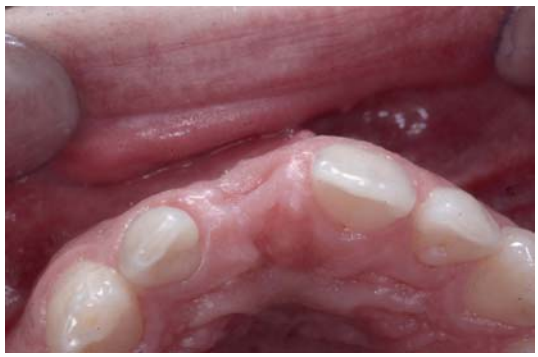
Part I

Effect of rapidly resorbable bone substitute materials on osteoblastic cell differentiation *in vitro*

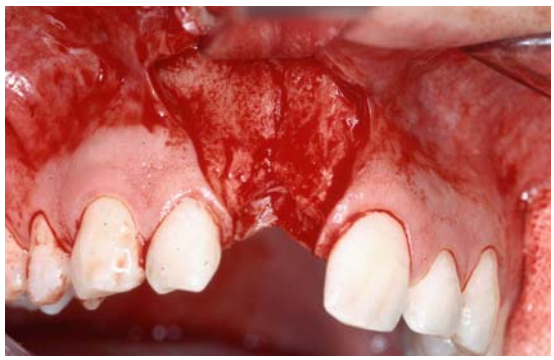
I/1 Introduction Part I

The use of oral implants has become a common treatment to replace missing or lost teeth (Belser et al. 2000, Bornstein et al. 2003). When teeth are missing, the surrounding bone and soft tissue is challenged as a result of the natural resorptive process subsequent to extraction. Consequently, resorption of the alveolar ridge after tooth extraction frequently mandates site development by augmentation before implants can be placed (Belser et al. 2000, Ganz & Valen 2002, Winkler 2002).

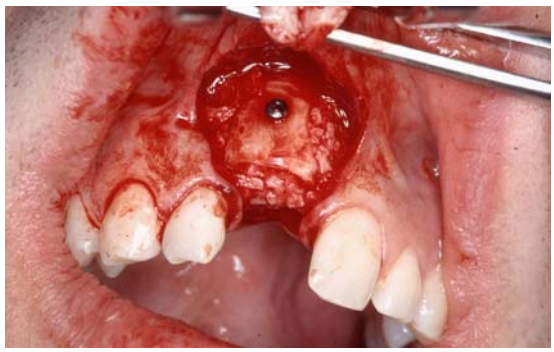
High success rates have been demonstrated for dental implants placed in bone that was previously augmented with autografts and barrier membranes. Thus, the clinical results of implants in regenerated bone have been found to be comparable to those of implants in non-regenerated bone (Buser et al. 2002). 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 (Belser et al. 2000, Winkler 2002). 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 prothesis and not solely by bone anatomy (Belser et al. 2000) (Fig. 1). This



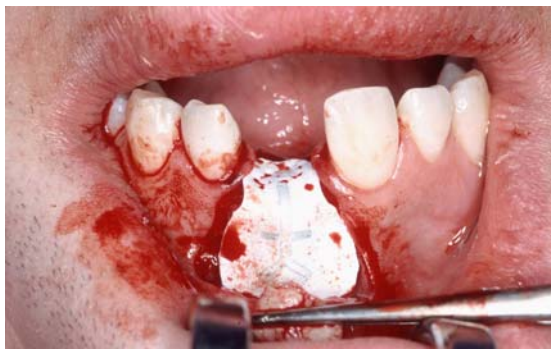
(a) Patient presenting with missing maxillary right central incisor. Ridge contour is concave as a result of the natural resorptive process subsequent to extraction. Occlusal view demonstrates horizontal deficiency of the alveolar ridge requiring site development by augmentation before an implant can be placed.



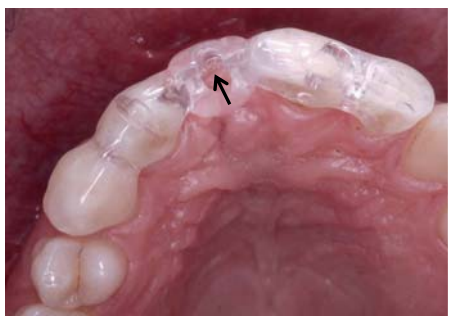
(b) The ridge is exposed for bone grafting. Frontal view shows horizontal deficit. Ridge contour is concave. Dimensions and extent of missing alveolar bone is easily visualized.



(c) The autogenous bone graft which has been harvested in the retromolar area of the mandible is placed to augment the labial aspect of the ridge, and secured in position with a retaining screw.

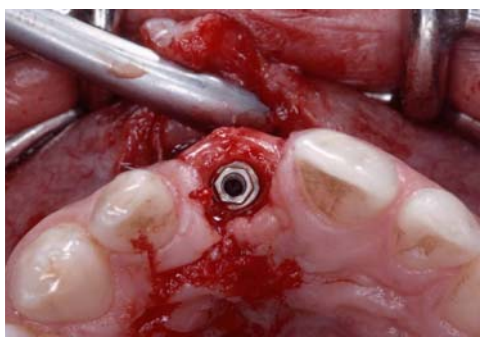


(d) A nonresorbable membrane is then placed over the graft.



(e) After the graft has been allowed to heal, the patient returns for implant placement. The ridge form shows an adequate contour to place the implant in the position (arrow) prescribed by the surgical guide stent which has been fabricated according to the prosthodontic requirements for the design of the implant superstructure.

(f) Following the removal of the membrane and the retaining screw, the implant bed is prepared. The pilot hole is drilled into the bone with aid of the surgical template to guide the accurate placement of the implant.



(g) Occlusal view shows implant in position at appropriate depth. The implant position has been dictated by the surgical guide stent. The ridge form shows an adequate contour. If grafting had not been performed, it would have been impossible to have the implant placed in the ideal location to allow fabrication of an aesthetic implant-borne restoration.

Figure 1. Clinical photographs demonstrating the principle of "Restoration-driven implant placement." Surgical photographs are a courtesy of PD Dr. Dr. M. Stiller.

implies diagnostic waxing on articulated diagnostic casts and fabrication of surgical templates (Spiekermann 1994, Belser et al. 2000, Taylor et al. 2000). Consequently, the prosthodontic requirements for the design of the implant superstructure (rather than the bone volume available) dictate the position in which the dental implants have to be placed (Fig. 1). This has been called "Restoration-driven" implant placement (Garber 1995, Garber & Belser 1995, Belser et al. 2000, Taylor et al. 2000) (Fig. 1). Thus, augmentation of the alveolar ridge before implant placement is frequently required in implant dentistry (Belser et al. 2000, Ganz & Valen 2002, Winkler 2002, Eisig et al. 2003).

The current gold standard for bone reconstruction in implant dentistry is the use of autogenous bone grafts (Buser et al. 1994, 1998a, 1999a, von Arx et al. 2001, 2002). Among the various techniques to reconstruct or enlarge a deficient alveolar ridge, the concept of guided bone regeneration (GBR) (Buser et al. 1994) has become a predictable and well-documented surgical approach (Buser et al. 1999a). The need for localized ridge augmentation prior to the placement of dental implants has been one of the clinical indications for GBR (Buser et al. 1994). At present, autogenous bone grafts are preferably combined with barrier membranes (Buser et al. 1996, Buser et al. 1999a, von Arx et al. 2001). These autografts have been used to reduce the defect volume, thereby stabilizing the blood clot (Friedmann et al. 2002), and to support the membrane as a space-maintaining device, thus preventing their collapse into large defects (Buser et al. 1994, Buser et al. 1998a, Hämmerle et al. 1998, Kohal et al. 1999). 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 (Stricker et al. 2003, Timmenga et al. 2003). 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 (Kalk et al. 1996, Wheeler 1997, Kaptein et al. 1998, Orsini et al. 2004). Using synthetic biodegradable bone substitutes as a membrane-supporting device would simplify GBR, since it avoids second-site surgery for autograft harvesting (Buser et al. 1998a, von Arx et al. 2001). This is also true for sinus floor elevation procedures (Kalk et al. 1996, Wheeler 1997, Kaptein et al. 1998).

Relatively rapid biodegradation of synthetic biodegradable bone substitutes is desirable, especially prior to dental implant placement, because ideally new bone should form leaving no residual particles that may interfere with preparation of the implant bed at surgery (von Arx et al. 2002). Bioactive calcium phosphate ceramics and bioactive glasses are candidate biomaterials which qualify as bone substitutes for this kind of application, since they are widely used in orthopaedics (Metsger et al. 1982, Hollinger et al. 1996, Yaszemski et al. 1996, Ducheyne 1998, Hench 1998). Synthetic, i.e. alloplastic bone substitute materials, are superior to freeze-dried human allografts and bovine deproteinized bone xenografts due to their safety in terms of disease transmission and immunological aspects (von Arx et al. 2001, Orsini et al. 2004). With biomaterials of human or animal origin it is not possible to completely eliminate the risk of virus and prion contamination (Ouhayoun 1995, Hildebrandt 2002).

Among the ceramics most commonly investigated for use in bone regeneration are β-tricalcium phosphate (β-TCP) (Metsger et al. 1982, Saffar et al. 1990, Hollinger et al. 1996, Yaszemski et al. 1996), hydroxyapatite (HA) (Ducheyne & de Groot 1981, Jarcho 1981, Eggli et al. 1988, Daculsi et al. 1989, LeGeros 1994, Yaszemski et al. 1996, Ducheyne 1998) and bioactive glass (Hench & Paschall 1973, Ducheyne 1998, Hench 1998). All of these materials are biocompatible (Hollinger et al. 1996, Metsger et

al. 1982, Yaszemski et al. 1996) and osteoconductive (Klawitter & Hulbert 1971, Ducheyne & de Groot 1981, Metsger et al. 1982, Eggli et al. 1988, Hollinger et al. 1996, Yaszemski et al. 1996, Ducheyne 1998, Hench 1998, Merten et al. 2001, Schliephake & Kage 2001, Zerbo et al. 2001, Cordioli et al. 2002, Tadjoedin et al. 2002, Wiltfang et al. 2002). However, they differ considerably in the rate of resorption. HA resorbs very slowly compared to β-TCP (Holmes et al. 1987, Eggli et al. 1988, Yaszemski et al. 1996) and bioactive glass (Schepers & Ducheyne 1997, Ducheyne 1998, Hench 1998, Tadjoedin et al. 2002). Recently, the use of tricalcium phosphate 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 (Tadjoedin et al. 2000, 2002, Cordioli et al. 2001, Merten et al. 2001, Schliephake & Kage 2001, Zerbo et al. 2001, Wiltfang et al. 2002). Even with β-TCP, biodegradation has been reported to be incomplete 9.5 months after grafting in the human mandible (Zerbo et al. 2001). Histologic examination of these biopsies revealed that 34% of the biopsy consisted of mineralized bone tissue and 29% of remaining β-TCP (Zerbo et al. 2001). Biopsies sampled at 8 months after sinus floor augmentation consisted of 20% mineralized bone and 44% remaining β -TCP (Zerbo et al. 2001). With respect to Bioglass 45S5 (BG) particles of a narrow size range, Tadjoedin et al. (2002) reported that after grafting in the human sinus floor BG particles appeared to resorb within 1-2 years. This was by dissolution rather than by osteoclastic activity (Tadjoedin et al. 2002). When using mixtures of 80, 90, and 100% BG particles and 20, 10 and 0% autogenous bone, histomorphometric analysis of biopsies harvested at 4, 6 and 15 months showed the grafts to consist of 27% of mineralized bone tissue at 4 months, of 36% of bone at 6 months and of 42% of bone at 15 months. 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

(Tadjoedin et al. 2002). Thus, compared to the bone substitute materials which are currently clinically available (Tadjoedin et al. 2000, 2002, Cordioli et al. 2001, Zerbo et al. 2001), there is a significant need for bone substitute materials which degrade more rapidly, but still stimulate osteogenesis at the same time (Hürzeler et al. 1996, Wallace et al. 1996, Wetzel et al. 1996, Valentini & Abensur 1997, Lorenzetti et al. 1998, Groeneveld et al. 1999, von Arx et al. 2001). Particularly in non-load-bearing applications such as alveolar ridge augmentation, a biomaterial used as a bone substitute should be a temporary material serving as a scaffold for bone remodeling. The material must degrade in a controlled fashion into non-toxic products that the body can metabolize or excrete via normal physiological mechanisms (Yaszemski et al. 1996). Moreover, this substance should be rapidly resorbable and should undergo complete remodeling and substitution by newly formed functional bone tissue in view of placing dental implants in such augmented sites (Hürzeler et al. 1996, Wallace et al. 1996, Wetzel et al. 1996, Valentini & Abensur 1997, Lorenzetti et al. 1998, Groeneveld et al. 1999, von Arx et al. 2001, Wiltfang et al. 2002).

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 calcium-alkali-orthophosphate materials and glass ceramics (Berger et al. 1995a, 1995b, 2003, Reif et al. 1998, Ignatius et al. 2001). These are β-Rhenanite and its derivatives; glassy crystalline calcium alkali orthophosphates, which exhibit stable crystalline $Ca_2KNa(PO_4)_2$ phases; and novel glass ceramics (Schneider et al. 1994, Berger et al. 1995a, 1995b, 2003, Ignatius et al. 2001, Reif et al. 1998). These materials have a higher solubility than tricalcium phosphate (TCP). They are designed to exhibit a higher degree of biodegradability compared to TCP (Berger et al. 1995a,

1995b, 1997, 1998, Reif et al. 1998) and therefore could be excellent alloplastic materials.

To fill bone defects, calcium phosphates are mainly applied as granules. Bone substitutes with improved surgical handling properties include moldable calcium phosphate cements in paste form that can be either introduced into a bony defect with a spatula or injected with a syringe. They set in situ thereafter, which makes them an intriguing group of new materials for bone reconstruction (Khairoun et al. 1997, 1999, Schmitz et al. 1999, Niedhart et al. 2001, Nilsson et al. 2002, Ooms et al. 2002, 2003). A novel, calcium phosphate cement was developed whereby calcium deficient hydroxyapatite (CDHA) is formed during setting (Khairoun et al. 1997). This cement was designed for high biodegradability (Khairoun et al. 1997, 1999, Knabe et al. 2000). Compared to the rapidly resorbable calcium-alkali-orthophosphates the surgical handling is easier but degradation is slower. Thus, this material is advantageous for one stage procedures when dental implant placement and bone augmentation can be performed simultaneously.

Ideally, bioactive ceramics 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 (Ohgushi et al. 1990, Hench 1998, Ducheyne & Qiu 1999). The use of *in vitro* osteogenic cell cultures has proven valuable for initial biological testing of endosseous implant materials (Davies 1996). Quantitative evaluation of bone-related genes and their respective proteins in putative osteoblast grown on different biomaterials facilitates gaining insight into the effect of endosseous implant materials on osteoblastic cell differentiation (Zreiqat & Howlett 1999, Zreiqat et al. 1999a). This is related to the quantitative *in situ* hybridization and immunocytochemical techniques which permit study of the expression of markers of the osteoblast phenotype (Zreiqat et al. 1996), therefore

generating valuable information concerning the osteogenic capacity of candidate implant materials (Zreiqat & Howlett 1999). Consequently, such an *in vitro* assay is useful for screening novel bioactive bone substitute materials. Furthermore the information gained may have important implications in the design of novel biomaterials (Zreiqat & Howlett 1999).

Differentiating osteoblasts are known to synthesize and secrete type I collagen, alkaline phosphatase (ALP), a marker of preosteoblasts (Rodan & Rodan 1984, Sodek & Cheifitz 2000) and other non-collagenous extracellular matrix proteins such as osteonectin, osteocalcin, osteopontin and bone sialoprotein (BSP) (Table 1) (Termine et al. 1981, Fisher & Termine 1985, Fisher et al. 1990, Strauss et al. 1990, Pockwinse et al. 1992, Sodek et al. 1991, Aubin 1998a, 1998b, 2000, Sodek & Cheifitz 2000). These non-collagenous matrix proteins are known to play an important role in the process of bone matrix mineralization (Fisher & Termine 1985, Owen et al. 1990, Sodek et al. 1991, Aubin 1998a, 2000, 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). Osteoblast culture studies demonstrated that mRNA of collagen I 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 and osteocalcin occurs later during the third period of extracellular-matrix mineralization (Owen et al. 1990, Strauss et al. 1990, Gerstenfeld et al. 1990, Pockwinse et al. 1992, Sodek & Cheifitz 2000). Osteopontin peaks twice during proliferation and then later but prior to BSP and osteocalcin (Aubin 2000, Sodek & Cheifitz 2000). Osteonectin is found in preosteoblasts, osteoblasts, osteocytes and newly formed osteoid in matrix (Termine et al. 1981, Bianco et al. 1988). This protein

Table 1 List of major collagenous and non-collagenous proteins found in bone

Extracellular Matrix Proteins Synthesized By Human Bone-Derived Cells in vitro

Protein	**MW**	**Properties**	**Reference**
Type I Collagen (Col I)	~ 32kDa	Principal structural component of the bone ECM, cell attachment via RGD sequence	Eyre 1980, Glimcher 1989
Type III Collagen (Col III)		Low levels (2-5% total)	Auf'mkolk et al. 1985
Osteonectin (ON)	33 kDa (from sequence)	Phosphorylated glycoprotein, binds calcium	Termine et al. 1981, Lan & Sage 1990
Osteopontin (OP)	32 kDa (from sequence)	Phosphoprotein, cell attachment via RGD sequence, binds to hydroxyapatite	Franzen & Heinegard 1985, Flores et al. 1982
Osteocalcin (OC)	5,8 kDa	Binds Ca^{2+} and hydroxyapatite, vitamin D responsive, mineralization related, involved in osteoclast recruitment/differentiation	Price et al. 1981, Hauschka et al. 1989
Bone Sialoprotein (BSP)	33,6 kDa (from sequence)	Phosphoprotein binds to cells and hydroxyapatite through RGD sequence	Franzen & Heinegard 1985
Fibronectin (Fn)	550 kDa	Cell attachment via RGD sequence, interacts with glycosaminoglycans	Weiss & Reddi 1980, 1981, Heinegard & Oldberg 1989, Grezik & Robey 1994

Important Cellular Protein

Alkaline Phosphatase (ALP)	140 kDa (homodimer)	Mineralization, a marker of preosteoblasts	Eyre 1980, Glimcher 1989

has the ability to bind to collagen and promote hydroxyapatite formation *in vitro* (Termine et al. 1981). Bone sialoprotein (Franzen & Heinegard 1985) is characterized by its ability to mediate initial formation of hydoxyapatite crystals (Oldberg et al. 1988) and is transiently expressed very early (Malavel et al. 1999, Aubin 2000, Sodek & Cheifitz 2000) and then upregulated again in differentiated osteoblasts actively involved in mineralization (Chen et al. 1992, Malavel et al. 1999, Aubin 2000, Sodek & Cheifitz 2000). These bone-matrix proteins have proven to be particularly useful osteogenic markers (Sodek & Cheifitz 2000). Consequently, because there is no specific single marker for osteoblasts, relevance has to be placed upon the cellular expression of a range of non-collagenous and collagenous bone-related proteins as well as alkaline phosphatase, when examining cellular differentiation.

These studies investigate the effect of a series of novel rapidly resorbable calcium phosphates, glass ceramics and a calcium phosphate bone cement as compared to α-TCP on the expression of bone-related genes and proteins by human bone-derived cells (HBDC). Thereby the effect of these novel bone substitute materials on osteoblastic cell differentiation is evaluated.

I/2 Materials and Methods

I/2.1 Test Materials

I/2.1.1 Test Materials Study A

In study A, four calcium phosphate materials which were created from β-Rhenanite ($CaNaPO_4$) and its derivatives were tested and compared to α-TCP ($Ca_3(PO_4)_2$): β-Rhenanite ($CaNaPO_4$) was denominated R1. Other materials resulted from modification of $CaNaPO_4$ using magnesium or potassium phosphate or silicate.