cultured on CTP-S3 expressed significantly more mRNA for OP and OC than cells on Ti-DPS, HA, CTP-S1, CTP-S2 and CTZP-S1 ($p<0.04$). mRNA expression for Col Iα2 ($p<0.05$) and ON ($p<0.023$) was highest on CTZP-S1, followed by CTP-S3. On CTP-S1 surfaces, more ON mRNA was noted compared to Ti-DPS, HA, CTP-S2, CTZP-S2 ($p<0.05$). At the protein level a different pattern was observed. Cells grown on Ti-DPS, CTP-S1 and CTP-S3 expressed more OP protein than cells on HA, CTP-S2, CTZP-S1 and CTZP-S2 ($p<0.03$) (Fig. 22(d)). Protein expression by HBDC cultured on HA and CTP-S1 was significantly higher for OC than in cells on CTZP-S1, CTZP-S2 and CTP-S3 ($p<0.05$) (Fig. 22(d)). The same was true comparing BSP protein levels on HA and CTP-S1 to these on CTZP-S1 ($p<0.02$). Furthermore, protein production for Col I was significantly higher in cells grown on HA ($p<0.018$) and CTP-S3 ($p<0.002$) compared to all other surfaces. Protein expression for ON was similar for all substrata tested (Fig. 22(d)). HA had the highest cell numbers at the end of the incubation period followed by CTP-S1 (Fig. 19).

## II/4 Discussion and Conclusions Part II

### II/4.1 Discussion Study D

In implant dentistry there has been an ongoing effort to enhance and accelerate osseointegration of dental implants by optimizing their implant surface design (Keller 1998). This is related to the fact that treatment outcomes in dental implantology are critically dependent on implant surface designs that optimize the biological response during the different mechanisms by which bone becomes juxtaposed to an endosseous implant surface (Davies 1998, Keller 1998). The mechanisms by which endosseous implants become integrated in bone can be subdivided into three distinct phases (Davies 1998). The first, osteoconduction relies

on the migration of differentiating osteogenic cells to the implant surface, through a temporary connective tissue scaffold. Anchorage of this scaffold is a function of implant surface design. The second phase, *de novo* bone formation results in a mineralized interfacial matrix, being laid down on the implant surface. Implant surface topography will determine, if the interfacial bone formed is bonded to the implant. A third tissue response is that of bone remodeling, also resulting in *de novo* bone formation at discrete sites (Davies 1998). In this context, an important aspect is the influence of the implant surface on osteoblastic cell differentiation. To enhance osseous integration dental implant surfaces should possess the ability to stimulate differentiation of osteogenic cells and matrix formation at their surface. The results of a previous study examining the same implant surfaces as used in the current study showed that these materials facilitated growth of rat bone marrow cells and formation of mineralized extracellular matrix. However these experiments also pointed out the need for further functional characterization of the cellular response (Knabe et al. 2002).

Thus, this study investigated the effect of different dental implant surfaces on the temporal phenotype of HBDC. Previously, this technique to quantitatively relate the expression of bone-related mRNAs to their respective proteins as a measure of phenotypic differentiation was established (Zreiqat et al. 1996, Zreiqat & Howlett 1999).

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, Aubin 2000). Differentiating osteoblasts are known to synthesize and secrete type I collagen, alkaline phosphatase and additional non-collagenous extracellular bone matrix proteins such as osteonectin, osteocalcin, osteopontin and bone sialoprotein (Owen et al. 1990, Sodek et al. 1991, Sodek &

Cheifitz 2000, Aubin 1998a, 1998b, 2000). These bone-matrix proteins have proven to be particularly useful osteogenic markers (Sodek & Cheifitz 2000). 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. 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, 1998b, 2000). Thus, the present study quantitatively records the response of HBDC to different dental implant surfaces at four time points in terms of osteogenic genes and their translated proteins as a measure of phenotypic differentiation (Zreiqat et a. 1996, Zreiqat & Howlett 1999). Significant differences in cellular growth and the temporal expression of an array of bone-related genes and proteins were observed when identical cells were grown on the different implant surfaces. Repeatedly, it was observed that HBDC grown on a specific implant surface expressed different levels of osteogenic mRNA and the translated protein for a specific bone-related marker at a given time point so that in these instances there was no correlation in levels of mRNA and proteins expressed for the same surface. For example, at 3 days, RNA levels for Col Iα2 expressed by HBDC cultured on Ti-DPS were low (Fig. 17(a)), whereas protein expression for Col I was enhanced (Fig. 18(a)). This can be due to a number of reasons. 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). Furthermore, it is possible that the mRNA expressed at a given time point might not have been fully translated into protein yet. This is in agreement with previous findings (Zreiqat & Howlett 1999, Zreiqat et al. 1999a).

In study D, HA-coated surfaces had the most effect on the proliferation and differentiation of HBDC, inducing enhanced mRNA expression for OP and ON or OC at all time points. Further, a significantly enhanced mRNA and protein expression of all osteogenic markers tested including osteocalcin, osteopontin, osteonectin and bone sialoprotein occurred at day 7 in culture. A pattern which was also maintained at day 14 on the protein level. These four markers are characteristic for the later stages of differentiated osteoblast function and have been tightly linked to osteoid production and matrix mineralization. In addition, cell proliferation on HA surfaces was highest. Thus taken together, results obtained here suggest that HA-coated surfaces may possess a higher potency to promote osteogenesis and matrix calcification compared to the different titanium surfaces examined here in spite of the relatively lower surface roughness of HA. Thus our observations are in agreement with these of Perizzolo et al. (2001) who found increased differentiation and matrix mineralization on HA-coated surfaces. Further, our findings correspond to these of ter Brugge et al. (2002a, 2003) who also reported enhanced osteoblast differentiation with HA-coated surfaces compared to smooth and grit-blasted titanium. Moreover, our results correlate with those documented *in vivo*, whereby various histological and clinical studies showed an enhanced initial rate of osseous integration with HA-coated implants (Ducheyne 1988, Davies 1998, Caulier et al. 1997, Lacefield 1998, Keller 1998, Cochran 1999, Cochran & Buser 2000, Jansen et al. 2000, Ong & Chan 2000, Geurs et al. 2002). However, our results are in contrast to the findings of our previous study employing rat bone marrow cells in which we encountered a delayed growth pattern with HA (Knabe et al. 2002). These differences may be related to the different cells used, since reports in the literature indicate that various cell types may react differently to surface changes of HA coatings of varying crystallinity induced by

liquid immersion in tissue culture media (Frayssinet et al. 1994, Hulshoff et al. 1995, DeSantis et al. 1996, Anselme et al. 1997, Hott et al. 1997).

Ti-DPS showed a rise in expression of BSP and OC protein levels at day 3. Both bone-related proteins are phenotypic markers of the later stages of osteoblast differentiation. At this early time point protein expression of all osteogenic markers was higher on Ti-DPS compared to HA surfaces. A tendency which was not seen at later time points. This phenomenon may reflect that a lag phase is encountered with HBDC on the bioactive HA surface within the first three days of culture. Although Ti-DPS and Ti-TPS have similar surface roughness, cell numbers on Ti-DPS were higher at day 7, 14 and 21 compared to Ti-TPS, and at day 3 there was an upregulation in OC and BSP protein levels on Ti-DPS surfaces. A pattern which was maintained at 21 days at the mRNA level for OP, OC and ON expression. Furthermore, cell numbers on Ti-DPS were higher at day 7, 14 and 21 compared to Ti-TPS. These observations correspond to previous *in vitro* (Martin et al. 1995) and *in vivo* findings whereby higher alkaline phosphatase activity and increased bone-implant contact was reported for sandblasted and acid-etched surfaces compared to Ti-TPS surfaces (Buser et al. 1991, Martin et al. 1995, Kieswetter et al. 1996, Cochran et al. 1998).

Compared to the smooth titanium surfaces tested, cells cultured for 3 days on Ti-DPS expressed enhanced mRNA levels for alkaline phosphatase and osteonectin, and enhanced protein levels for osteopontin, osteocalcin and bone sialoprotein. A pattern which was maintained at day 14 at the protein level. This was accompanied by higher cell numbers at day 14 and 21, suggesting a higher potency to enhance osteogenesis. Consequently, our findings are in agreement with those noted by Mustaffa and colleagues (2001) who reported enhanced proliferation and

differentiation of cells derived from human mandibular bone when cultured on grit-blasted surfaces compared to smooth titanium.

Our observations correlate with the findings of others (Groessner-Schreiber & Tuan 1992) who reported enhanced mineralization of extracellular matrix *in vitro* for Ti-TPS surfaces compared to smooth titanium. When comparing the cellular response on Ti-TPS to that on the smooth machined surfaces, we report here the enhanced expression levels of alkaline phosphatase, osteonectin mRNA and osteopontin protein at day 3 characterizing the change from the proliferative to the post-proliferative period. The enhanced expression of osteocalcin protein or mRNA at 7, 14 and 21 days on Ti-TPS further confirms our findings.

To understand the osseous integration mechanism of implant materials, the atomic and molecular phenomena occurring at the material surface and their effects on osteoblast responses must be elucidated (Gronowicz & McCarthy 1996, Davies 1998, Keller 1998). This involves attachment of the cell to the implant material followed by osteoblast proliferation and differentiation. In our study considerable differences were detected between the amounts of osteogenic markers expressed, when identical cells were grown on the different titanium and HA-coated implant surfaces. Consequently, further exploration of the materials dependent effects reported here should involve the study of cell adhesion and intracellular signaling mechanisms (Gronowicz & McCarthy 1996, Krause et al. 2000, ter Brugge & Jansen 2002, ter Brugge et al. 2002b, Zreiqat et al. 2002) which lead to the observed differences in osteoblastic differentiation generated by the different implant surfaces. This should include characterization of integrin expression and intracellular signal transduction pathways (Gronowicz & McCarthy 1996, Krause et al. 2000, ter Brugge & Jansen 2002, ter Brugge et al. 2002b, Zreiqat et al. 2002). The nature of dental implant surfaces influences osteoblast adhesion, growth and phenotypic expression.

However, the intracellular signaling events that follow osteoblast attachment to these surfaces, are not known. Furthermore, it would be rewarding, if this was combined with physiochemical characterization of the solution-mediated surface reactions and serum protein adsorption events occurring at the surfaces of the hydroxyapatite coating, since the mechanisms underlying the enhanced *in vitro* cell responses to HA coatings are not totally understood (Keller 1998). Recent studies indicate that these cell responses appear to be associated, in part, to the degradation properties and release of calcium and phosphate ions into the biological environment (Chang et al. 2000). Furthermore, it has been hypothesized that calcium phosphate-based implant materials may increase both the adsorption and retention of macromolecular species from the biological milieu, and thus potentiate osteoconduction (Davies 1998).

In our current study, the HA-coated implant surfaces induced greater osteoblast proliferation and phenotypic expression than the roughened titanium implant surfaces. Moreover, with the sandblasted and acid-etched surfaces enhanced osteoblast differentiation and growth was found compared to the Ti-TPS surfaces. These findings are in agreement with *in vivo* observations by Wong et al. (1995) who reported superior osseointegration in terms of both pushout failure load and bone-implant contact with HA-coated implants compared to titanium implants with sandblasted and acid-etched surfaces subsequent to implantation in trabecular bone. Currently several titanium dental implant surfaces with sandblasted and acid-etched or dual acid-etched surfaces are in clinical use. These surfaces differ in their surface microtopography (Cochran & Buser 2000, Lazzara 2000, Knabe et al. 2002, Gehrke & Neugebauer 2003) by, for example, exhibiting two levels of roughness, a first level of 20-40 μm (Cochran & Buser 2000, Knabe et al. 2002, Gehrke & Neugebauer 2003) or 5-8 μm (Lazzara 2000) roughness and a superimposed second level at 8-9 μm (Gehrke & Neugebauer 2003), 2-4 μm (Cochran & Buser 2000), or 1-

3 µm (Lazzara 2000) roughness. However, experimental and clinical data comparing these surfaces to each other as well as to HA-coated surfaces are extremely scarce. To achieve implant surfaces which optimize cell and tissue responses, continued acquisition of fundamental knowledge of cell and tissue responses to specific materials characteristics is necessary (Keller 1998). Consequently, further comparative *in vitro* and *in vivo* studies evaluating these implant surfaces in the same experimental set up would appear to be of great value.

An additional novel approach to further enhance bone bonding to endosseous implants is to attach biological functional molecules covalently to the implant surface (Nanci & Puleo 1999). As outlined above, the organic component of bone is comprised of numerous extracellular matrix proteins that serve multiple roles in bone formation and homeostasis ranging from simple cell attachment (fibronectin, osteopontin, bone sialoprotein, vitronectin) to serving as nucleators for mineralization (osteopontin, bone sialoprotein) (Sodek et al. 1991, Davies 1996, Sodek & Cheifitz 2000). These extracellular matrix proteins interact with integrin receptors (Hynes 1992). Over the past decade, many of the protein segments targeted by specific integrins have been identified. The sequence arginine-glycine-aspartic acid (RGD) is the most extensively studied integrin stimulating peptide. The RGD sequence is a ubiquitous adhesive motif found in many bone extracellular matrix proteins such as fibronectin, vitronectin, type I collagen, osteopontin and bone sialoprotein. RGD-related peptides (i. e. peptides containing the RGD sequence) increase the overall adhesiveness of implant surfaces for osteoblasts. Stimulation of integrins by RGD-related peptides causes enhanced cell attachment and activation of intracellular signal transduction pathways which eventually lead to increased gene expression in the nucleus (Gerstenfeld 1999).

Over the past decade there have been increasing efforts to create biologically functional implant materials which have biological factors chemically or physically immobilized on their surfaces in order to induce specific cellular and tissue responses, and thus to create new tailored biomaterials targeted at specific cell populations in order to optimize implant integration *in vivo* and to promote long-term device integration (Hoffman 1996, Rezania et al. 1997, Yukna et al. 1998, Bhatnagar et al. 1999, Rezania & Healy 1999a, Healy et al. 2000, Ferris et al. 1999, Eid et al. 2001). However, considerable difficulties had to be overcome with regard to binding biological factors to metal substrata (Puleo & Nanci 1999). Adequate methods for binding RGD peptides covalently to titanium or titanium alloy have been developed only recently (Howlett et al. 2000, Puleo et al. 2002). Numerous *in vitro* studies have demonstrated that RGD-coated surfaces have a significant influence on osteoblast cell behavior. They cause increased cell attachment, influence cytoskeletal reorganization, cell migration and cause enhanced osteoblastic differentiation and mineralization of the extracellular matrix produced by osteoblasts (Rezania et al. 1997, Rezania & Healy 1999b, 2000, Healy et al. 2000, Kantlehner et al. 2000, Cavalcant-Adam et al. 2002, Huang et al. 2003, Zreiqat et al. 2003). The RGD motif does not target osteoblasts specifically. When short linear RGD-containing peptides are used such as RGDC (Arg-Gly-Asp-Cys) or RGDS (Arg-Gly-Asp-Ser), these peptides do not selectively stimulate a specific integrin receptor but several receptors simultaneously, viz. alpha1beta1, alpha2beta1 (the so-called collagen receptors); alpha5beta1 and the so-called vitronectin receptor alphavbeta3. In spite of these considerations a significant increase in new bone formation around RGDC peptide-modified implants was found *in vivo* (Ferris et al. 1999). These results indicated that peptide-modified metal implants may be effectively used to enhance (dental and orthopaedic) implant integration *in vivo*.

Since non-specific attachment of cells to RGD-modified surfaces is a concern, there have been ongoing research efforts to develop surfaces modified with cell attachment peptides that are selective for only osteoblastic cells and to use RGD-containing peptides that possess a higher potency than the short linear peptides as RGDC (Arg-Gly-Asp-Cys) or RGDS (Arg-Gly-Asp-Ser) (Kantlehner et al. 2000). Binding peptides to implant materials offers several advantages over binding larger biomolecules like growth factors (as for example bone morphogenetic proteins). The use of large, intact growth factors and extracellular matrix proteins for coating applications is limited by stability, availability, and expense. Small peptides can be fabricated synthetically to very high purity, are not dependent on tertiary structure for bioactivity, and are relatively inexpensive to produce (Ferris et al. 1999). In general, RGD-containing peptides are widely applied in various medical fields. For example, they are used for tumor targeting to inhibit tumor metastasis and angiogenesis in tumors (Pasqualini et al. 1997, Dechantsreiter et al. 1999), to treat renal failure by inhibiting tubular obstruction (Noiri et al. 1994), are used to modify biomaterials for cardiovascular applications (Tweden et al. 1995), in dermatology to improve woundhealing (Greziak et al. 1997) and to treat ophtalmologic sequelae arising from autoimmunodiseases (Pierschbacher et al. 1994). In addition to short linear RGD-containing peptides such as RGDC (Arg-Gly-Asp-Cys) or RGDS (Arg-Gly-Asp-Ser), a number of longer linear and cyclic RGD-containing peptides (i.e. peptides containing a larger number of amino acids) have been developed. These peptides target specific integrin receptors, for example the collagen receptor alpha2beta1 (Aumailley et al. 1991, Cardarelli et al. 1992, Ivaska et al. 1999) or alphavbeta3 (the vitronectin receptor) (Koivunen et al. 1995, Dechantsreiter et al. 1999, Kantlehner et al. 2000). Several studies have shown that, if osteoblasts attach to surfaces via the collagen receptor alpha2beta1, enhanced gene expression of various bone-related proteins

representing the osteoblastic phenotype takes place in addition to enhanced cell attachment (Xiao et al. 1997, 1998, Gerstenfeld 1999). If osteoblasts attach via the vitronectin receptor alphavbeta3, only increased cell attachment was observed (Gerstenfeld 1999). Furthermore, a manifold higher potency was found for certain cyclic peptides compared to linear peptides (Aumailley et al. 1991, Koivunen et al. 1995, Kumagai et al. 1991).

The mechanisms underlying the enhanced cell and tissue responses to RGD-coated surfaces are currently not fully understood. Further research efforts are required to elucidate the intracellular signaling events that mediate these cell and tissue responses. Consequently, the use of RGD peptides may constitute another relevant approach for optimizing the surface characteristics of endosseous implant materials.

### II/4.2 Conclusions Study D

All examined dental implant surfaces significantly affected cellular growth and the temporal expression of an array of bone-related genes and proteins. HA-coated titanium had the most effect on osteoblastic differentiation inducing a greater expression of an array of osteogenic markers than recorded for cells grown on Ti-DPS and Ti-TPS, thus suggesting that the HA-coated surface may possess a higher potency to enhance osteogenesis. Furthermore, Ti-DPS surfaces induced greater osteoblast proliferation and differentiation than Ti-TPS.

### II/4.3 Discussion Study E

Cell and tissue response to implant materials is one of the most important themes in the field of biomaterials (Lemons 1988, Ducheyne et al. 1992, de Bruijn et al. 1994, Geesink 1994, Davies 1996, Ducheyne 1988, 1998, Keller 1998, Jansen et

al. 2000, de Groot et al. 2002, Ong et al. 2002, Barrrere et al. 2003). Implanted bioactive calcium phosphate ceramics are known to bond directly to bone (Ducheyne 1988, 1998, Lemons 1988, Denissen et al. 1991, 1996, Smukler-Moncler et al. 1992, de Bruijn 1994, Dhert 1994, Geesink 1994, Davies 1996, Keller 1998, Burgess et al. 1999, Jansen et al. 2000, de Groot et al. 2002, Ong et al. 2002). However, differences in these materials are reflected in the rate of bone formation on their surfaces (Lemons 1988, Ohgushi et al. 1990, Ducheyne 1998, Ducheyne & Qui 1999, Jansen et al. 2000, de Groot et al. 2002). Ideally, bioactive ceramics for use in bone regeneration should possess the ability to stimulate bone formation (Ohgushi et al. 1990, Davies 1996, Ducheyne & Qui 1999, Jansen et al. 2000). This in turn requires the ability to cause differentiation of stromal bone marrow cells into osteoblasts at their surfaces (Ohgushi et al. 1990, Ducheyne & Qui 1999). This study investigated the temporal molecular phenotype of HBDC grown on various novel bioactive calcium titanium and calcium titanium zirconium phosphates. Previously, the technique to quantitatively relate the expression of bone-related mRNAs to their respective proteins as a measure of phenotypic differentiation was established (Zreiqat et al. 1996, 1999, Zreiqat & Howlett 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, Aubin 1998a, 2000, Sodek & Cheifitz 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). mRNA for 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, Aubin 1998a, 2000, Sodek & Cheifitz 2000). Thus, study E quantitatively records the response of HBDC to various endosseous implant materials at four time points in terms of osteogenic genes and their translated proteins as a measure of phenotypic differentiation (Zreiqat et al. 1996, 1999a, Zreiqat & Howlett 1999).

The different ceramic substrata tested, significantly affected cellular growth and the temporal expression of an array of bone-related genes and proteins. In agreement with previous findings (Zreiqat et al. 1996, 1999a, 1999b, Zreiqat & Howlett 1999), we repeatedly found no correlation between mRNA and protein expressed for the same material tested at a given time point. For example, in the present study and at 7 days, mRNA levels for osteocalcin expressed by HBDC cultured on HA were low, whereas protein expression for osteocalcin was enhanced. The lack of correlation between levels of mRNA and proteins expressed can be due to a number of reasons. 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. Furthermore, it is possible that the mRNA expressed at a given time point might not have been fully translated into protein yet. In the current study, protein levels on the plasma-sprayed HA surfaces peaked at day 7. These observations are similar to those reported previously. When examining different bioceramic substrata, Zreiqat et al. (1999a) found that protein levels on dense polycrystalline HA peaked at day 10 and decreased at day 14, when at the same time differences in mRNA levels were not as pronounced. These findings correlate with those reported by ter Brugge and

colleagues (2003) who found that alkaline phosphatase levels of rat bone marrow cells cultured on various HA coatings peaked at day 8.

In the study E, CTP-S3 had the most effect on the differentiation of HBDC, inducing osteopontin, bone sialoprotein and osteonectin protein expression at day 3 in culture. These 3 markers are characteristic for the later stages of differentiated osteoblast function. In general, HBDC cultured on CTP-S3 resulted in an enhanced expression of osteopontin, osteocalcin and osteonectin mRNA compared to the other substrata. These three bone-related proteins have been tightly linked to osteoid production and matrix mineralization, suggesting that CTP-S3 may possess a higher potency to promote osteogenesis and matrix calcification than Ti-DPS and the other ceramics tested. Moreover, CTP-S3 induced enhanced protein expression of all osteogenic markers at day 7, while, at 7 and 14 days, cell numbers on CTP-S3 were lower than on Ti-DPS, indicating that stimulation of cell differentiation over proliferation had occurred.

Cell numbers on CTP-S1 were higher compared to all other novel ceramic substrata tested, this was accompanied by an enhanced expression of osteopontin mRNA and protein at 3 days characterizing the change from the proliferative to the post-proliferative period. Furthermore, at day 7 CTP-S1 induced enhanced osteopontin and osteonectin mRNA expression as well as enhanced protein expression of all osteogenic markers. Also at day 21, enhanced protein levels for osteopontin, osteocalcin and bone sialoprotein, the 3 markers characteristic for the later stages of osteoblast differentiation, were noted suggesting that CTP-S1 might possess the potency to induce osteogenesis.

All novel calcium titanium and calcium titanium zirconium phosphates facilitated the expression of the osteoblastic phenotype at least as much as Ti-DPS rendering them candidate implant materials for producing calcium phosphate

coatings on titanium substrata. As a result, these data provide evidence for the biocompatibility of the novel calcium titanium and calcium titanium zirconium phosphates at a molecular level. Furthermore, these findings are in agreement with those reported previously (Knabe et al. 1999). When employing a rat bone marrow cell culture system and tetrazycline labeling to determine ECM mineralization, we observed good cell growth and elaboration of mineralized extracellular matrix on CTP-S1, CTP-S2 and CTZP-S1 after 14 days (Knabe et al. 1999). Here it would appear rewarding to pursue the temporal changes in extracellular-matrix mineralization on the different substrata by applying a quantitative tetracycline labeling assay as proposed by Todescan et al. (1996) or $^{45}Ca$ labeling.

Both surface chemistry, and surface morphology and roughness are important properties which influence cell-biomaterials interactions and cell differentiation (Lacefield 1999, Boyan & Schwartz 2000, Howlett et al. 2000). However, it is not always easily possible to dissociate the roughness parameters from the surface chemistry or solution-mediated surface reactions of calcium phosphate ceramics (Keller 1998, Perizzolo et al. 2001). In study E, HA supported the expression of the osteoblastic phenotype to a higher degree than Ti-DPS, although HA exhibited a considerable lower degree of surface roughness than Ti-DPS. Furthermore, although CTP-S1, CTP-S2, CTZP-S2 and CTP-S3 specimens were very similar in surface roughness, considerable differences in gene and protein expression of osteogenic markers were noted. Thus the differences in cellular differentiation observed were likely to be related to the effect of surface chemistry of these materials rather than their surface roughness. Whereas CTP-S1 and CTP-S3 had a greater surface roughness and a greater effect on osteoblast differentiation than Ti-DPS and HA, suggesting a possible synergistic effect of surface roughness and surface chemistry.

To understand the bone biomaterials bonding mechanism, the atomic and molecular phenomena occurring at the material surface and their effects on the reaction 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. It is plausible to speculate that observed differences in osteoblastic differentiation generated by the different bioceramics seen in this study are related to differences in integrin expression levels which in turn regulate specific intracellular signal transduction pathways (Gronowicz & McCarthy 1996, Krause et al. 2000, Zreiqat et al. 2002). First studies to address this issue are currently underway. Furthermore, it would be rewarding, if this was combined with characterization of the solution-mediated surface reactions and protein adsorption events occurring at the surfaces of these novel bioactive ceramic substrata (Ducheyne & Qui 1999), since taken together this could significantly contribute to unraveling the complexity of the reactions at the bioactive ceramic-bone interface.

Equally important is the correlation between the *in vitro* data reported here with *in vivo* findings (Keller 1998). First *in vivo* experiments studying the amount of bone formation and the expression of these osteogenic markers subsequent to implantation of these novel calcium phosphate coatings in the sheep femur are currently underway.

### II/4.4 Conclusions Study E

All substrates examined in study E had a significant effect on cellular growth and the temporal expression of an array of osteogenic genes and proteins. Since all novel calcium titanium and calcium titanium zirconium phosphates facilitated the expression of the osteoblastic phenotype at least as much as the titanium surface,

these ceramics can be regarded as potential implant materials for producing calcium phosphate coatings on titanium substrata. Hence, their biocompatibilty has been demonstrated at a molecular level. Surfaces of CTP-S1 and CTP-S3 had the most effect on osteoblastic differentiation inducing a greater expression of an array of osteogenic markers than recorded for cells grown on Ti-DPS and HA, thus suggesting that these novel implant materials may possess a higher potency to enhance osteogenesis. Consequently, CTP-S1 and CTP-S3 appear to be promising bioceramics for producing calcium phosphate coatings on titanium substrata.