formation in HBDC cultured on GB9N was significantly higher for Col I and ALP than in cells on all other substrata ($p<0.02$) (Fig. 10(e)). The same was true when comparing OC ($p<0.05$), BSP ($p<0.05$) and ON ($p<0.01$) protein production on GB9N and Mg5 to that on TCP, Co and AP40, and OP protein levels on GB9N to these on TCP and Co surfaces ($p<0.05$) (Fig. 10(e)). GB9N had the highest cell numbers at the end of the incubation period (Fig. 8).

I/4 Discussion

I/4.1 General Discussion Part I

Cell and tissue response to implant materials is one of the most important themes in the field of biomaterials (Jarcho 1981, Klein et al. 1983, LeGeros & Daculsi 1990, Gross et al. 1991, Kotani et al. 1991, Ducheyne & Cuckler 1992, Neo et al. 1992, Davies 1996, Hollinger et al. 1996, Yaszemski et al. 1996, Hench 1998, Ducheyne & Qui 1999, Oonishi et al. 1999). Implanted calcium phosphate ceramics (Jarcho 1981, Klein et al. 1983, LeGeros & Daculsi 1990, Ohgushi et al. 1990, Gross et al. 1991, Kotani et al. 1991, de Bruijn et al. 1994, Hollinger et al. 1996, Ducheyne 1998) and glass ceramics (Hench & Paschall 1973, Gross et al. 1991, Schepers et al. 1991, 1993, 1998, Schepers & Ducheyne 1997, Hench 1998, Ducheyne & Qui 1999) are known to bond directly to bone. However, differences in these materials are reflected in the rate of bone formation on their surfaces (Ohgushi et al. 1990, Gross et al. 1991, Neo et al. 1992, Schepers & Ducheyne 1997, Hench 1998, Ducheyne & Qui 1999). Ideally bioactive calcium phosphate ceramics for use in bone regeneration should possess the ability to activate bone formation (Hench & Paschall 1973, Ohgushi et al. 1990, Davies 1996, Hench 1998, Ducheyne & Qui 1999). This in turn requires the ability to differentiate stromal bone marrow cells into osteoblasts on

their surface (Ohgushi 1990, Ducheyne & Cuckler 1992, Davies 1996, Hench 1998, Schepers et al. 1993, Schepers & Ducheyne 1997, Ducheyne & Qui 1999).

Differentiating osteoblasts are known to synthesize and secrete type I collagen, alkaline phosphatase, and other non-collagenous extracellular bone matrix proteins such as osteonectin, osteocalcin, osteopontin and bone sialoprotein (Sodek et al. 1991, Sodek & Cheifitz 2000, Aubin 1998a, Aubin 2000). These bone-matrix proteins have proven to be particularly useful osteogenic markers (Sodek & Cheifitz 2000). Differential gene expression of osteogenic cells can be defined by three principal biological periods: cellular proliferation, cellular maturation and focal mineralization (Owen et al. 1990). Type I collagen is expressed during the initial period of proliferation and extracellular-matrix biosynthesis, whereas ALP is expressed during the post proliferative period of extracellular-matrix maturation, and the expression of osteopontin, osteocalcin and bone sialoprotein occurs later during the third period of extracellular-matrix mineralization (Sodek et al. 1991, Sodek & Cheifitz 2000, Aubin 1998a, Aubin 2000). Consequently, because there is no specific single marker for osteoblasts, the cellular expression of a range of non-collagenous and collagenous proteins as well as alkaline phosphatase has to be investigated, when examining cellular differentiation. Therefore, when studying the effect of candidate biomaterials for bone regeneration on cellular behavior it is important to examine cell proliferation and differentiation, first because these materials should possess the ability to differentiate osteoprogenitor cells into osteoblasts and second because proliferation and differentiation of osteoblasts are affected by the chemistry of substrata (Schwartz & Boyan 1994, Zreiqat et al. 1999a, 1999b, Ducheyne & Qui 1999, Howlett et al. 2000). Thus, the present studies quantitatively record the response of HBDC to various bone substitute materials at four or five time points in terms of osteogenic

genes and their translated proteins that form an array of osteogenic parameters as a measure of phenotypic differentiation (Zreiqat et al. 1996, Zreiqat & Howlett 1999).

Different calcium phosphates tested, significantly affected cellular growth and the temporal expression of an array of bone-related genes and proteins in all three studies. Repeatedly, it was observed that HBDC grown on a specific biomaterial expressed different levels of osteogenic mRNA and the translated protein for a specific osteoblastic marker at a given time point. For example in study A, at 14 days, mRNA levels for OP on R1 were low (Fig. 3(d)), whereas protein expression for OP was enhanced (Fig. 4(d)). This can be due to a number of reasons. The mRNA expressed might not have been fully translated into protein yet. Also the protein might still be present, while the mRNA has already been degraded due to the fairly long half-life of the targeted protein compared to the much shorter half-life of the respective mRNA molecule. For instance, the half-life of procollagen mRNA was reported to be approximately 5-10 h (Kähari & Vuorio 1987). This is in agreement with the findings of Zreiqat et al. (1996, 1999a, Zreiqat & Howlett 1999).

I/4.2 Discussion Study A

In study A, R1 had the most effect on the differentiation of HBDC, inducing expression of all bone-related proteins including osteopontin, osteocalcin, osteonectin, and bone sialoprotein protein at 3 and 5 days indicating later osteoblast differentiation. A pattern which was maintained at later time points (14 and 21 days), where R1 induced greater expression of osteopontin, osteocalcin, osteonectin and bone sialoprotein protein than any other material. These four proteins have been tightly linked to osteoid production and matrix mineralization, suggesting that R1 may possess a higher potency to promote osteogenesis and matrix calcification, than TCP and the other calcium phosphates tested. At 5, 7 and 14 days cell numbers on R1

were lower than on TCP, indicating that stimulation of cell differentiation over proliferation had occurred. These findings are in agreement with those reported previously. When employing a rat bone marrow cell culture system we observed reduced cell growth on R1 compared to TCP after 14 days (Knabe et al. 1997). R1+SiO_2 showed a rise in expression of Col Iα2, ALP and ON mRNA at 3 days, characteristic for the change from the proliferative to the post-proliferative period, before this feature was seen in TCP or the control. After two and 3 weeks, cells on R1+SiO_2 displayed significantly enhanced expression of all osteogenic proteins in combination with the highest cell numbers. Thus enhanced cellular proliferation and differentiation was observed, suggesting that R1+SiO_2 may possess a higher potency to enhance osteogenesis than TCP, R17 and R1/M2. Furthermore, at day 21 cells grown on R1, R17 and R1+SiO_2 expressed significantly higher levels of ALP mRNA, this is indicative that these surfaces are continuing to maintain cells in a pre-osteogenic phase. R17 and R1/M2 supported the expression of the osteoblastic phenotype mostly to the same or a higher degree than cells grown on TCP. Consequently, these biomaterials can be regarded as potential bone substitutes. As a result, these data provide evidence for the biocompatibility of these novel calcium phosphates at a molecular level.

Apart from surface chemistry, another surface property influencing cell-biomaterials interactions is surface morphology (Brunette 1998). All tested calcium phosphate ceramics displayed a similar surface roughness, therefore the differences detected in the amount of osteogenic markers expressed between these surfaces suggest that the differences seen have to be attributed to compositional features rather than to differences in surface roughness.

I/4.3 Discussion Study B

In study B, the most pronounced effect on osteoblast differentiation was encountered with GB9, inducing expression of osteopontin, osteocalcin and osteonectin protein at 3 and 14 days indicating later osteoblast differentiation. A pattern which was maintained at a later time point (day 21) for osteopontin, osteocalcin and bone sialoprotein. These three proteins have been tightly linked to osteoid production and matrix mineralization, suggesting that this material may possess a higher potency to promote osteogenesis and matrix calcification, than TCP and the other calcium phosphates tested.

GB14 showed a rise in expression of Col Iα2 and ALP mRNA at 3 days characterizing the change from the proliferative to the post-proliferative period, before this feature was seen in TCP or the control. After one week, cells on Biocement displayed significantly enhanced expression of all osteogenic markers and thus enhanced cellular differentiation, while proliferation was reduced. Also at the end of the incubation period greater gene expression for the osteoblastic markers characterizing the later stages of osteoblast differentiation was noted in HBDC on Bioc and GB14 compared to cells on TCP, while cell numbers were lower. Thus, with the Bioc and GB14 surfaces, stimulation of cell differentiation over proliferation was observed. These findings are in agreement with those previously reported by Oreffo et al. (1998a, 1998b) who also documented stimulation of cell differentiation over proliferation when culturing human bone marrow osteoprogenitors on Biocement D. Also in a previous study in which a rat bone marrow cell culture system was employed we observed reduced cell growth on GB14 and Bioc compared to TCP (Knabe et al. 1998, 2000).

When studying cell-biomaterials interactions a parameter that has to be taken into consideration is the surface morphology of the specimens. Although TCP, GB9

and GB14 displayed a similar surface roughness (Knabe et al. 1998), considerable differences were detected between the amount of osteogenic markers expressed, when identical cells were grown on these materials, whereas the differences in osteoblastic phenotype expression between GB14 and Bioc were less striking in spite of the greater surface roughness of the GB14 specimens. These observations suggest that the differences in cellular differentiation might be attributed to compositional features rather than to differences in surface roughness. Since GB14 and Bioc supported the expression of the osteoblastic phenotype mostly to the same or a higher degree than cells grown on the control and TCP, these biomaterials can be regarded as potential bone substitutes. As a result, these data provide evidence for the biocompatibility of these novel calcium phosphates at a molecular level.

First *in vivo* studies with GB14 and Biocement D showed favorable bone apposition without any foreign body reaction (Ooms et al. 2002, 2003, Müller-Mai et al. 1997, Müller-Mai 2000, 2003, Khairoun et al. 2002). Moreover, histomorphometric results after 84 days of implantation of GB14 showed more than 84% bone contact at the interface, i.e. good bone bonding behavior. At the same time particles displayed a high degradation rate due to different processes, mainly leaching and particulate degradation leading to a guided bone regeneration by meeting the balance between degradation and osteogenesis (Müller-Mai et al. 1997, Müller-Mai 2000, 2003). In addition, the histomorphometric analysis revealed significant higher bone contact after 7, 28 and 84 days of implantation in the rabbit femur compared to different bioglass particles 45S5, 52S, 55S and also a higher degree of degradation after 84 days of implantation (Müller-Mai 2000, 2003).

I/4.4 Discussion Study C

In study C, GB9N stimulated osteoblast differentiation to the greatest extent. GB9N elicited mRNA expression of all osteogenic markers including osteopontin,

osteocalcin, osteonectin at 3 days. These 3 markers are characteristic for the later stages of differentiated osteoblast function. A pattern which was also maintained at later time points (day 7, 14 and 21) at both the mRNA and protein level. At day 7 and 14, GB9N induced greater expression of osteopontin, osteocalcin and osteonectin mRNA and protein than any other bioactive ceramic. These three bone-related proteins have been tightly linked to osteoid production and matrix mineralization, suggesting that GB9N may possess a higher potency to promote osteogenesis and matrix calcification than TCP and the other ceramics tested. At 5, 7 and 14 days cell numbers on GB9N were lower than on TCP, indicating that stimulation of cell differentiation over proliferation had occurred. This is in contrast to the findings of Mayr-Wohlfarth et al. (2001) who reported enhanced cell proliferation but reduced differentiation of SaOS-2 cells on GB9N compared to α-TCP specimens. However, they studied the expression of osteoblastic markers only at the mRNA level at only one time point (5 days). Furthermore, they did not use primary cells but a cell line (SaOS-2 cells), examined three-dimensional scaffolds and concluded that apart from the chemical composition of the substrata the porosity and the pore size of the scaffold were additional factors having an influence on the cellular response.

Mg5 showed a rise in expression of ALP mRNA at 3 days characterizing the change from the proliferative to the post-proliferative period and a rise in expression of OC mRNA and OC and BSP protein at day 5 indicating the later stages of osteoblast differentiation. These features were not yet seen in TCP or the control at day 5. At day 14, greater OP and OC mRNA expression in comparison to TCP was noted. After 3 weeks, Mg5 induced enhanced expression of OP and ON mRNA and of OC, ON and BSP protein. Cell numbers, however, were lower than on TCP suggesting that cell differentiation had taken place at the expense of proliferation. Since this novel glass ceramic supported the expression of the osteoblastic

phenotype to a higher degree than TCP, it can be regarded as a promising bone substitute material. Gene and protein expression of bone-related markers on AP40 was mostly similar to that on TCP. Furthermore, enhanced protein formation for Col I was noted on AP40 at day 3 and for BSP at day 5 compared to TCP. After 2 weeks AP40 induced enhanced protein production for ALP, OP and BSP compared to TCP and Mg5. This is suggestive that AP40 facilitated the expression of the osteoblastic phenotype at least as much as TCP rendering it a potential bone substitute material. As a result, these data provide evidence for the biocompatibility of the novel glass ceramics GB9N, Mg5 and AP40 at a molecular level.

As mentioned above, another important surface property that influences cell-biomaterials interactions is surface morphology and roughness (Brunette 1988, Boyan & Schwartz 2000). Although AP40 exhibited a considerable lower degree of surface roughness than TCP, it facilitated the expression of the osteoblastic phenotype at least as much as TCP. Also Mg5 specimens supported the expression of the osteoblastic phenotype to a higher degree than TCP in spite of their lower surface roughness. Furthermore, although GB9N and TCP specimens were very similar in surface roughness, considerable differences in gene and protein expression of osteogenic markers were noted suggesting that these differences were more related to the effect of surface chemistry of these materials rather than their surface roughness.

I/4.5 General Discussion Part I (continued)

In determining the effect of substrata on cellular behavior, it is necessary to investigate the sequential cascade of cellular attachment, multiplication and differentiation. Understanding the mechanisms whereby biomaterials influence molecular signaling of cells may give insight into the relationships between the

surface characteristics of implant materials and tissue differentiation. These studies investigated the temporal molecular phenotype of HBDC grown on various novel bone substitute materials. Zreiqat et al. (1996) and Zreiqat & Howlett (1999) were the first to quantitatively relate the expression of bone-related mRNAs to their respective proteins as a measure of phenotypic differentiation.

To understand the bone biomaterials bonding mechanism, the atomic and molecular phenomena occurring at the material surface and their effects on the reaction and signaling pathways of cells and tissues must be elucidated (Ducheyne & Qui 1999). This implies any of the cellular activities leading up to tissue formation, including cell attachment, differentiation and extracellular-matrix formation. In the studies reported here, considerable differences were detected between the amount of osteogenic markers expressed when identical cells were grown on the different test materials. Hence, it is important to gain further insight into the mechanisms of cell attachment and intracellular signaling which eventually lead to these differences in osteoblastic differentiation.

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 occuring at the bioactive ceramic surface (Ducheyne et al. 1993, Radin & Ducheyne 1993, 1996, Radin et al. 1997, Ducheyne & Qui 1999, El-Ghannam et al. 1999, Kaufmann et al. 2000a, 2000b). A key element of bone bioactive behaviour of calcium phosphate ceramics is the development of a carbonated apatite surface after immersion in biological fluids (Kokubo et al. 1992, Ducheyne et al. 1993, Kokubo 1993, Ducheyne & Qui 1999). 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 (Davies 1998, El-Ghannam et al. 1999). Thus, to decipher the complexity of the reactions at the surface of these novel bone substitute materials, it would be logical to first analyze the surface transformation and protein adsorption events and then to study the cell adhesion and intracellular signaling events which eventually lead to the differences in osteoblastic differentiation observed with the different bone substitute materials tested.

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 (Gronowicz & McCarthy 1996, Su et al. 1996, Rezania & Healy 1999a, Krause et al. 2000, Cowles et al. 2000). Integrins are transmembrane heterodimers composed of an α- and a β-subunit and are connected to the cytoskeleton (Hynes 1987, 1992). Bone cells express the following integrin receptors $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ (Hughes et al. 1993, Saito et al. 1994, Gronthos et al. 1997). The interaction of osteoblasts with implant surfaces is mediated largely by the β1 subfamily (Gronowicz & McCarthy 1996, Rezania & Healy 1999a, Krause et al. 2000, Zreiqat et al. 2002). 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 (Juliano & Haskill 1993, Rosales et al. 1995) resulting in the activation of signaling molecules and regulation of gene expression, thus modulating cellular migration, proliferation, differentiation, and apoptosis (Fig. 11) (Hynes 1992, Bates et al. 1995, Faull & Ginsberg 1995, Hildebrand et al. 1995, Yamada & Miyamato 1995, Wary et al. 1996, Shakibaei et al. 1999).

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) (Krause et al. 2000) (Fig. 11). Activation of FAK is considered to play a critical role in

control of adhesion dependent cell survival and proliferation (Richardson & Parson 1995, Yamada & Miyamoto 1995).

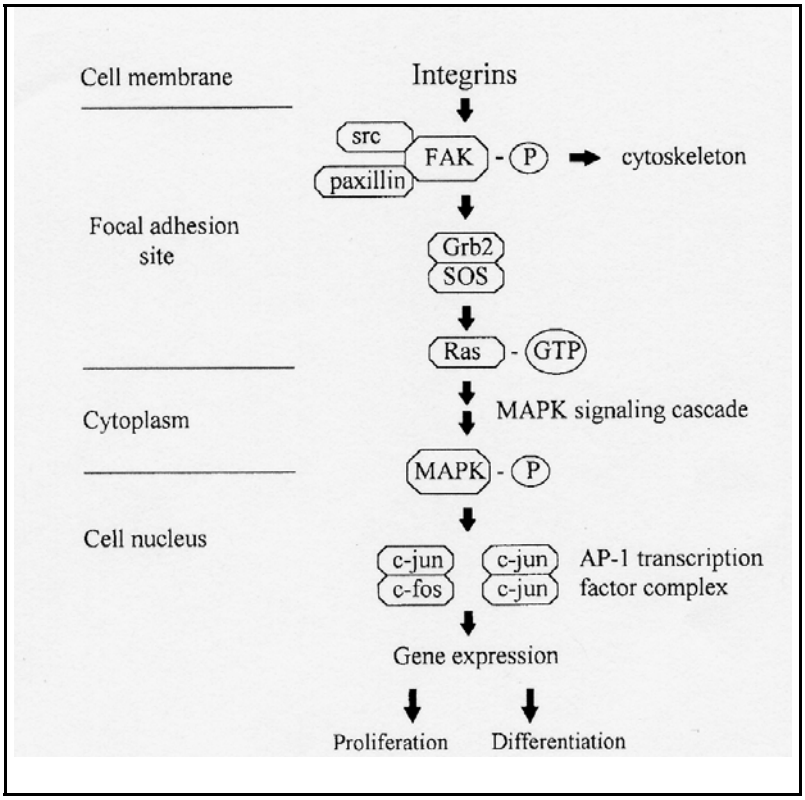


Figure 11. Integrin-mediated intracellular signaling pathway (Taken from Cowles et al. 2000).

Recent studies have demonstrated that osteoblast adhesion to titanium alloy and fibronectin resulted in activation of FAK and mitogen-activated protein (MAP) kinase signal transduction pathways (Krause et al. 2000). FAK associates with several signaling proteins (e.g. the src-family of protein-tyrosine kinases: Src-homology collagen (Shc) and growth factor receptor-bound protein 2 (Grb2)) (Fig. 11). One downstream signaling protein in the integrin-generated signaling pathway is the adaptor protein Shc. Mitogen-activated protein kinase (MAPK) p44 (Extracellular signal-regulated kinase 1 - Erk1) and p42 MAPK (Extracellular signal-regulated kinase 2 - Erk2) are important mediators of cellular responses to intracellular signaling proteins. FAK interaction with Shc creates a Grb2-binding site thus linking

FAK to the Ras/MAPK (mitogen activated protein kinase) pathway (Dedhar 1995) (Fig. 11). The Shc-Grb2-complex induces Ras activation via Grb2-associated son of sevenless (SOS), a cytoplasmic GTP (guanosine triphosphate) exchange protein (Harmer & DeFranco 1997) (Fig. 11). Ras then stimulates the Erk1/Erk2 MAP kinase cascade (Marshall 1994) (Fig. 11), which plays an important role in cellular differentiation and growth. Activated MAP kinases (Erk1/2) translocate to the nucleus and are able to phosphorylate and activate transcription factors including c-fos and c-jun, members of the activator protein-1 (AP-1) transcription factor complex, which control gene expression (Cowles et al. 2000, Krause et al. 2000) (Fig. 11). The AP-1 complex initiates early transcription events which lead to cell proliferation and/or can affect differentiation (Fig. 11). Furthermore, AP-1 has an important role in osteoblast differentiation and bone development. AP-1 sites are known to be present in the promoters of many bone-specific genes such as type I collagen, alkaline phosphatase, osteocalcin and osteopontin (Cowles et al. 2000, Krause et al. 2000). In addition, possible interaction with the p38 pathway is of interest (Noth et al. 2003, Nohe et al. 2004). Ivaska and coworkers (1999) reported a novel signaling mechanism in human osteosarcoma cells (SaOS-2) mediated by $\alpha_2\beta_1$ integrin involving isoform-specific activation of the p38 signaling protein. Recent data suggest a possible interaction between the Ras/MAPK pathway and the p38 pathway (Nohe et al. 2004).

Furthermore, the effect on apoptosis is of importance. Activation of the PI3K/Akt (phosphatidylinositol-3-kinase/Protein Kinase B) survival pathway results in depression of apoptosis (Adams & Shapiro 2003). Consequently, activation of this pathway would be expected by biomaterials which stimulate osteogenesis.

Previously, it was demonstrated that surface modification of alumina by Mg^{2+}-ion beam implantation resulted in significantly enhanced cell adhesion and

expression of osteogenic genes and proteins by HBDC as well as upregulation of key signaling proteins of the MAP kinase signaling pathway compared to the native alumina (Zreiqat et al. 2002). Consequently, further exploration of the materials dependent effects reported here will involve the study of cell adhesion mechanisms and the intracellular signal transduction pathways which lead to the observed gene and protein expression of osteogenic markers. This way, marked differences in gene and protein expression can be correlated with differences in integrin expression and intracellular signal transduction pathways. Adequate techniques to study integrin mediated adhesion and the subsequently activated intracellular pathways have been established only recently (Gronowicz & McCarthy 1996, Shenker et al. 1997, Krause et al. 2000, Cowles et al. 2000, Adams & Shapiro 2003, Noth et al. 2003, Razzouk & Shapiro 2003). These techniques have been used in just a few studies of orthopaedic biomaterials (titanium alloy and alumina) (Gronowicz & McCarthy 1996, Krause et al. 2000, Zreiqat et al. 2002), however not for bioactive ceramics, and not related to repair in the oral and maxillo-facial domain. Furthermore, it would be rewarding, if this was combined with investigations of the solution-mediated surface reactions and protein adsorption events occurring at the surface of these novel bioactive ceramic substrata (Ducheyne & Qui 1999), since taken together these data could significantly contribute to unraveling the complexity of the reactions at the bioactive ceramic-bone interface. This also includes analysing the effect of soluble ions released from these bioactive substrata on the cellular response (Xynos et al. 2000a, 2001). Xynos et al. (2000b) reported that bioactive glass 45S5 stimulated osteoblast proliferation and differentiation when culturing primary human osteoblasts on the bioactive-glass ceramic substrate. Previously, Ducheyne et al. (1994, 1999) and El-Ghannam et al. (1997a) had shown this effect with a rodent cell line and shown it to be associated with cell membrane receptor activation. These findings of Xynos et al. (2000b) were

similar to our results for GB9. In order to determine whether this stimulation of osteogenesis by bioactive glasses occurs through direct contact between substrate and cells or through ions released during their resorption, they studied the relationship between this stimulation and ionic products of bioactive glass dissolution (Xynos et al. 2000a, 2001). They observed that these ionic products increased osteoblast proliferation (Xynos et al. 2000a). They suggested that this effect was mediated by insulin-like growth factor II whose expression was upregulated (Xynos et al. 2000a). They also examined the effect of ionic products on the gene expression profile of human osteoblasts and demonstrated significant up-regulation of genes including cell surface receptors, signal transduction factors, transcription factors, growth factors and extracellular matrix regulators (Xynos et al. 2001).

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 (Radin & Ducheyne 1993, 1996, Radin et al. 1997, Ducheyne & Qui 1999, El-Ghannam et al. 1999, Kaufmann et al. 2000b). Consequently, combining these two powerful analytical methodologies (advanced surface analysis methods and techniques to examine integrin mediated cell adhesion and signaling mechanisms) to study and elucidate the mechanisms by which some of these rapidly resorbable bone substitute materials induce enhanced osteoblastic differentiation, and applying this to the field of implant dentistry would be of great value, since the mechanisms by which these bioactive bone substitutes interact with bone tissue on these signaling pathways in osteoblast differentiation are at present not understood. Once these factors are identified and studied, it should be possible to alter biomaterial molecular components and surface characteristics in ways that promote optimal cell adhesion,

proliferation and differentiation, and thus, to create bone substitute materials whose surface chemistry preferentially boosts the osteogenic cascade, leading to more expeditious and enhanced bone formation in combination with rapid biodegradation of the material**.**

Another important avenue of inquiry is the correlation between the *in vitro* data reported here with *in vivo* phenomena, since it is hypothesized that enhanced expression of osteogenic markers *in vitro* leads to more and more expeditious bone formation at the bone-biomaterial interface *in vivo*. In order to test this hypothesis and, thereby, validate the *in vitro* assay employed here, correlation of the *in vitro* and *in vivo* data is required. This implies first correlating quantitative gene and protein expression of the osteogenic markers *in vitro* with quantitative histomorphometric evaluation of the amount of bone formed after biomaterials implantation. This is in addition to determining the decrease in particle size. And second quantifying the expression of these markers in histologic sections obtained from *in vivo* experiments is critical to comparing the expression of the various markers *in vitro* and *in vivo.* Histologic evaluation of the bone-biomaterial interface requires undecalcified poly-methylmethacrylate (PMMA) sections. However, until recently, it was not possible to perform immunohistochemistry and *in situ* hybridization on implant materials embedded in PMMA. To avoid these difficulties Reis et al. 1996 and Neo and coworkers (Neo et al. 1996, 1998a, 1998b) performed *in situ* hybridization on decalcified sections of bone using procollagen I (α1) and osteonectin, osteocalcin and osteopontin probes to analyze the tissue response around TCP or calciumcarbonate particles at a molecular level by visualizing active osteoblasts in the different stages of differentiation (Neo et al. 1996, 1998a, 1998b, Reis et al. 1996, Oshawa et al. 2000). This implied, however, that the implant material had to be removed from the specimens during the decalcification process. Only recently have

novel embedding resins and embedding techniques become available that permit immunohistochemical analysis on undecalcified implant materials containing sections of bone (Johansson et al. 1999, Röser et al. 2000, Gross & Kraska 2002). Figure 12 shows an example of a light micrograph of an undecalcified section of mandibular sheep bone stained for alkaline phosphatase. Immunohistochemical labeling reveals active osteoblasts expressing alkaline phosphatase.

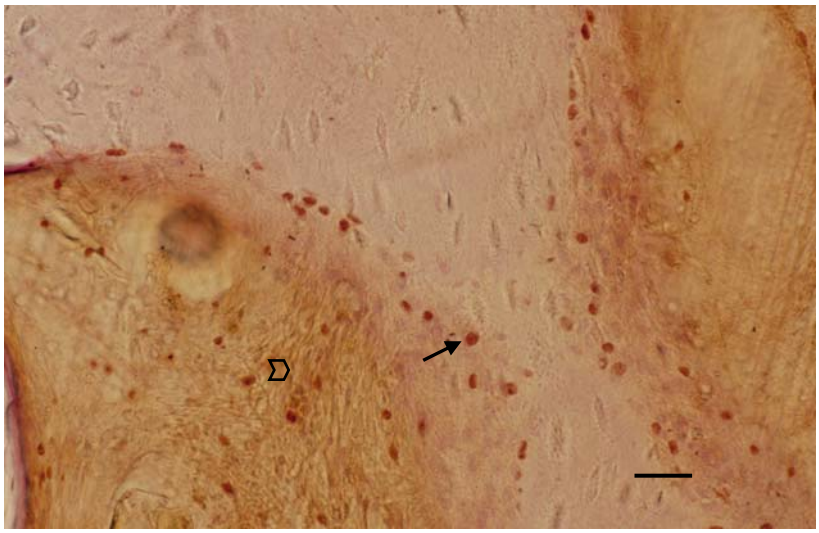
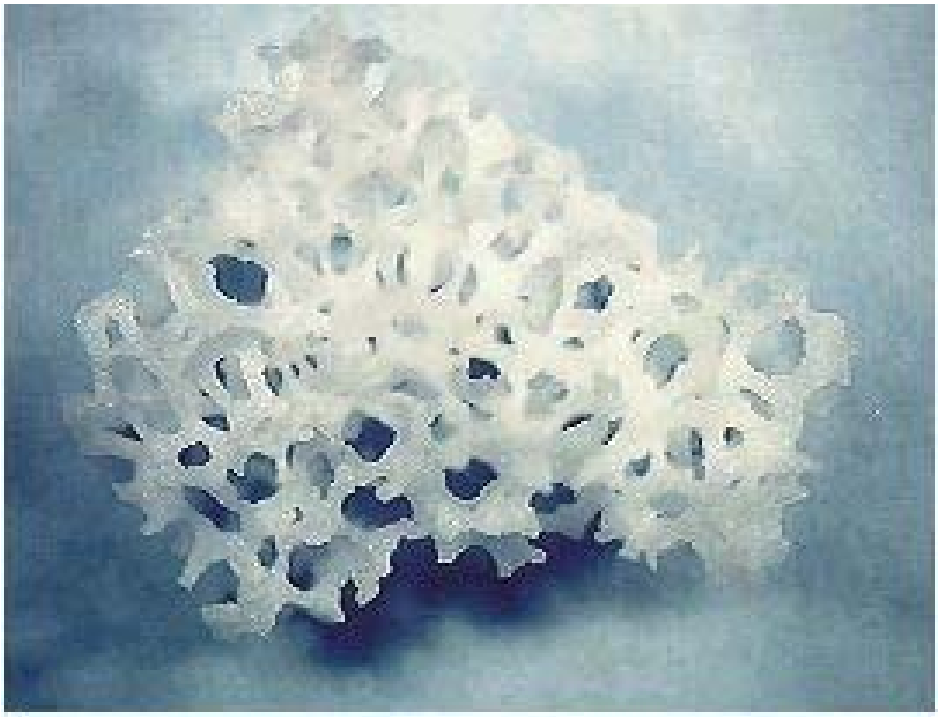


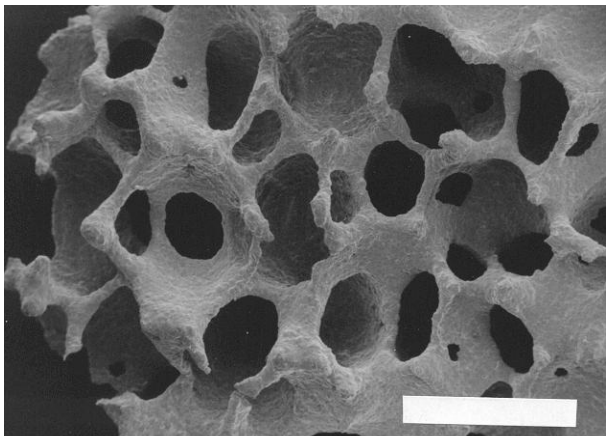
Figure 12. Light micrograph of mandibular sheep bone stained for alkaline phosphatase. The red signal indicates positive staining for alkaline phosphatase. Intensely stained osteoblasts are present indicating active osteoblasts expressing alkaline phosphatase (arrow). In addition, some diffuse matrix staining can be observed (arrowhead). Sawed section. The section was deacrylized prior to immunolabeling. Staining with hematoxylin (Appendix B6); bar, 12 µm (original magnification: x200).

These recent advances in histologic techniques facilitate characterizing the tissue response at the bone-biomaterial interface *in vivo* at a molecular level and, thus, can contribute significantly to enhancing our understanding of tissue integration of endosseous implant materials. An excellent animal model for studying these novel rapidly resorbable bone substitute materials applicable for localized ridge augmentation prior to the placement of dental implants are membrane protected critical size defects as described by von Arx et al. (2001).

An additional attractive feature of these novel calcium alkali orthophosphates is that they can be used for fabricating three-dimensional scaffolds with various pore sizes for tissue engineering purposes (Berger et al. 2003) (Fig. 13).



(a)



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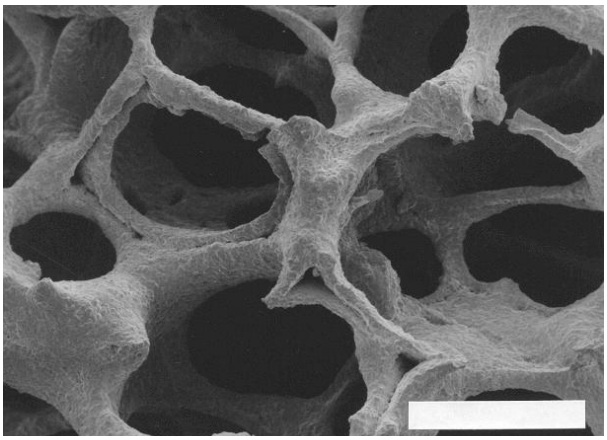

(c)

Figure 13. Three-dimensional cancellous ceramic scaffolds with various pore sizes fabricated from rapidly resorbable calcium alkali orthophosphates. (a) Photograph, (b) and (c) scanning electron micrographs. Bar = 1 mm.