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Effect of rapidly resorbable bone substitute materials on the temporal expression of the osteoblastic phenotype *in vitro*

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Abstract: Ideally, bioactive ceramics for use in alveolar ridge augmentation should possess the ability to activate bone formation and, thus, cause the differentiation of osteoprogenitor cells into osteoblasts at their surfaces. Therefore, in order to evaluate the osteogenic potential of novel bone substitute materials, it is important to examine their effect on osteoblastic differentiation. This study examines the effect of rapidly resorbable calcium–alkali–orthophosphates on osteoblastic phenotype expression and compares this behavior to that of β-tricalcium phosphate (TCP) and bioactive glass 45S5. Test materials were three materials (denominated GB14, GB9, GB9/25) with a crystalline phase $Ca_2KNa(PO_4)_2$ and with a small amorphous portion containing either magnesium potassium phosphate (GB14) or silica phosphate (GB9 and GB9/25, which also contains $Ca_2P_2O_7$); and a material with a novel crystalline phase $Ca_{10}[K/Na](PO_4)_7$ (material denominated 352i). SaOS-2 human bone cells were grown on the substrata for 3, 7, 14, and 21 days, counted, and probed for an array of osteogenic markers. GB9 had the greatest stimulatory effect on osteoblastic proliferation and differentiation, suggesting that this material possesses the highest potency to enhance osteogenesis. GB14 and 352i supported osteoblast differentiation to the same or a higher degree than TCP, whereas, similar to bioactive glass 45S5, GB9/25 displayed a greater stimulatory effect on osteoblastic phenotype expression, indicating that GB9/25 is also an excellent material for promoting osteogenesis. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 00A: 000–000, 2007

Key words: bone substitute materials; calcium–alkali–orthophosphate ceramics; SaOS-2 cells; osteoblast differentiation; bioactive glass

INTRODUCTION

The use of dental implants has become a common treatment to replace missing or lost teeth.[1] However, when teeth are missing, the natural resorptive process subsequent to extraction frequently results in an alveolar ridge with deficient bone volume.[1–3] In addition, the fabrication of an esthetically and functionally successful implant prosthesis generally can be accomplished only if the implants are placed in the ideal position with regard to the anticipated restorative design.[1,2] As the implant should ultimately represent the apical extension of an optimal prosthetic superstructure, the implant position should primarily be determined by the planned, future prosthesis, and not solely by bone anatomy.[1,2] Thus, augmentation of the alveolar ridge before implant placement is frequently required in implant dentistry.[1–3]

The current gold standard for bone reconstruction in implant dentistry is using autogenous bone grafts.[4–6] Among the various techniques to recon-

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struct or enlarge a deficient alveolar ridge, the concept of guided bone regeneration (GBR)[7] has become a predictable and well-documented surgical approach.[4] The need for localized ridge augmentation prior to the placement of dental implants has been one of the clinical indications for GBR.[7] At present, autogenous bone grafts are preferably combined with barrier membranes.[4,5] These autografts have been used to reduce the defect volume, thereby stabilizing the blood clot, and to support the membrane as a space-maintaining device, thus preventing their collapse into large defects.[4,7] Furthermore, augmentation of the maxillary sinus floor with autogenous bone grafts has become a well-established pre-implantology procedure for alveolar ridge augmentation of the posterior maxilla.[8] The main disadvantages of autogenous bone grafts have been the need for an additional surgical site, increased donor site morbidity, insufficient volume of (intraorally) harvested bone, and the need to use general anesthesia for extraoral bone harvesting.[9–12] Using biodegradable bone substitutes as a membrane-supporting device would simplify GBR, since it avoids second-site surgery for autograft harvesting.[4,5] This is also true for sinus floor elevation procedures.[9–12]

A bone substitute material for alveolar ridge augmentation must be rapidly resorbable and should undergo remodeling and complete substitution by newly formed functional bone tissue in view of placing dental implants in such augmented sites.[5,13,14] Alloplastic bone substitute materials are superior to freeze-dried human allografts because of their safety in terms of disease transmission and immunological aspects.[5,12]

Recently, the use of tricalcium phosphate (TCP) and bioactive glass (Bioglass 45S5) particles as alloplastic bone graft materials for alveolar ridge augmentation and sinus floor elevation procedures has received increasing attention in implant dentistry.[6,14–19] With β-TCP, biodegradation has been reported to be incomplete 9.5 months after grafting in the human mandible. Histologic examination of these biopsies revealed that 34% of the biopsy consisted of mineralized bone tissue and 29% of remaining β-TCP.[16] Biopsies sampled at 6 months after sinus floor augmentation consisted of 38% mineralized bone and between 8 and 26% remaining β-TCP.[19] With respect to bioactive glass 45S5 (BG) particles of a narrow size range, Tadjoedin et al. reported that, after grafting in the human sinus floor, BG particles appeared to resorb within 1–2 years.[15] This was by dissolution rather than by osteoclastic activity.[15] The volume of the biologically transformed BG particles in the biopsies decreased from 29% at 4 months to 15% at 6 months and 8% at 15 months.[15] Thus, compared with the bone substitute materials that are currently clinically available,[14–19] there is a significant need for bone substitute materials that degrade more rapidly, but still stimulate osteogenesis at the same time.[5,13,14] Thus considerable efforts have been undertaken to produce rapidly resorbable bone substitute materials, which exhibit good bone bonding behavior by stimulating enhanced bone formation at the interface in combination with a high degradation rate. This has led to the development of a series of novel, bioactive, rapidly resorbable glassy crystalline calcium–alkali–orthophosphate materials.[20–22] These are glassy crystalline calcium–alkali–orthophosphates, which exhibit stable crystalline $Ca_2KNa(PO_4)_2$ or $Ca_{10}[K/Na](PO_4)_7$ phases.[20–23] These materials have a higher solubility than TCP. They are designed to exhibit a higher degree of biodegradability compared to TCP[20–22] and, therefore, could be excellent alloplastic materials for alveolar ridge augmentation. Another approach to increase the solubility and biodegradability of calcium-orthophosphates is by adding more phosphates, resulting in the formation of crystalline or amorphous diphosphates. Diphosphates have a higher solubility than orthophosphates and $Ca_2P_2O_7$ is transiently formed *in vivo* during the mineralization process of the bone matrix.[24] A number of *in vitro* and *in vivo* studies, which examined calcium phosphates to which diphosphates were added, yielded favorable results regarding their potential to regenerate bone.[25,26] Therefore, a calcium–alkali–orthophosphate ceramic was recently developed, which also contains a small portion of diphosphates ($Ca_2P_2O_7$).[22] Furthermore, three-dimensional scaffolds can be fabricated from these calcium–alkali–orthophosphate ceramic materials[27] (with and without addition of diphosphates), which is advantageous for tissue engineering purposes.

Ideally, bioactive biomaterials for use in bone regeneration should possess the ability to activate bone formation and, thus, cause the differentiation of osteoprogenitor cells into osteoblasts at their surfaces. Therefore, in order to evaluate the osteogenic potential of novel implant materials, it is important to examine the effect of these materials on osteoblastic differentiation. Consequently, over the last decade, various cell culture assays have been developed which permit studying the effect of biomaterials on the expression of markers of the osteoblast phenotype *in vitro*.[28–32] These *in vitro* assays have proven valuable for screening novel biomaterials, since they facilitate gaining insight into how osteoblastic cell differentiation is influenced by endosseous implant materials.

This study examines the effect of novel rapidly resorbable calcium–alkali–orthophosphates when compared with currently clinically used materials (β-TCP and bioactive glass 45S5) on the expression of an array

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TABLE I
Composition of Novel Calcium–Alkali–Orthophosphate Ceramic Bone Substitute Materials, TCP, and Bioactive Glass 45S5

	Composition (wt %)						
Material	CaO	P_2O_5	Na_2O	K_2O	MgO	SiO_2	$Ca_3(PO_4)_2$
GB14	30.67	43.14	9.42	14.32	2.45		
GB9	32.25	40.81	8.91	13.54	2.57	1.92	
GB9/25	29.25	44.81	8.41	13.04	2.57	1.92	
352i	40-45	45-48	5-8	1-2	1-1.5	1-2	
TCP							100
BG	24.5	6.0	24.5			45.0	

TCP, tricalcium phosphate; BG, bioactive glass 45S5.

of osteogenic markers by human osteoblast-like cells (SaOS-2).

MATERIALS AND METHODS

Test materials

Four novel rapidly resorbable calcium–alkali–orthophosphate ceramic bone substitute materials[21–23,31,33] were studied in comparison to currently clinically used synthetic bone substitute materials (β-TCP, bioactive glass 45S5). Table I summarizes their composition. These materials are glassy-crystalline materials with a higher solubility than β-tricalcium phosphate and their main crystalline phase is either the new phase $Ca_2KNa(PO_4)_2$[20–23] or $Ca_{10}[K/Na](PO_4)_7$.[22] Test materials were three materials with the crystalline phase $Ca_2KNa(PO_4)_2$ and with a small amorphous portion containing either magnesium potassium phosphate (material denominated GB14) or silica phosphate (materials denominated GB9 and GB9/25, GB9/25 also contains $Ca_2P_2O_7$); and a calcium phosphate material with the crystalline phase $Ca_{10}[K/Na](PO_4)_7$ (material denominated 352i).[20–22] Preparation of GB9 and GB14 has been described in detail elsewhere.[20,21] Importantly, GB9/25[22] also contains a small portion of crystalline and amorphous diphosphates ($Ca_2P_2O_7$), unlike GB9, which does not.[20,21,31,33] Crystallization occurs spontaneously from the melt, and thus, the bioceramics can easily be fabricated. The dissolution rate of the calcium–alkali–orthophosphates depends on the amount of added MgO, SiO_2 and P_2O_5. In a simulated physiological solution (0.2*M* Tris-HCl), GB14, GB9, GB 9/25, and 352i dissolve faster than TCP.[20–22] GB14, GB9, GB9/25, and 352i discs were made using reagent-grade $CaHPO_4$, Na_2CO_3, K_2CO_3, $MgCO_3$, SiO_2, and H_3PO_4. These compounds were mixed, and a melt was formed in a platinum crucible at about 1550°C for 2 h. The material was cast and crushed to produce granules. β-TCP, GB14, GB9, GB9/25, and 352i specimens were prepared by compressing granules (grain size 40 μm) followed by sintering to form 10-mm diameter discs, as described previously.[31,33,34]

Bioactive glass 45S5 (BG) discs, 12 mm in diameter and 1-mm thick, were cut from cast rods (MO-SCI, Rolla, MO) using a slow-speed diamond wheel saw (South Bay Technology, Clemente, CA) and acetone as a coolant. After cutting, discs were polished with 600- and 800-grit silicon carbide papers and then ultrasonically cleaned, first in acetone and then in ethanol. A layer of carbonate apatite (c-Ap) was formed on the BG surface using a treatment in Tris buffered solution (pH 7.4 at 37°C) supplemented with electrolytes typical for plasma (TE),[35] as described recently by Radin et al.[36] In brief, each disc was placed in a separate vial and immersed at a BG surface-to-solution-volume ratio of 0.1 cm^{-1}. The vials were placed on a shaker table in a water-jacketed incubator (Thermo Forma) at 37°C with a humidified atmosphere containing 5% CO_2. The immersion time was 3 days. Subsequently, the discs were rinsed with ethanol, dried, and stored in a desiccator.[36] This procedure resulted in the bioactive glass surface to be fully and uniformly covered by a layer of fine, densely packed precipitates composed of crystalline carbonate calcium phosphate apatite as evidenced by SEM/EDX/FTIR analyses as previously described.[36] Prior to cell seeding, BG discs were sterilized by immersion in ethanol for 30 min followed by exposure to UV-light for 30 min. This was followed by immersion in tissue culture medium for 2 h at 37°C in order to facilitate serum protein adsorption as previously described.[28,35,36] Previously it was shown by El-Ghannam et al.[28,37] that this type of pretreatment (creation of a c-Ap layer and subsequent immersion in tissue culture medium prior to cell seeding) resulted in the greatest stimulatory effect of Bioglass specimens on osteoblast differentiation, and thus lead to more enhanced osteoblast differentiation compared with untreated Bioglass discs or BG discs solely immersed in tissue culture medium. The ceramic specimens were sterilized at 300°C for 3 h. Phase transformations do not occur below 600°C.

Surface roughness measurements

The surface roughness of the various specimens was characterized by profilometry using a Hommel T 8000 Surface Profile Measuring System with a diamond stylus (Hommel, Germany). Parameters used to quantify surface roughness were R_a (the arithmetic mean of departures of the roughness profile from the mean line) and R_z (the average of five consecutive values of roughness height,

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which is defined as the distance between the top of the highest peak and the bottom of the deepest valley).

Scanning electron microscopy

The surface morphology of the various specimens was characterized by scanning electron microscopy (SEM). SEM analysis of the different bioceramic surfaces was performed after fabrication and after the preincubation treatment in fully supplemented cell culture medium. Preparation for SEM analysis included rinsing the different samples three times in 0.1*M* cacodylate-buffered solution, pH 7.2, and fixing in 4% glutaraldehyde in 0.1*M* sodium cacodylate-buffered solution at 4°C for 15 min. Subsequently, the specimens were washed with cacodylate buffer 0.1*M*, pH 7.2, three times and dehydrated in ascending concentrations of ethanol, viz. 30, 50, 70, 80, 90, and 96%, finally immersed in absolute ethanol for 10 min each, after which the specimens were immersed for 10 min each in three baths of hexamethyldisilazane (Sigma, St. Louis, MO). Each specimen was then air-dried for 24 h. The dried specimens were glued onto aluminium stubs, sputter-coated with gold, and examined in a CamScan MaXim at an accelerating voltage of up to 20 kV.

Cell cultures

The SaOS-2 human osteoblast-like osteogenic cell line,[38,39] derived from a human osteosarcoma, was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were cultured in a modified McCoy's 5A medium (Sigma-Aldrich, Taufkirchen, Germany) containing 10% FCS (Gibco, Paisley, UK), 1% penicillin (10,000 U)/streptomycin (10 mg/mL, Gibco), 1% 200 m*M* L-glutamine (Gibco), and 0.1*M* ascorbic acid phosphate magnesium salt (Wako Pure Chemicals, Osaka, Japan) and maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The culture medium was changed three times per week. When cells reached confluence, a trypsin-EDTA solution (0.5 g/L trypsin and 0.2 g/L EDTA, Gibco) was used to detach cells from the bottom of the culture flasks, and 1/3 of the total cells were transferred into a new tissue culture flask. These osteogenic human cells (SaOS-2) were used for the cell culture experiments described later.

Cellular quantitative immunocytochemistry assay

All calcium phosphate ceramics were preincubated in 500 μL of culture medium for 24 h without cells. Previously, it was demonstrated that preincubation of calcium–alkali–orthophosphate ceramics in cell culture medium for 24 h prior to cell seeding resulted in greater bone nodule formation compared to seeding cells on the native ceramic specimens.[33,34] The bioactive glass specimens were immersed in tissue culture medium and incubated for 2 h, as described earlier. SaOS-2 cells of the 5th to 8th passage were seeded at a density of 8.49×10^4 cells/cm^2 on the different substrates (10 specimens per material) and cultured for 3, 7, 14, and 21 days. A modified version of the cellular quantitative immunocytochemistry assay described by Wang et al.[32] was used to quantify the intracellular protein expression of an array of osteogenic markers characteristic of the osteoblast phenotype. In brief, at the predetermined time-points, cells were harvested from the test surfaces by trypsinization using 0.02% trypsin/0.02% EDTA in phosphate-buffered saline solution (PBS) and counted with a hemocytometer as described previously.[29,31,32] A prescribed aliquot of cells (1×10^4) was placed into wells of 96-well plates and centrifuged at 1000 rpm for 10 min. The supernatant from each well was vacuum-aspirated using a fine needle and cells were dried to the plates for 30 min in a fan-forced incubator at 37°C. Cells dried to the plates were fixed by immersing the entire plates for 1 min in methanol:acetone (9:1) at room temperature as previously described.[29,31,32] Fixation of cells was required in order to avoid loss of cells during the detection protocol. Subsequently, cells were air-dried and then washed in two changes of PBS. This was followed by incubation in 0.25% Triton 100-X (ICN, USA) and 0.25% Nonidet NP40 (Sigma) in PBS for 5 min to permeabilize the cell membrane. This solution was decanted and cells were washed in two changes of 0.05% (v/v) Triton 100-X in PBS. In order to reduce nonspecific binding, 50 μL of the blocking solution consisting of 2% (w/v) bovine serum albumin, heat inactivated (Sigma-Aldrich) in Hanks' balanced salt solution (Sigma-Aldrich), was added to each well, and the plates were incubated for 20 min at room temperature in a humidified chamber. The blocking solution was then decanted and the wells drained, before the monoclonal or polyclonal antibodies were added. The expressed intracellular proteins were detected using monoclonal antibodies for ALP (Sigma), OC (Santa Cruz), as well as polyclonal antibodies for Col I (LF-39), OP (LF-124), ON (BON-I), and bone sialoprotein (BSP, LF-83).[40] The polyclonal antibodies used in this study were generously provided by Dr. Larry Fisher (NIDCR, Bethesda, MD).

The presence of a biotin label on the $F(ab)_2$ fragment of the secondary antibody was quantitated using a one-step application of a soluble complex of streptavidin and biotinylated alkaline phosphatase[41] (Dako Cytomation, Denmark) and, subsequently, visualization by the p-NPP assay.[41] An amount of 5 m*M* of levamizole was added to inhibit endogenous ALP activity. Quantitation was performed by measuring the optical density of the yellow color (p-NP), and it was read by a SPECTRA MAX 340PC plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. The results were normalized to the internal control β-actin protein.[29,31,32]

Statistical analysis

Two runs of experiments were performed and assays were run in quadruple. The measurements from the two different experimental runs were pooled. To compare the different substrata to each other, multiple testing was performed using Student's *t*-test, which included applying the Bonferroni-adjustment according to the number of tests performed. Significance was assumed achieved for $p < 0.05$.

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TABLE II
Surface Roughness of the Different Bioceramics Examined

	Surface Roughness	
Biomaterial	R_z, Mean (μm)	R_a, Mean (μm)
TCP	13.89	2.30
GB14	13.03	2.17
GB9	13.37	2.21
GB9/25	13.24	2.18
352i	13.43	2.22
BG	4.02	0.43

TCP, tricalcium phosphate; BG, bioactive glass 45S5.

RESULTS

Surface roughness measurements

T2 Table II lists the results of the surface roughness assessment. Owing to the difference in fabrication process, the TCP, GB14, GB9, GB9/25, and 352i specimens exhibited a significantly higher surface roughness than the BG specimens (Table II).

SEM analysis

F1 The SEMs in Figure 1 illustrate the surface morphology of the various bioceramic discs after fabrication. Figure 2 shows the various bioceramic surfaces F2 after completion of the preincubation treatment in fully supplemented cell culture medium. The Bioglass 45S5 surface is uniformly covered by a layer of fine, densely packed precipitates [Fig. 2(B)]. Previously, these precipitates were shown to be composed of a crystalline carbonate calcium phosphate apatite (c-Ap).[35]

Cellular proliferation

All substrates supported continuous cellular growth for 21 days (Fig. 3). By day 21, the number F3 of cells on GB9 was higher than on all other materials. This was followed by GB9/25, which displayed higher cell numbers than 352i, bioactive glass 45S5 (BG), and TCP. Furthermore, 352i had more cells than BG and TCP, while GB14 had less cells than BG and TCP. And cell numbers on BG were higher than on TCP after 3 weeks of incubation.

Cellular differentiation

At day 3, SaOS-2 cells cultured on GB9 expressed significant higher protein levels levels for ON and BSP, compared with the other surfaces ($p < 0.0006$) [Figs. 4(A) and 5(E,F)]. Also protein production for F4, F5 Col I and OP was significantly higher when cells were grown on GB9 than for cells cultured on BG,

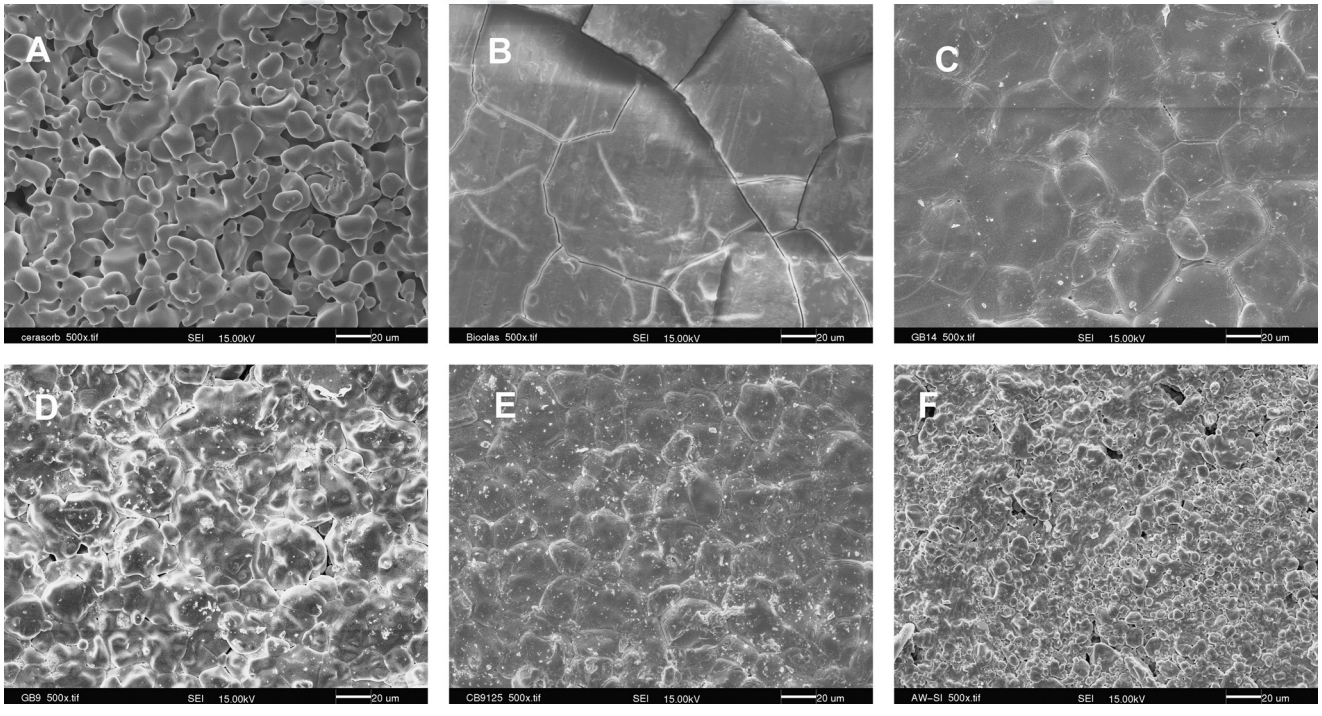

Figure 1. Scanning electron micrographs of the various calcium phosphate specimens after fabrication of the disc-shaped substrata: (A) tricalcium phosphate; (B) Bioglass 45S5; (C) GB14; (D) GB9; (E) GB9/25; (F) 352i.

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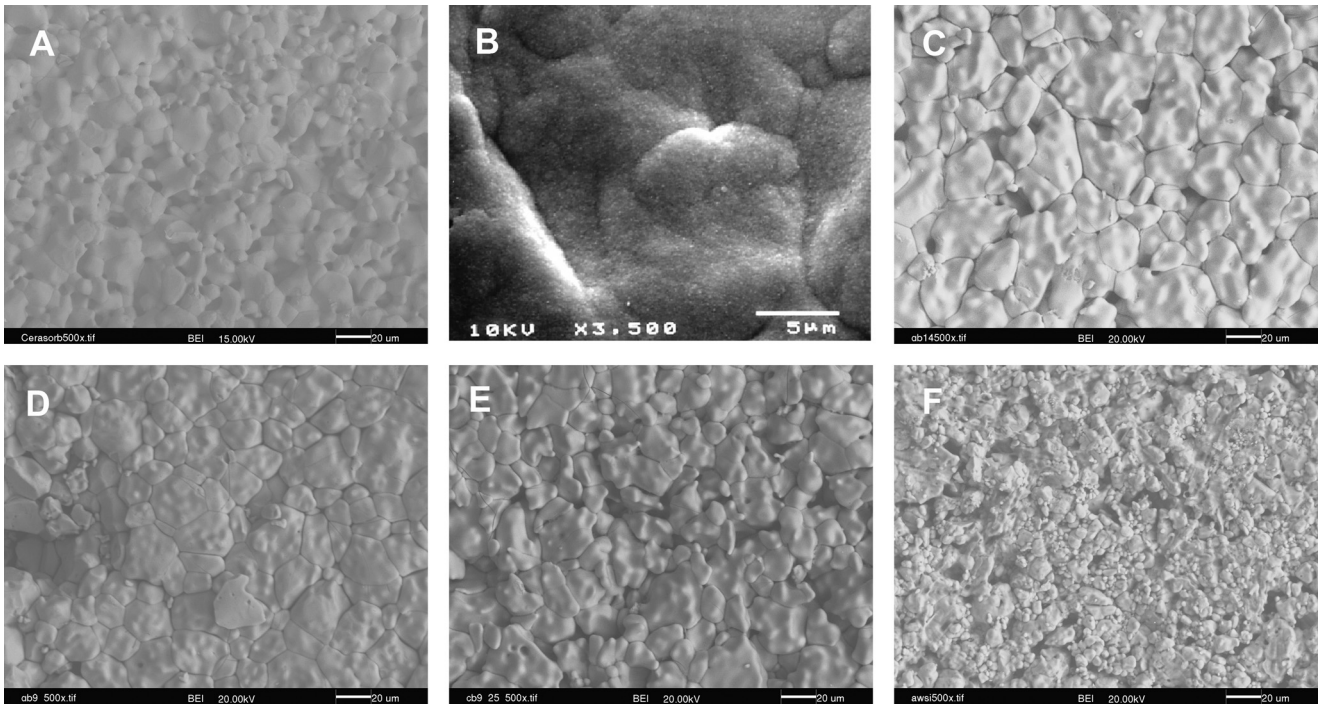

Figure 2. Scanning electron micrographs of the various bioceramic surfaces after preincubation in fully supplemented cell culture medium: (A) tricalcium phosphate; (B) Bioglass 45S5; (C) GB14; (D) GB9; (E) GB9/25; (F) 352i.

GB9/25, and 352i ($p < 0.002$) [Fig. 5(A,C)]. Furthermore, SaOS-2 cells grown on the novel biomaterials, GB9/25 and 352i, and on BG had significantly higher levels of ALP compared with cells cultured on GB14, GB9, and TCP ($p < 0.002$) [Fig. 5(B)].

At day 7, protein formation in SaOS-2 cells cultured on BG and GB9 was significantly higher for Col I, ON, and BSP than in cells grown on all other materials ($p < 0.003$) [Figs. 4(B) and 5(A,E,F)]. The same was true when comparing protein expression for Col I, ON, and BSP in cells on GB9, GB9/25, and GB14 to that in cells cultured on TCP specimens ($p < 0.002$) [Fig. 5(A,E,F)]. Moreover, cells cultured on all calcium–alkali–orthophosphate materials expressed significantly higher protein levels for OP than cells cultured on TCP ($p < 0.006$) [Fig. 5(C)]. Protein production by SaOS-2 cells for ALP was similar on all biomaterials studied [Figs. 4(B) and 5(B)]. Furthermore, SaOS-2 cells grown on BG had significantly higher levels of OC compared with cells cultured on GB9, GB9/25, TCP, and 352i ($p < 0.002$).The same was true when comparing OC production in cells on GB14 and GB9 to that in cells cultured on 352i ($p < 0.006$) [Figs. 4(B) and 5(D)].

At day 14, cells grown on GB9 expressed significantly higher protein levels of Col I, ON, and BSP than do cells cultured on all other surfaces ($p < 0.0095$) [Figs. 4(C) and 5(A,E,F)]. The same was true when comparing OP formation in cells on GB9 to that on GB9/25, GB14, 352i, and TCP) [Figs. 4(C) and 5(C)]. Moreover, cells cultured on GB9 also expressed significantly higher levels of OC than SaOS-2 cells grown on TCP [Figs. 4(C) and 5(D)]. Furthermore, BG displayed more cells expressing significantly higher protein levels of OC than cells grown on all other materials as well as significantly higher protein levels of Col I, ON, and BSP compared with cells on GB9/25, GB14, 352i, and TCP [Figs. 4(C) and 5(A,E,F)]. Moreover, protein production by SaOS-2 cells for Col I, OP, OC, ON, and BSP was higher when these cells were cultured on any of the calcium–alkali–orthophosphate ceramics than for the same cells grown on TCP [Figs. 4(C)

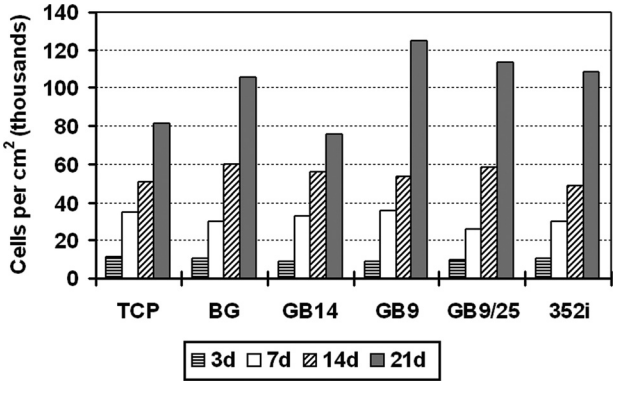

Figure 3. Number of SaOS-2 cells cultured over 21 days on different bioceramics.

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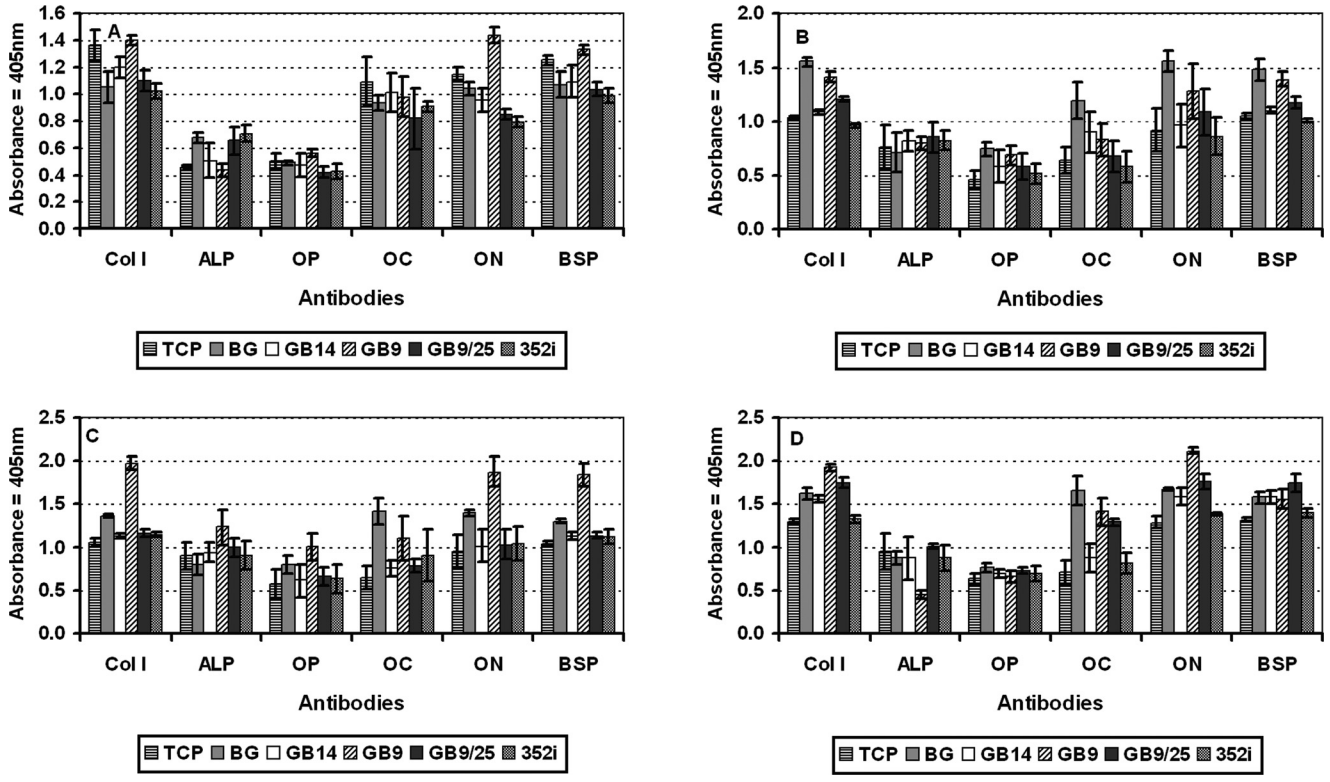

Figure 4. The temporal expression of bone-related proteins by SaOS-2 cells cultured on different calcium phosphate materials for 3 weeks. (A) Day 3, (B) day 7, (C) day 14, (D) day 21. Intracellular protein expression by SaOS-2 cells is at (A) 3, (B) 7, (C) 14, and (D) 21 days of culture on tricalcium phosphate (TCP), bioactive glass 45S5 (BG), GB14, GB9, GB9/25, and 352i. Results are normalized to the internal control β-actin protein for each time point and each substratum. All values are mean ± standard deviation of eight measurements. Col I, Type I collagen; ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; ON, osteonectin; and BSP, bone sialoprotein.

and 5(A,C–F)]. However, these differences in protein production were only statistically significant for Col I and BSP ($p < 0.004$). Also cell growth was higher on GB9/25, GB14, and GB9 specimens compared with the TCP surfaces. Furthermore, cells cultured on GB9 and GB9/25 also expressed significantly

Author Proof

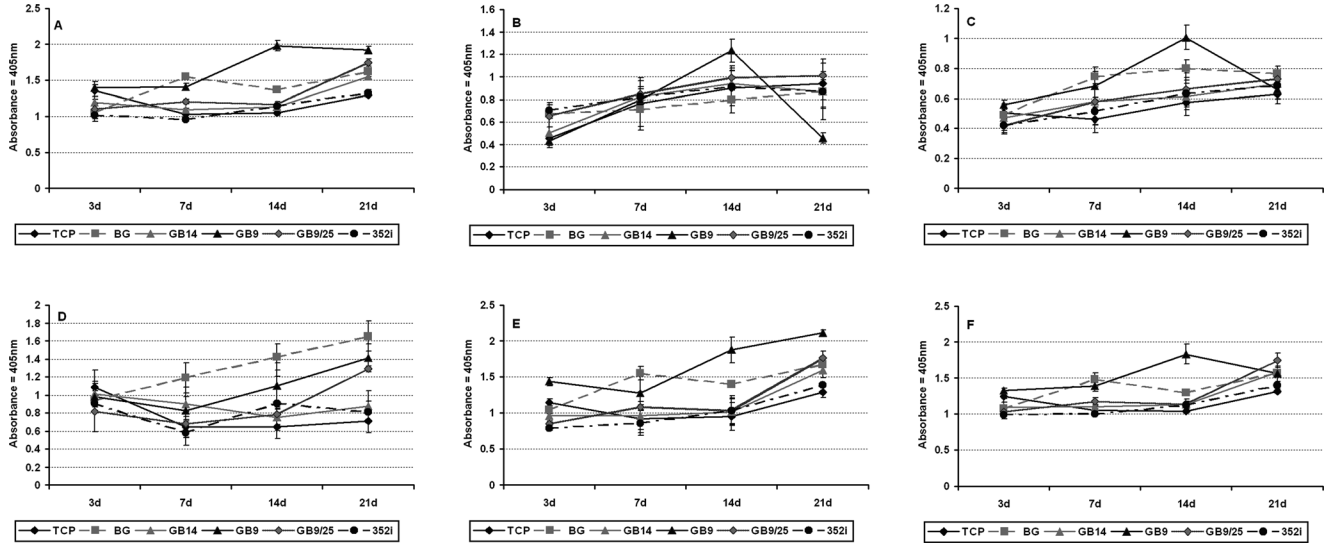

Figure 5. The expression of various osteogenic proteins by SaOS-2 cells cultured on different bioceramics for 3 weeks depicted as a function of time. (A) Type I collagen, (B) alkaline phosphatase, (C) osteopontin, (D) osteocalcin, (E) osteonectin, and (F) bone sialoprotein. Results are normalized to the internal control β-actin protein for each time point and each substratum. All values are mean ± standard deviation of eight measurements. TCP, tricalcium phosphate; BG bioactive glass.

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higher levels of ALP than cells grown on BG ($p < 0.01$) [Figs. 4(C) and 5(B)].

At day 21, GB9 had the highest cell numbers (Fig. 3) expressing significantly higher protein levels of Col I and ON than do cells cultured on all other surfaces ($p < 0.002$) [Figs. 4(D) and 5(A,E)]. The same was true when comparing OC formation in cells on GB9 to that on 352i and TCP surfaces ($p < 0.0003$) [Figs. 4(D) and 5(D)]. Furthermore, SaOS-2 cells cultured on GB9/25 displayed greater cell numbers and significantly higher levels of Col I and BSP than cells grown on GB14, BG, 352i, and TCP ($p < 0.005$), and significantly higher levels of ON than cells on GB14, 352i, and TCP ($p < 0.0003$) [Figs. 4(D) and 5(A,E,F)]. Also OC and OP formation was greater in cells cultured on GB9/25 compared with the same cells grown on TCP ($p < 0.0123$) [Figs. 4(D) and 5(C,D)]. Protein production by SaOS-2 cells for Col I, OP, OC, ON, and BSP was higher when these cells were grown on BG ($p < 0.003$) and GB14 than for the same cells grown on 352i and TCP [Figs. 4(D) and 5(A,C–F)], while cell number was lower (Fig. 3). However, with regard to GB14, these differences in protein production were only statistically significant for Col I, ON, and BSP ($p < 0.003$). Cells cultured on 352i expressed higher levels of Col I, OP, OC, ON, and BSP than cells on TCP [Figs. 4(D) and 5(A,C–F)]. However, these differences in protein expression were only statistically significant for BSP ($p < 0.003$).

In cells grown on GB9, protein expression of Col I, ALP, OP, and BSP peaked at 14 days, while protein production for OC and ON increased further at 21 days (Fig. 5).

DISCUSSION

Ideally, a biomaterial used as bone substitute material should be a temporary material serving as a scaffold for bone formation. As such, it should undergo complete substitution by newly formed functional bone tissue.[42,43] Especially for applications such as alveolar ridge augmentation, this substance must be rapidly resorbable in view of placing implants as quickly as possible in such augmented sites.[5,9,13,14] Bone bioactivity has been defined as the ability of materials to form a bond with the adjacent tissues.[44,45] Ideally, this bond consists of bone laid down by osteoblastic cells recruited to the implant surface. Consequently, bioactive bone substitute materials for use in alveolar ridge augmentation should possess the ability to activate bone formation in combination with a high degradation rate.[5,13,14,30] This in turn requires the ability to differentiate osteogenic cells into osteoblasts on their surface.[30,45] Therefore, in order to evaluate the osteogenic potential of bioactive bone substitute materials it is important to examine the effect of these materials on osteoblastic differentiation.

Osteoblast differentiation is defined by three principal biological periods: cellular proliferation, cellular maturation, and matrix mineralization.[46–48] Differentiating osteoblasts are known to synthesize and secrete type I collagen, alkaline phosphatase, and other noncollagenous extracellular bone matrix proteins such as osteonectin, osteocalcin, osteopontin, and bone sialoprotein.[46–49] These bone–matrix proteins have proven to be particularly useful osteogenic markers characterizing the different stages of osteoblast differentiation.[46] Type I collagen is expressed during the initial period of proliferation and extracellular-matrix biosynthesis, whereas ALP is expressed during the postproliferative period of extracellular-matrix maturation, and the expression of osteopontin, osteonectin, osteocalcin, and bone sialoprotein occurs later during the third period of extracellular-matrix mineralization.[46–49] Consequently, since there is no specific single marker for osteoblasts, the cellular expression of a range of noncollagenous and collagenous proteins as well as alkaline phosphatase has to be investigated when examining cellular differentiation. Thus, the present study quantitatively records the response of human osteogenic cells to novel rapidly resorbable calcium–alkali–orthophosphates when compared with currently clinically used bone substitute materials (β-TCP and bioactive glass 45S5) in terms of protein expression of an array of osteogenic markers as a measure of phenotypic differentiation.[29,31]

Different calcium phosphates tested, significantly affected cellular growth and the temporal expression of an array of bone-related proteins. In the current study, GB9 had the most effect on proliferation and differentiation of SaOS-2 cells, inducing type I collagen formation as well as expression of osteopontin, bone sialoprotein, and osteonectin protein at 3, 7, and 14 days, indicating later osteoblast differentiation. This pattern was maintained at a later time point (day 21) for Col I, ON, and OC. OP, ON, OC, and BSP have been tightly linked to osteoid production and matrix mineralization,[46–49] thereby suggesting that this material may possess a higher potency to promote osteogenesis and matrix calcification, than do TCP, BG, and the other calcium phosphates tested.

GB9/25, BG, and 352i induced enhanced ALP expression at 3 days characterizing the change from the proliferative to the postproliferative period, before this feature was seen in TCP. This suggests that these materials are capable of promoting more rapid differentiation of osteogenic cells into osteoblasts compared with TCP.

After 1 and 2 weeks, BG showed enhanced expression of Col I, BSP, ON, and OC compared with

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GB9/25, GB14, 352i, and TCP. Also at 3 weeks, similar to GB9, cell numbers and Col I, BSP, ON, and OC production were higher than on TCP and 352i. These findings underline the capability of bioactive glass 45S5 to induce osteoblast differentiation early on. Furthermore, they are in agreement with previous studies, which showed that bioactive glass 45S5 stimulates the osteoblast phenotype *in vitro*.[28,30,36,50–53] This was due to surface-mediated[28,30,36,44,50,53] as well as solution-mediated effects.[30,51–53]

At 7 days, cells on GB9/25 displayed greater expression of Col I and BSP than cells on TCP. This pattern was maintained at 14 days and was accompanied by enhanced cellular proliferation compared with the TCP surfaces. Moreover, at 21 days, similar to GB9 and BG, GB9/25 surfaces displayed greater cell numbers expressing higher protein levels of OP, ON, OC, and BSP compared with cells on TCP. Since these osteoblastic markers characterize the later stages of osteoblast differentiation, this is suggestive that GB9/25 may possess a higher potency to promote osteogenesis and matrix calcification than does TCP. Furthermore, at 21 days also greater cell numbers and protein expression for Col I and BSP were noted on GB9/25 compared with GB14, 352i, and BG, suggesting that GB9/25 is also an excellent material for promoting osteogenesis and bone regeneration.

At 7, 14, and 21 days GB14 showed greater protein expression of ColI, ON, and BSP than TCP. This is indicative that GB14 stimulates the expression of the osteoblastic phenotype to a greater extent than TCP.

At 3 days, 352i induced greater ALP expression than TCP. At 7 days, cell cultured on 352i expressed significantly higher OP levels than cells on TCP, while ALP and OC expression was similar. Moreover, at 14 days 352i showed greater protein expression of ColI and BSP than TCP, a pattern which was maintained for BSP at 21 days, while at 14 and 21 days, cells on 352i expressed similar protein levels for OP, OC, and ON compared with cells on TCP. Cell numbers, however, were higher on 352i after 3 weeks of incubation. These findings suggest that this novel calcium–alkali–orthophosphate material 352i is capable of supporting the expression of the osteoblastic phenotype to a similar degree as TCP.

Apart from surface chemistry, another surface property influencing cell–biomaterials interactions is surface morphology. Although TCP, GB9, GB9/25, GB14, and 352i displayed a similar surface roughness, considerable differences were detected between the amount of osteogenic markers expressed, when identical cells were grown on these materials, whereas osteoblastic phenotype expression of cells on BG was greater compared to that of cells cultured on 352i and TCP in spite of the lower surface roughness of BG specimens. These observations suggest that the differences in cellular differentiation might be attributed to compositional features rather than to differences in surface roughness.

Of the various bone substitute materials studied, GB9 had the greatest stimulatory effect on osteoblastic proliferation and differentiation This is suggestive that this materials possesses the highest potency to enhance osteogenesis and matrix calcification. Furthermore, similar to bioactive glass 45S5, GB9/25 exhibited a stimulatory effect on osteoblastic phenotype expression, which indicates that GB9/25 is also an excellent material for promoting osteogenesis and bone regeneration. Our observations for GB14 suggest that this material is capable of eliciting greater osteoblast differentiation than TCP. These observations are in agreement with a previous study in which primary human bone derived cells were employed,[31] as well as with *in vivo* findings after implantation of GB14 particles in the rabbit femur. Histomorphometric analysis revealed significant higher bone contact after 7, 28, and 84 days of implantation compared with different bioactive glass particles 45S5, 52S, 55S, and also higher degree of degradation after 84 days of implantation. The GB14 particles displayed a high degradation rate due to different processes, mainly leaching and particulate degradation leading to a GBR by meeting the balance between degradation and osteogenesis[54,55] After 84 days of implantation, GB14 particles showed more than 84% bone contact at the interface, i.e. good bone-bonding behavior.[54,55] Since 352i supported the expression of the osteoblastic phenotype mostly to the same degree as cells grown TCP, this novel bioceramic can be regarded as potential bone substitutes. As a result, these data provide evidence for the biocompatibility of this novel calcium–alkali–orthophosphate-material at a molecular level.

These findings are clinically very significant, as histological evaluation of biopsies, sampled from patients 6 months after augmentation of the sinus floor using TCP particles, have shown that TCP particles supported bone regeneration[16–19,56,57] and had a stimulatory effect on osteoblastic differentiation. It was demonstrated that by 6 months TCP particles attracted osteoprogenitor cells that migrated into the interconnecting micropores of the bone substitute material.[17,18] These cells differentiated into osteoblasts and thus brought about bone deposition. Furthermore, good bone-bonding behavior as well as bone formation within the degrading particles has been observed, as well expression of the osteogenic markers Col I, BSP, and OC in the newly formed bone matrix in contact with the TCP particles.[56,57] The histologic data also indicated that the TCP particles degraded by chemical dissolution, and that the role played by osteoclasts was only minor.[19]

Calcium phosphate ceramics and glasses are known to stimulate bone tissue formation.[30,44,45]

However, the underlying mechanisms are not fully understood. To obtain a fundamental understanding of the mechanisms by which bone tissue formation is stimulated, the atomic and molecular phenomena occurring at the materials surface and their effects on the reaction and signaling pathways of cells and tissues must be elucidated.[30] This implies any of the cellular activities leading to tissue formation, including cell attachment, differentiation, and extracellular-matrix formation. With bioactive ceramics, solution-mediated surface reactions take place after immersion in biological fluids. These reactions include dissolution, reprecipitation, and ion-exchange phenomena in combination with protein adsorption events occurring at the bioactive ceramic surface.[30,35,58] A key element of bone bioactive behavior is the development of a carbonated apatite surface after immersion in biological fluids.[30,58,59] It is also an important aspect that the biomaterial surface composition and structure influences serum protein adsorption. There is support for the view that the enhanced cellular and tissue responses to bioactive ceramics are related to enhanced fibronectin adsorption at their surfaces.[37,60] Also it is noteworthy that surface chemistry of the biomaterial surface modulates the structure and activity of adsorbed fibronectin.[61] The cellular interactions between osteoblasts and the biomaterial surface are thought to be mediated primarily by membrane-associated adhesion receptors belonging to the integrin superfamily.[61–64] In addition to their role as adhesion receptors, integrins are also involved in transducing signals from the extracellular matrix to the interior of the cell[65] resulting in the activation of signaling molecules and regulation of gene expression, thus modulating cellular migration, proliferation, differentiation, and apoptosis.[62–66] Thus, to decipher the complexity of the reactions at the bioactive ceramic–bone interface, it is logical to first analyze the surface transformation and protein adsorption events, and then to study the osteoblast responses to these bioactive surfaces. Although numerous studies have investigated cellular responses to bioactive ceramics, little is known about the intracellular events that take place in osteoblasts at these bioceramic surfaces. In our study, considerable differences were detected between the amount of osteogenic markers expressed, when identical cells were grown on the different test materials. Furthermore, we were able to show that several of the rapidly resorbable calcium–alkali–orthophosphate ceramics tested were able to stimulate osteoblast differentiation. However, the intracellular signaling events that follow osteoblast attachment to these bioactive ceramics, leading to differences in osteoblast activity, are not known. Because cell signaling affects cell proliferation and differentiation, it is important to understand the cell signaling pathways induced by osteoblast–implant material interactions.[63] Interaction between osteoblasts and bone matrix components via integrins leads to a rearrangement of cytoskeletal components and activation of specific signaling proteins localized at focal adhesions and focal adhesion kinase (FAK).[63] Recent studies have demonstrated that osteoblast adhesion to titanium alloy and fibronectin resulted in activation of FAK and the mitogen-activated protein kinase signal transduction pathway, which modulates cellular differentiation.[63] Furthermore, the effect on apoptosis is of importance.[66] Activation of the PI3K/Akt (phosphatidylinositol-3-kinase/protein kinase B) survival pathway results in depression of apoptosis.[67] Consequently, activation of this pathway would be expected by biomaterials, which stimulate osteogenesis. The effect of bioactive bone substitute materials interacting with bone tissue on these signaling pathways in osteoblast function and differentiation is at present not understood. Developing this understanding has been hampered by the inadequacy of the experimental techniques that could be used. Adequate techniques to study integrin-mediated adhesion and the subsequently activated intracellular differentiation and cell survival pathways have been established only recently.[62–64,66–68] In addition, over the last decade, advanced surface analysis methods have been developed and combined with molecular techniques, in order to facilitate a better understanding of the surface transformations of bioactive, resorbable ceramics and the protein adsorption events associated with immersion in biological fluids.[28,30,35,60,69] Consequently, studies combining these two powerful analytical methodologies (advanced surface analysis methods and techniques to examine integrin-mediated cell adhesion and signaling mechanisms) to elucidate the mechanisms by which some of these rapidly resorbable bone substitute materials induce enhanced osteoblastic differentiation are currently underway. These studies involve SEM/EDX and FTIR analyses as well as immersion experiments in fluids with and without serum and characterization of protein adsorption applying ELISA and Western blotting techniques.

Furthermore, the correlation between the *in vitro* data with *in vivo* phenomena is important, since it is hypothesized that enhanced osteoblastic cell differentiation *in vitro* leads to more and more expeditious bone formation at the bone–biomaterial interface *in vivo*. In order to test this hypothesis, correlation of the *in vitro* and *in vivo* data is required. This includes (1) correlating quantitative gene and protein expression of the osteogenic markers *in vitro* with the amount of bone formed after biomaterials implantation. (This is in addition to determining the decrease in particle size.) (2) Quantifying the expression of these markers in histological sections obtained from

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in vivo experiments is critical to comparing the expression of the various markers *in vitro* and *in vivo*. To this end, an *in vivo* study has been performed, in which the bone substitute materials described in the current study were implanted in the sheep mandible and sinus floor to regenerate membrane-protected critical size defects.[5,70] Histological assessment is currently underway. Histological evaluation of the bone–biomaterial interface requires undecalcified PMMA sections. Only recently have novel embedding resins and embedding techniques become available that permit performing immunohistochemistry on undecalcified sections.[71,72] Moreover, a methodology has been developed that facilitates immunohistochemical analysis of osteogenic markers on PMMA-embedded sawed sections of bone which contain bone substitute materials.[56,70,72] Correlating *in vivo* results with the data from the *in vitro* studies, which elucidate the mechanisms by which these rapidly resorbable bone substitute materials induce enhanced osteoblastic differentiation would be of great value, since taken together, these studies would facilitate characterizing the tissue response at the bone–biomaterial interface *in vitro* and *in vivo* at a molecular level and, thus, could contribute significantly to obtaining a fundamental understanding of the processes involved in tissue integration of bioactive implant materials.

In conclusion, all calcium phosphate materials studied significantly affected cellular growth and the expression of the osteoblastic phenotype by SaOS-2 cells. Of the various bone substitute materials studied, GB9 had the greatest stimulatory effect on osteoblastic proliferation and differentiation, suggesting that this material possesses the highest potency to enhance osteogenesis. Furthermore, similar to bioactive glass 45S5, GB9/25 displayed a stimulatory effect on osteoblastic proliferation and phenotype expression, which indicates that GB9/25 is also an excellent candidate bone substitute material for promoting osteogenesis and bone regeneration. Since GB14 and 352i supported the expression of these osteoblastic phenotype to the same or a higher degree than cells grown on TCP, these novel bioceramics can be regarded as potential bone substitutes. As a result, these data provide evidence for the biocompatibility of these novel calcium–alkali–orthophosphate-materials at a molecular level. Further research exploring the material dependent effects reported here is currently underway. These research efforts involve the study of cell adhesion mechanisms and the intracellular signal transduction pathways, which lead to the observed differences in osteoblastic phenotype expression. Equally important is the correlation between *in vitro* data with *in vivo* phenomena, by focusing on the detection of the same osteogenic markers in the tissue surrounding the present bone substitute materials subsequent to implantation in the sheep mandible. Such measurements are currently made on the same specimens as those used for quantifying the bone biomaterial contact.

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