# Forschung · Wissenschaft
# Recherche · Science




***Publisher***
***Herausgeber***
***Editeur***
*Schweizerische Zahnärzte-Gesellschaft SSO*
*Société Suisse d'Odonto-Stomatologie*
*CH-3000 Bern 7*



***Adresse der wissenschaftlichen Redaktion***
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# Surface-induced modulation of human mesenchymal progenitor cells

*An in vitro model for early implant integration*


## Summary

*Clinical experience indicates that the surface architecture of dental implants has an important impact on their integration. This has been related to the finding that differentially treated substrates can modulate the expression of osteogenic markers in various bone-related cell lines and primary cells. Here, we investigated the influence of surface architecture on the differentiation of human mesenchymal progenitor cells (HMPC) from adult bone marrow, i. e. the cells likely involved in initial bone synthesis at the bone-implant interface. Cells were seeded on machine surfaced (MS) or sandblasted/acid etched (SE) titanium discs in agarose-coated dishes, and on polystyrene (PS) controls. On all substrates cell densities did not change between days 7 and 14. Cell numbers were higher on SE, likely due to increased attachment to the rougher material. Alkaline phosphatase activity (ALP) was similar on all substrates, whereas mRNA expression of bone sialoprotein (BSP) at day 14 was about tenfold higher on SE ($p<0.05\%$). The SE-related increase of BSP in progenitor cells indicates an earlier differentiation of immigrated cells and could thus explain earlier implant integration and shorter time to functional loading observed in the clinic. The in vitro model and BSP quantification could be used to screen for changes in osteogenic cell differentiation induced by specific implant surfaces, with potential relevance on the prediction of bone-implant integration.*


*Schweiz Monatsschr Zahnmed 117: 906–910 (2007)*

*Key words: dental implant, surface, bone, mesenchymal progenitor cells, bone sialoprotein*

*Accepted for publication: 29 May 2007*

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## Introduction

Dental implants anchored directly in the jaw are increasingly becoming an integral part of dental treatment (Meffert et al. 1992, Belser et al. 1998, Esposito et al. 2005). The various available systems differ in material, shape and surface texture, which all may influence the velocity of integration especially during the early phase as indicated by experimental studies assessing mechanical stability and histomorphology (Buser et al. 1991, Cochran 1999, Kasemo & Gold 1999, Shalabi et al. 2006a). Depending on the site of implantation, the quality of bone and the design of the implant, loading is possible within a few weeks

after implantation (Buser et al. 1999, Salvi & Lang 2001, Klinger et al. 2006). In parallel, implant surfaces have been screened in vitro for their capacity to modulate cell functions relating to osseointegration, such as cell adhesion, differentiation or mineralization (Schwartz & Boyan 1994, Schwartz et al. 1999). Indeed, when grown on rougher surfaces a variety of mainly immortalized and non-human primary cells have been observed to increase markers related to bone formation, such as cellular alkaline phosphatase (ALP), deposition of extra-cellular calcium and expression of bone matrix proteins such as collagen type I, osteonectin, osteopontin or bone sialoprotein (Schneider et al. 2003, Schwartz et al. 2005). Though surface-related stimulation of osseonintegrative functions in vitro may be consistent with clinical reports on surface-related modulation of implant integration, the varying cell sources, changing seeding densities and different culture conditions used in such studies make it difficult to relate them to each other and eventually to specific stages of osseointegration.

Since the in vitro models used so far may not well resemble the situation of early osseointegration in humans, we aimed at investigating early cell-implant interactions in an in vitro model better emulating these conditions at the bone-implant interface. This model is based (i) on the use of human primary mesenchymal progenitor cells (HMPC) known to migrate to the implant site (Davies 1996, Davies 2003, Franchi et al. 2005), (ii) on avoiding excessive seeding densities to prevent early cell confluence and (iii) on cultures on Ti-discs placed in agarose-coated wells, where redistribution of cells detached from the adjacent surface area is unlikely.

## Material und Methods

### Chemicals and cell cultures

Unless mentioned specifically, chemicals were from Fluka/Sigma (CH-9470 Buchs), cell culture media from Gibco (Life Technologies, Gaithersburg, MD 20877) or from Seromed (Biochrom KG, Berlin, D) and culture dishes and plastic ware were from Falcon or Nunc (Life Technologies, Gaithersburg, MD 20877).

### Cultivation of human mesenchymal progenitor cells (HMPC)

Bone marrow aspirates were obtained from two non-related male donors, 50 and 56 years old, following exposure of the iliac crest during maxillofacial routine intervention after informed consent and in accordance with the local ethical committee. HPMC from each donor were expanded separately in complete medium (CM) containing alpha-MEM with 10% fetal bovine serum (GIBCO-BRL Life Technologies, Basel, CH), 4.5 mg/ml D-Glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml Streptomycin and 0.29 mg/ml L-glutamine supplemented with 5 ng/ml FGF-II and 10 nmol of Dexamethasone for two passages as described in detail (Frank et al. 2002). Before use, six-well polystyrene cell culture plates (Falcon) were coated with low-melting agarose (1% w/v%) in order to avoid reattachment of detached cells. Briefly, culture plates were quickly rinsed with molten agarose and the excess removed before hardening. Culture plates left to dry over night under ultraviolet light displayed a thin layer of agarose on the bottom and the walls of the wells. Pure titanium discs (height 2 mm, diameter 10 mm, gift from Friatec AG, Mannheim, Germany), which were machine surfaced (MS: Cat. 10-5010) or sandblasted/acid etched (SE: Cat. 10-5016) were then fixed on the bottom of the coated well with a drop of fibrin glue (100 mg Fibrinogen/ml, 500 IU Thrombin/ml, Baxter, Austria). Cells were seeded at 3000 cells/cm$^2$ into the disc-containing wells and into the uncoated wells of the control cultures.

Cells from each donor were grown on titanium discs and controls in duplicate samples at osteogenic conditions, i.e. in CM additionally containing 10 nM Dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate and 10 mM beta-glycerophosphate. Cultures were harvested after seven days and 14 days, respectively.

### Determination of DNA content, alkaline phosphatase (ALP) activity and bone sialoprotein (BSP) expression

After removing Ti-disc cultures from the six-well polystyrene culture plates, they were rinsed with PBS and then placed into wells containing 0.01% SDS where their cells were scraped off. Control dishes were rinsed with PBS before adding 0.01% SDS and scraping-off the cells from the bottom of control culture wells. While Ti-discs were regenerated as described earlier (Baschong et al. 2001), appropriate aliquots of the SDS-lysates were used for DNA quantification and determination of ALP activity. DNA was quantified by means of a Cy-Quant kit (Molecular Probes, Leiden, NL) as indicated by the manufacturer and with calf thymus DNA as a standard. ALP activity was measured as the rate of conversion of p-nitrophenyl phosphate using the SIGMA-Kit 104 (Sigma-Aldrich, Buchs SG, Switzerland ) accordingly and expressed as nanomoles of p-nitrophenol/min/microgram DNA (for details: Frank et al. 2002).

Gene expression was assessed using real-time RT-PCR as described previously (Frank et al. 2002). In short, RNA was extracted from scratched-off cells using Trizol (Life Technologies, Basel, CH), treated with DNAse using the DNA-free™ Kit (Ambion, USA) and reverse-transcribed into cDNA by using random hexamers and Stratascript™ reverse transcriptase (Stratagene, NL). PCR reactions were performed and monitored using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Rotkreuz, CH). In the same reaction, cDNA samples were analyzed both for BSP and the housekeeping gene (18-S rRNA) using a multiplex approach (Perkin Elmer User Bulletin No. 2), as previously described (Frank et al. 2002). Expression levels of each gene of interest were calculated by normalizing the quantified RNA amount to the 18-S rRNA and by further dividing the resulting value by that previously obtained in human osteoblasts (average of five donors) using an identical procedure ($2^{\Delta\Delta Ct}$ formula, Perkin Elmer User Bulletin No. 2). Cells from each donor were assessed separately in each condition and in at least two experimental series.

### Scanning electron microscopy

Titanium surfaces were imaged using a Hitachi-9000 scanning electron microscope at 20 kV.

### Statistics

In each experimental series, values were normalized as n-fold differences of the PS control at day 14. Differences between values obtained at the various conditions were analysed for significance ($p < 0.05$) using the Mann-Whitney test for non-parametric comparison of independent samples (SPSS Software, SPSS, CH-8044 Zurich, Switzerland).

## Results

The effect of an implant's surface structure on the initiating phase of bone neosynthesis at the bone implant interstitium was emulated by directly culturing HMPC cells under osteogenic conditions

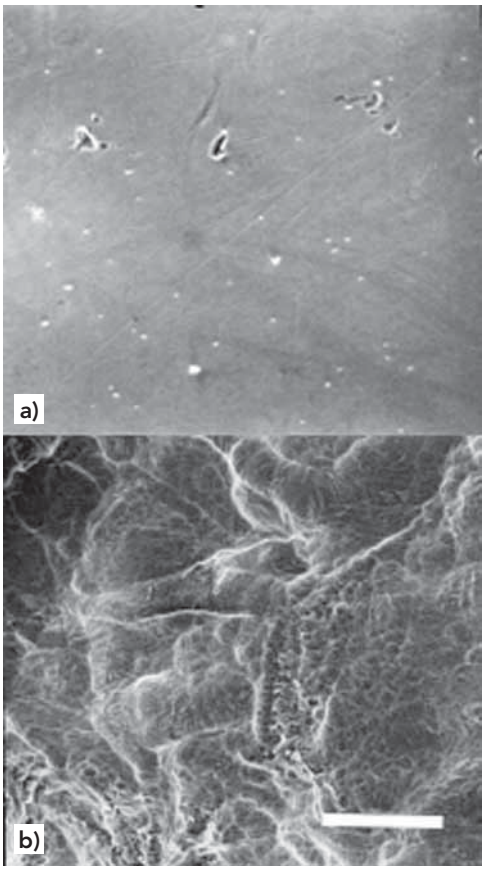


*Fig 1 Surface architecture of pure titanium discs. Scanning electron micrographs of a) machine surfaced (MS) and b) sandblasted/acid etched (SE) surfaces. Scalebar = 50 micrometers.*

on machine surfaced (MS, Fig 1, top) and sandblasted/acid etched (SE, Fig 1, bottom) pure titanium discs, i.e. on surface structures corresponding to those used also in commercial implants.

### Cell number

The number of cells growing on the three different surfaces was assessed via the amount DNA/cm$^2$ (Fig 2, top), i.e. DNA per base area of the titanium discs (MS, SE) or of the surface of the non-coated control dish, respectively. On any substrate, the number of cells did not change between days 7 and 14 in culture. The amount of cells growing on PS and MS were comparable; cell number on SE was about five-fold higher ($p < 0.05$).

### Alkaline phosphatase (ALP)

ALP activities (Fig 2, middle) were determined enzymatically and related to the amount of DNA determined in each substrate in order to exclude effects on cell growth or adhesion. At day 7, the average cellular ALP activities measured on MS or SE and on PS varied considerably, yet without statistical significance. After 14 days the average cellular ALP activities had attained practically identical values on any of the three surfaces (MS, SE, PS).

### Bone sialoprotein (BSP)

BSP expression was quantified by real-time RT-PCR. Values were related to the total amount of extracted RNA and further normal-

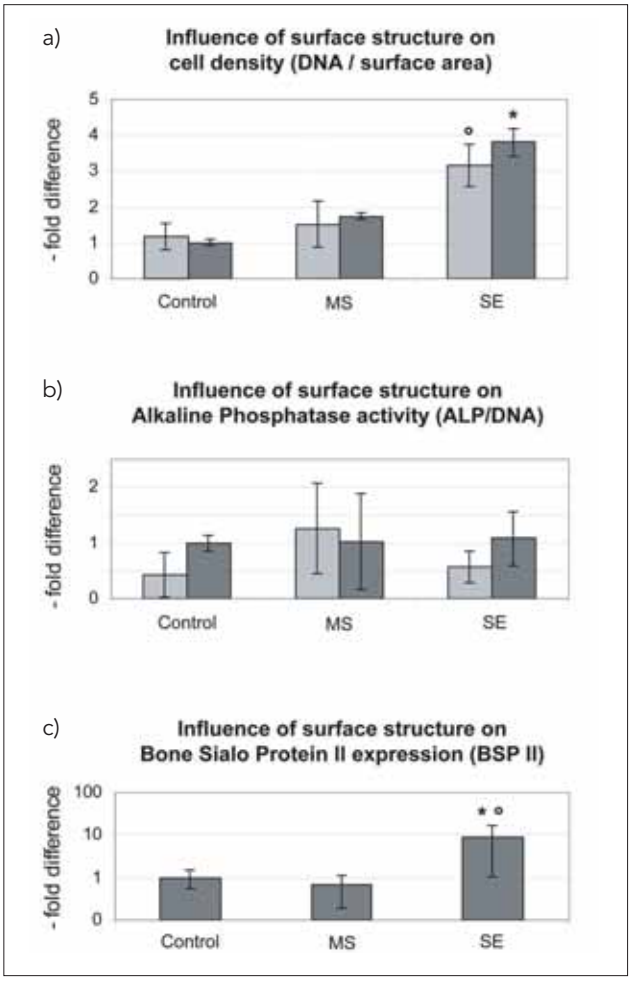


*Fig 2 Surface modulation of human mesenchymal progenitor cells (HMPC). HMPC isolates from two adult donors were grown under osteogeneic conditions on machine surfaced (MS) or sandblasted/acid etched (SE) titanium discs and on polystyrene (PS, control) separately and at least in two experimental series. Cell densities a) expressed as DNA/area and b) alkaline phosphatase acivitity (ALP) were measured in each series after 7 or 14 days in culture and normalized to the PS control at day 14 (PS-14) and then pooled ($n = 5$) and expressed as n-fold difference from PS-14. Expression of bone sialoprotein mRNA c) was measured only at day 14. White columns: day 7, black columns: day 14. Error bars correspond to standard deviations. Statistical evaluation: * difference significant ($p < 0.05$) versus PS-14, ° $p < 0.05$ versus PS-7.*

ized to the average expression level measured in normal osteoblasts from human adults. As shown in Figure 2 (bottom), BSP expression on MS compared well to that in the PS control, while BSP expression on the SE surface had attained almost tenfold higher values. This higher expression of BSP on the structured SE surface proved of statistical significance ($p < 0.05$) when compared to that on PS or on MS using the Mann-Whitney test for non-parametric comparison of independent samples.

## Discussion

The ease of surgical insertion, the capability to match function and aesthetics of the natural teeth, the durability of the implant

and the time required until functional loading influences form and structure of dental implants. On the one hand, it is obvious that optimal solutions for such interdependent requirements have to be thoroughly elaborated and that appropriate in vitro models would be advantageous for optimizing implant form and structure. On the other hand, it is also clear that it will be barely possible to design an in vitro model representative for all the processes involved in implant integration, although experimental evidence and clinical experience have documented time and again that appropriately structured surfaces can reduce the time to functional loading (Del Fabbro et al. 2006, Shalabi et al. 2006b).

In consequence, we focussed our investigation on the influence of the implant surface on bone neosynthesis between days 7 and 14 upon seeding and more specifically on surface-induced pre-osteoblast differentiation during this time window. In contrast to others, we mimicked this situation by using human primary mesenchymal progenitor cells (HMPC), i.e. the type of cells immigrating to colonize the implant surface upon implant insertion (Davies 1996, Davies 2003, Franchi et al. 2005). HMPC isolates were from two adult donors in their fifties, i.e. the typical age of implant recipients. We kept the initial seeding density at the few thousand cells/cm$^2$ applied also in monolayer cultures (Frank et al. 2002) and abstained from micro mass cultures (Schneider et al. 2003) to avoid the possibility of phenotypic modulation by early confluence. Moreover, by fixing the titanium discs into agarose coated culture wells, we aimed at avoiding reattachment of possibly surface-modulated cells.

In our model, cell densities did not change between days 7 and 14 indicating confluence and absence of cell proliferation. Expectedly, the density on SE was signifcantly higher than on MS or PS which agrees with a more efficient initial attachment to the larger surface area of SE-treated discs.

The lack of surface-related changes of ALP activity/DNA/area contrasts to the surface-dependent stimulation of ALP, typically observed at confluent conditions using osteoblast cultures or other cell systems (Boyan et al. 2002, Schwartz et al. 2005). Yet, in our model, a transient surface-related stimulation of ALP expression before the first measurement at day 7 cannot be excluded. Indeed, osteogenic differentiation of HMPC on titanium surfaces and thus peak levels of ALP have been reported to be anticipated in comparison to osteoblast cultures (Mendes et al. 2004). Thus, regulation of ALP activity may differ in cells from different sources or grown at different conditions.

In contrast to the absence of demonstrated ALP modulation, BSP mRNA expression on SE was about tenfold higher than on MS or PS. Elevated BSP expression under osteogenic conditions was reported already for HMPC monolayer cultures. There, it proved to be a more relevant marker for progressing differentiation along the osteogenic lineage than ALP (Frank et al. 2002). Furthermore, expression levels of BSP together with Osteopontin and the transcription factor Osterix were demonstrated to be predictive for in vivo bone formation, in particular when HMPC cultures were grown in three-dimensional ceramic scaffolds (Jaquiery et al. 2005). This however could not be shown for expression of ALP (Mendes at al. 2004).

In our experiments, up-regulation of BSP expression on SE confirmed the many reports on surface-related osteogenic differentiation (Boyan et al. 2002, Schwartz et al. 2005, Franchi et al. 2005, Del Fabbro et al. 2006). This higher expression of BSP on SE may correlate with a selective initial attachment of more susceptible HMPC, an effect more evident in our model since cell redistribution over time is restricted by the agarose coating. Alternatively, a similar effect could be expected by SE absorbing higher amounts of autocrine and serum factors, thus achieving local concentrations high enough to induce further commitment of the HMPC growing on the SE surface. An enhancement of osteoblast-related genes such as BSP by a SE Ti-surface was observed also in vivo with fetal bone marrow isolates from rat (Ogawa & Nishimura 2003), yet not in vitro when cultivating established human palatal mesenchymal cells as micro mass cultures (Masaki et al. 2005).

In conclusion, we report an in vitro model and a read-out parameter (i.e., expression of BSP) emulating the early phase of bone formation at the bone-implant interface. Using this model, we demonstrated that SE-treatment of titanium surfaces promotes osteogenic differentiation of HMPC, which could explain in vivo observations of enhanced bone/implant integration. The proposed model could be used to screen for implant surfaces inducing specific changes on osteogenic cell differentiation, as a possible alternative to more costly and time-consuming in vivo studies.

## Zusammenfassung

Beobachtungen in der Klinik weisen darauf hin, dass die Oberflächenbeschaffenheit eines Zahnimplantates einen wesentlichen Einfluss auf dessen Integration ausübt. In der Tat zeigen verschiedenste Knochenzellen *in vitro* eine erhöhte Konzentration knochentypischer Marker, wenn sie auf raueren Oberflächen wachsen. Hier wurde der Einfluss der Oberflächenstruktur auf die Differenzierung mesenchymaler Vorläuferzellen (human mesenchymal progenitor cells, HMPC) aus dem Knochenmark adulter Spender untersucht, d.h. an den Zellen, die typischerweise nach Insertion vom Knochenmark zur Implantatoberfläche einwandern. HMPC wurden auf maschinenpolierten (MS) oder sandgestrahlten/säuregeätzten (SE) Titanplättchen in mit Agarose ausgekleideten Kulturschalen aus Polystyrol kultiviert. Leere Schalen (PS) dienten als Kontrolle. Die Proliferation änderte sich zwischen Tag 7 und 14 auf keiner der drei Oberflächen. Auf SE befanden sich wesentlich mehr Zellen, wohl wegen erhöhter Haftung. Keine der Oberflächen beeinflusste zwischen Tag 7 und 14 die Aktivität der alkalischen Phosphatase (ALP). Die BSP-Expression auf SE war nach 14 Tagen rund zehnmal höher ($p < 0{,}05\%$).

Eine Stimulierung der BSP-Synthese durch rauere Oberflächen spricht, im Einklang mit den Beobachtungen in der Klinik, für eine frühere Differenzierung der einwandernden HMPC und in der Folge für eine frühere Integration und Belastbarkeit eines Implantates. Die Verwendung von HMPC und BSP als Differenzierungsmarker könnte als *In-vitro*-Modell zur Auswahl von Implantatoberflächen dienen.

## Résumé

L'expérience clinique indique que l'architecture de surface d'un implant dentaire a une influence importante pour son intégration. Au niveau de surfaces plus rugueuses, les cellulles osseuses montrent une activité élevée des marques des synthèse osseuse. L'objectif de l'étude était l'évaluation de cette modulation induite par des surfaces différentes sur des cellules humaines mésenchymateuses progénitrices (human mesenchymal progenitor cells, HMPC), c'est à dire les cellules qui migrent de la moelle osseuse vers la surface de l'implant pour y initier la néoformation osseuse. Des cellules HMPC ont été cultivées sur des disques en titane avec des surfaces usinées (MS) ou sablées et attaquées à l'acide

(SE). Sur l'ensemble des surfaces la densité des cellules n'augmentent pas entre sept et 14 jours. Le nombre de cellules était plus élevé sur SE, vraisemblablement en raison de l'amélioration de l'attachement sur une surface plus rugueuse. Entre les jours 7 et 14 l'activité de la phosphatase alcaline (ALP) n'était modulée par aucune surface. Au 14e jour l'expression de la Bone sialoprotéine (BSP) était presque dix fois plus élevée sur SE ($p < 0,05\%$).

L'augmentation du BSP sur SE pourrait correspondre à une différentiation plus avancée des cellules sur la surface rugueuse et pourrait expliquer l'intégration plus précoce de l'implant autorisant la charge mécanique plus rapide observée en clinique. Ce modèle utilisant les HMPC et le BSP comme marqueur de différention pourrait être utilisé pour la sélection d'une surface optimale des implants dentaires.

## Acknowledgement

We thank Martha Imholz for technical assistance and the M.E. Mueller Foundation and the Kanton Basel-Stadt for support.

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