogen) supplemented with 10^{-8} M Dexamethazone, (Fauldings, Adelaide) 10^{-8} M Vitamin D3 (Waiko, Japan) and M-25 ng/ml of Macrophage Colony Stimulating Factor (Chemicon, California) for the first 7 days. 100 ng/ml recombinant human RANKL (Research Diagnostics, New Jersey) was added for the following 10 days.

2.3. Staining for tartrate resistant acid phosphatase (TRAP)

The differentiated OCs were identified by staining the cells on the coverslips for TRAP. The cells were fixed with 4% (v/v) glutaraldehyde in HBSS for 10 min and stained for TRAP positive using the commercially available leukocyte acid phosphatase assay (Sigma – Aldrich) as per manufacturers' instructions. The coverslips were counter stained with 0.5% (w/v) methyl green in distilled water.

2.4. Cell attachment

The attachment of the OCs to each disc was evaluated after 17 days using scanning electron microscopy (SEM). Cells cultured on the biomaterials were rinsed three times in phosphate buffered saline (PBS) and fixed in 1.25% (v/v) glutaraldehyde, 4% (w/v) paraformaldehyde, and 4% (w/v) sucrose in PBS, for 1 h. The fixative was removed by washing with buffer containing 4% (w/v) sucrose in PBS and post fixed in 1% osmium tetroxide in PBS. The discs were rinsed with PBS after osmium fixation and sequentially dehydrated in graded ethanol and dried in critical point dryer.

2.5. Resorption lacunae analysis

Resorption pits/imprints excavated by the OCs on the discs and dentine were examined by the SEM. Cells were trypsinized and removed from the discs and dentine. The discs were washed 3 times in PBS and dried at room temperature. A thin layer of carbon was coated onto the samples prior to examination using a Philips XL-20 SEM operated at 10 kV.

3. Results

3.1. Osteoclast Formation

Human OCs were cultured *in-vitro* by isolating the normal human blood monocytes from buffy coats as previously described [17]. The formation of the OCs was confirmed by the expression of TRAP on the coverslips as well as analysis of the pits excavated by the OCs on

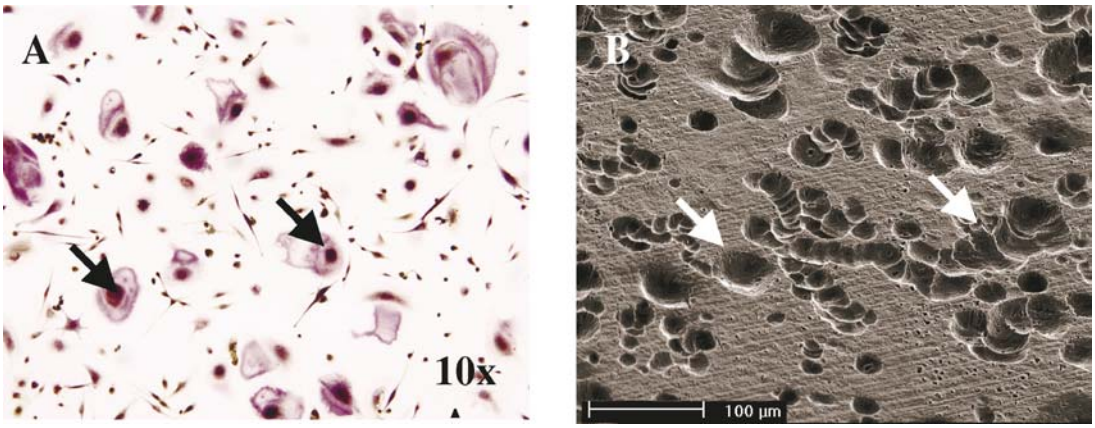

Figure 1 Formation of OC was confirmed by the expression of Tartrate Resistant Acid Phosphatase (TRAP) on the coverslips and resorption pits on dentine. (A) TRAP positive staining illustrating the formation of multinucleated osteoclasts (OCs) at day 17 (arrow). (B) OCs resorbed 75–80% of dentine indicating the functional activity of OCs (arrows denotes the resorbed area).

dentine at the end of day 17. This confirmed that the mononuclear cells from the buffy coats appeared to fuse and differentiate into mature OCs (Fig. 1(A)).The resorption pits were detected on dentine at the end of day17 and SEM images revealed that 75–80% of the area had clearly visible resorption pits (Fig. 1(B)).

3.2. Biomaterial resorption

SEM examinations revealed that the human osteoclasts cultured for 17 days on various material produced various patterns of resorption lacunae. The differences and the extent of degradation varied for different materials. The degraded areas, as a function of OCs, for all the five different types of materials were clearly distinguishable from the unresorbed areas. The native AW-Si, showed a loosely packed morphology (Fig. 2(A)),while the OC cultured on them excavated the surface superficially and produced resorption pattern resembling cracked ridges and crevices (Fig. 2(B)).

The native surfaces for GB9, GB14 and D9/25 have a similar morphological appearance in the form of large and well-connected granular matrix [(Fig. 2(C), (E) and (G)]. Resorptive function of the OCs on these surfaces also varied remarkably; GB9 showed some cracks and deeply etched resorption areas (Fig. 2(D)) whereas GB14 showed deep excavated lacunae (Fig. 2(F)). D9/25 exhibited clusters of small pits (Fig. 2(H)).

SEM micrographs of the native FA7Z showed surface morphology with tightly bound granules forming a smoother surface compared to the granular matrix of resorbable materials (Fig. 2I). Multinucleated OCs formed smooth circular imprints of resorption on FA7Z (Fig. 2(J)).

3.3. Osteoclast attachment on biomaterial

Osteoclasts attached to all five novel materials, regardless of the differences in the composition and the surface morphology, however there are differences in the pattern of OCs attachment to each of these materials. OCs attached to AW-Si are globular (Fig. 3(A)) with lamellipodia clearly visible but the size of OCs were small compared to the ones cultured on other resorbable materials.

OCs cultured on GB14 (Fig. 3(D)) and D9/25 (Fig. 3(B)), were large and extended compared to an elongated morphology when OCs were cultured on GB9 (Fig. 3(C)).

On FA7Z the multinucleated cells were large, flat and extended with well-defined peripheral hairy brush border (Fig. 3(E)). The cell surface around the multinucleation was smoother compared to the OCs on resorbable materials. OCs cultured on dentine (the control material) had a hairy lamellipodia around the resorption pits (Fig. 3(F)).

4. Discussion

Mimicking natural bone is a huge challenge faced by the biologists and material scientists alike. The success of bone substitute materials for skeletal tissue applications lies in the system where healthy bone remodeling occurs in contact with the biomaterial. Ideally this occurs when OCs resorb the material and then allow OBs to generate new bone.

The resorption cycle is a complicated coordinated series of events during which a moving OCs arrives to resorption site, attaches tightly to bone surface, dissolves the bone matrix, detaches and migrates to another resorption site. Resorbing OCs adhere to the bone through the sealing zone forming the ruffled border, which is involved in the dissolution of mineralized bone matrix [18]. Therefore, the design of the bone substitute materials should facilitate the cascade of the resorption cycle and the cellular mechanism to recognize an optimum bone substitute material.

The current study demonstrated that cultured human OCs attach and degrade the bioceramic materials in different forms. Various parameters are likely to influence material degradation: Chemical composition [19, 20], solubility [21], surface energies [22], surface morphology [23], crystalline structure, size and shape of the biomaterial particulate [24]. Materials used in this study varied largely in their chemical composition, solubility and the surface morphology. All the five novel materials tested here showed signs of OC activity, irrespective of the differences in their physical and chemical properties, suggesting a role played by the underlying

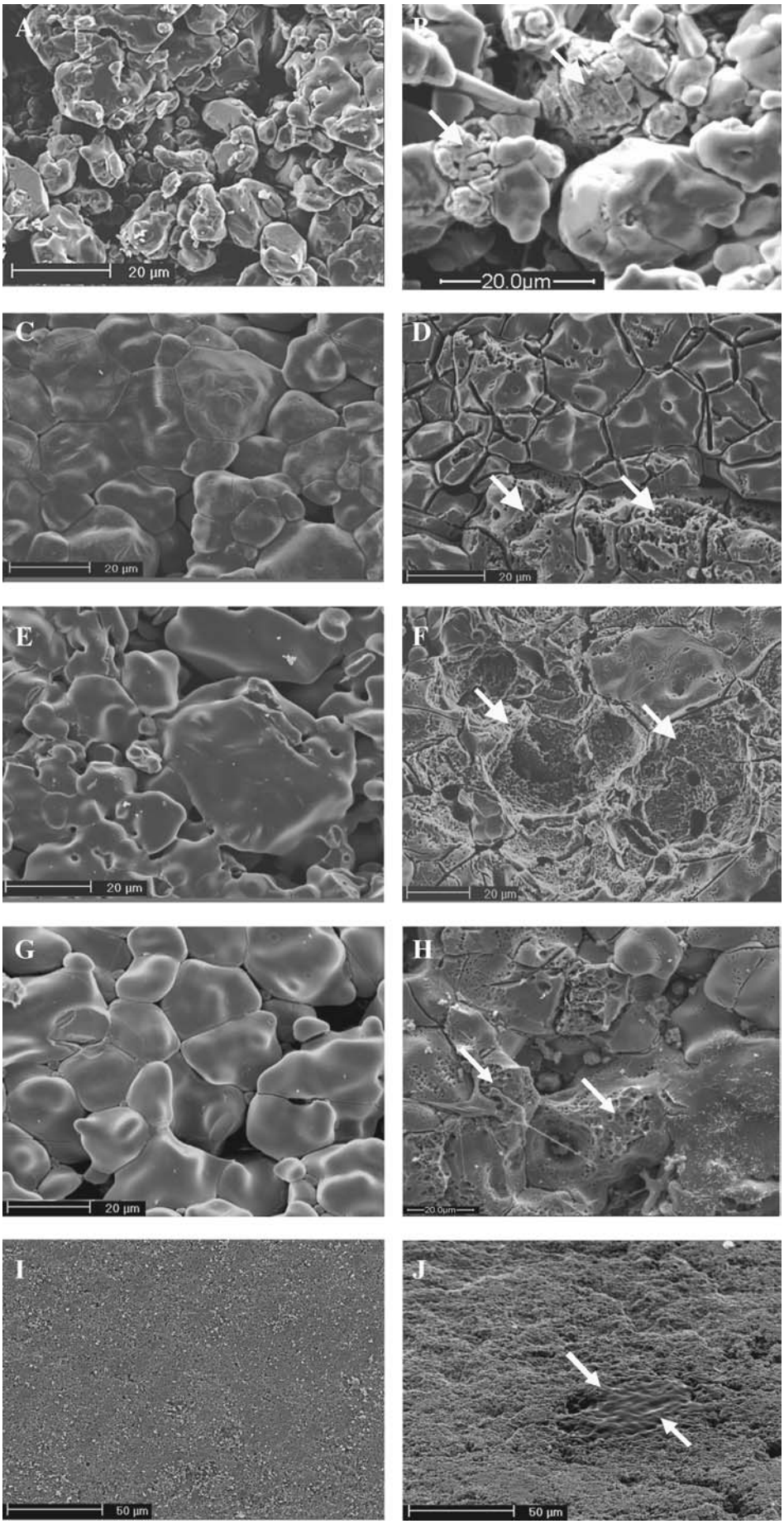

Figure 2 Summary of Scanning Electron Micrographs showing the surface morphology of novel materials prior to cell culture and comparing the change in morphology due to the functional activity of the osteoclast cultured for 17 days (A) Surface morphology of AW-Si (B) Cracked ridges of resorption on AW-Si (C) Surface morphology of GB9 (D) Cracks and etched resorption imprints on GB9 (E) Surface morphology of GB14 (F) Deep resorption lacunae on GB14 (G) Surface morphology of D9/25 (H) Clusters of small resorption pits on D9/25 (I) Surface morphology of FA7Z (J) Smooth patches of resorption on FA7Z (J).Arrows indicate the resorbed areas on each of the materials.

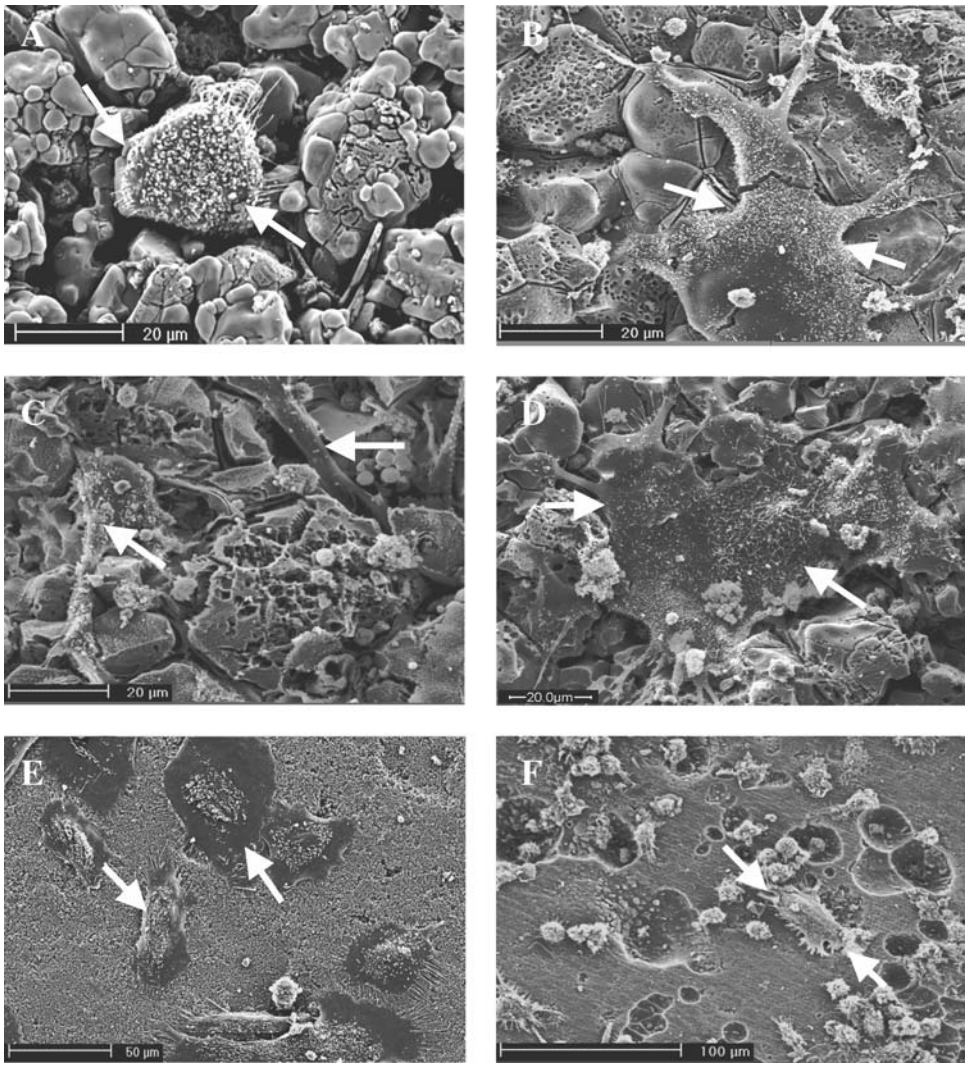

Figure 3 Summary of the Scanning Electron Micrographs showing the osteoclasts attachment on each of the material (A) globular forms of osteoclasts attachment on AW-Si (B) Large and extended osteoclasts attached to D9/25 (C) Large and elongated OC attached to GB9 (D) Large and extended OC on GB14 (E) OCs Flattened on FA7Z (F) OC around the resorption pits on Dentine. Arrows denote the multinucleated cells attached to the novel materials.

substrata in dictating how OCs function. The successful culture of the human OCs on these materials suggests that the composition and their surface morphology are conducive to the differentiation of the monocytes into functional human OCs. However, each of these materials exhibited differences in the OCs attachment and resorption pattern as a result of OCs activity, suggesting that surface morphology, composition and solubility play a major role in regulating OCs activity. SEM micrographs of AW-Si showed shallow resorption imprints with cracked ridges and crevices compared to the deeper craters and etchings observed on GB14 and GB9. AW-Si varies from the other resorbable materials in its crystallinity phase and solubility (Table I), suggesting that the crystalline phase combined with lower solubility may have caused poor dissolution of the calcium phosphate in the acidic microenvironment where OC attach. This phenomenon may have resulted in the formation of the shallow ridges of resorption imprints on AW-Si. GB14, GB9 and D9/25 share a similar crystalline phase but vary largely in their solubility (Table I),this difference in the solubility may have influenced the OCs to resorb the underlying substrata in various patterns. GB14 and GB9, showed deeper craters and etchings where as D9/25 showed clusters of small resorption pits indicating that the main crystalline phase of $Ca_2KNa(PO_4)_2$ with a higher solubility is more conducive to the OCs activity compared to the crystalline phase of $Ca_2KNa(PO_4)_2$ with a low solubility. Similarly OCs cultured on the long-term stable material (FA7Z) formed smooth and shallow resorption imprints instead of deeper craters also indicating that low solubility of FA7Z may have inhibited the OCs to resorb deeply. The variations in the resultant degradation pattern observed on the bioceramic materials indicate that the

TABLE I Crystalline phases and solubilities of novel materials

Sample code	Crystalline main phase	Solubility[a] (mm/g)
GB14	$Ca_2KNa(PO_4)_2$	19 ± 2
GB9	$Ca_2KNa(PO_4)_2$	30 ± 3
D9/25	$Ca_2KNa(PO_4)_2$	9 ± 2
AW-Si	$Ca_{10}[K,Na](PO_4)_7$	15 ± 2
FA7Z	$Ca_5(PO_4)3F + CaZr_4(PO_4)_6$	0.35 ± 0.05

[a]Determined according to DIN EN ISO 10993-14.

OCs activity may be related to the combined effect of material composition and solubility.

Apart from solubility, supplementation of the material with trace elements can induce substantial effect on OCs as others reported that material composition contribute significantly to the cell-material interaction [20, 21]; hence it is possible that the phase composition of the long-term stable and resorbable materials may have influenced the variations in the degradation patterns observed on the different materials. Zreiqat *et al*. [25, 26] reported that human bone-derived cells grown on the biomaterials implanted with magnesium ions enhanced their proliferation, differentiation [27, 28] whereby higher levels of integrin receptors were also expressed on the magnesium modified substrata [29]. Hence, it is plausible to relate the presence of magnesium phosphate in the composition of GB14 to the enhanced osteoclastic resorptive activity.

Similarly, the long-term stable material FA7Z has been supplemented with 30% of fluorapatite with traces of calcium zirconium phosphate. Fluorapatite provides long-term mechanical stability and helps in modifying the physiochemical and mechanical properties of the material [30]; an important factor for the optimum function of the long-term stable materials. Inclusion of zirconium to enhance the mechanical strength of the material as well as its fracture toughness has been previously reported [31]. Furthermore, Zreiqat *et al*. [26] demonstrated that bone cells cultured onto zirconia exhibited enhanced attachment compared to other bioceramics suggesting an important role of zirconia in bone cells adhesion.

Sabokbar *et al*. (1997) reported that the addition of zirconium di oxide (ZrO_2) to particles of polymethyl methacrylate increased TRAP expression and bone resorption in a system where mouse monocytes were co-cultured with the osteoblast like cells on bone slices [32]. Hence it is possible to speculate that the presence of zirconia in FA7Z may augment cellular activity to some degree despite low solubility indicating the influence of material composition on OCs resorption. Besides the chemical composition and solubility of the substrate, surface morphology may also contribute significantly to cellular behavior [33], particularly osteoclast attachment. The intricacies of cytoskeletal structure, protein binding, integrins and the physiological roles of intracellular signals linked to attachment would be affected by the surface morphology [34–36]. Generally OCs attach to bone surface and form a tight sealing zone which encloses the resorption lacuna where it secretes acid and erodes the surface [37, 38]. The surface morphology of the materials can have significant effect on the OCs to attach, spread and organize the cytoskeletal structure in order to trigger the complex process of resorption.

Monchau *et al*. [19] reported that tight OCs attachment is needed to create a contact with the mineralized surface and to build up a resorption area. SEM micrographs showed various forms of cellular attachment on each of the bioceramic materials tested in this study, a difference that can be attributed to surface morphology. AW-Si has a surface morphology with loosely packed, small granules whereas GB14, GB9 and D9/25 exhibit surface morphology with large and well-connected granules. FA7Z, which is a long-term stable material, has granules that are tightly bound forming a smoother surface compared to the granular matrix of resorbable materials.

Although AW-Si shows OCs attachment, the loosely packed small granular surface may have resulted in the inhibition of OCs spreading and the formation of a tight sealing zone, hence possibly minimizing the resorptive activity of the cell. This loosely packed granules may also have attributed to the cracked resorption imprint. Furthermore, reduced number of resorption sites (data not shown) was observed on AW-Si indicating that the loosely packed surface morphology may have limited OCs migration. Multinucleated cells were large and extended on GB14, GB9 and D9/25 indicating that large and well connected granular surface is likely to have assisted the OCs to spread and attach to the surface, thereby influencing the OCs activity.

OCs on FA7Z were large and flattened with characteristic hairy brush border and seemed to have attached tightly indicating that the surface morphology may have been amiable for cellular attachment. Smooth patches of OCs resorption found with the imprint margin were clearly evident. Langstaff *et al*. (2001) reported that the regular margins of the resorption pits correspond closely to the size and the shape of the ruffled border normally produced by the OCs in the resorptive phase [23]. Due to the characteristic cellular attachment with peripheral hairy brush border observed on FA7Z it is possible to remark that surface morphology complemented with trace elements in the phase composition is likely to support the resorptive activity of OCs in forming resorption imprints. However, due to the low solubility of FA7Z OCs may have only "smoothed the surface" instead of degrading it with deeper craters or etchings.

Studies presented here show that the chemical composition, solubility and/or surface morphology of the material may play a significant role in regulating the osteoclastic response. It is important to establish the co-ordinated effect of chemical composition, solubility and surface morphology to enhance the optimum function of biomaterials. Great attention has been devoted to study the mechanism by which biomaterials affect cellular behavior. We and others demonstrated that osteoblasts-like cell interactions with implant surfaces *in vitro* [29, 39, 40] and *in vivo* [41] are mediated by adhesion receptors belonging to the integrin superfamily. Integrins recognize binding domains within proteins of the extracellular matrix [42]. Moreover, integrins have been suggested to play a critical role in OCs activity by mediating their adhesion, thus regulating cytoskeletal organization required for both osteoclastic cell migration and formation of the sealing zone [37, 43]. Therefore, further exploration of the materials-dependent effects reported here should involve the study of cell adhesion mechanisms, which lead to the observed differences in osteoclastic behavior resulted by the different bioceramics.

5. Conclusions

The study show that human osteoclast attach and degrade the tested materials irrespective of the differences in their compositions and surface morphology. The different forms of OCs attachments and the resultant materials degradation imply that surface composition, solubility and morphology is likely responsible for this difference. Further studies relating to signaling pathways involved in OCs interaction with these bioceramics can give insight into the mechanisms that dictates OCs interaction with the material and how surface properties such as morphology, chemical composition and solubility modulate this interaction.

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