European Cells and Materials Vol. 2. 2001 (pages 1-9) DOI: 10.22203/eCM.v002a01 ISSN 1473-2262

THE INFLUENCE OF PORE GEOMETRY IN CP TI-IMPLANTS – A CELL CULTURE INVESTIGATION

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Abstract

Biocompatibility testing of differently structured titanium implants was performed using an *in vitro* test system of a newly established human fetal osteoblastic cell line (hFOB 1.19). Cell adhesion of osteoblastic cells on the different porous geometries and the suitability of a copper vapor laser system for surface structuring was tested with the following parameters: cell-number, cell viability, alkaline phosphatase expression.

The analysis of the cell culture results demonstrated that 25µm and 200µm porous geometries showed similar or even better results than the negative control of polystyrene; there was no sign of toxic effects. However, the 100µm porous geometry showed an impressive negative influence on the calculated parameters. The reason for this effect is unclear.

The series with 50µm, 300µm, 400µm and 500µm showed a comparable, intermediate effect on the cell culture with respect to the different parameters. However, the results were worse than with the 25 and 200µm porous geometry. In conclusion, the 25µm and 200µm porous geometry seems to have the most positive effect on the human osteoblastic cell line hFOB 1.19.

Key Words: Topography, copper vapor laser, porous geometry, cell culture, titanium, osteoblasts.

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Introduction

The success of cementless total hip arthroplasty is dependent both on the measure of primary mechanical stability, related to the precise shaping of the bony cavity, and on secondary stability achieved through the direct adsorption of bone cells at the implant surface. Such secondary stability is affected by the material's biocompatibility, the design of the implant components, the osteogenic potential of the accommodating bone, the surgical technique and on the stress forces applied at the bone-implant interface (Skalak, 1983). It is completed in two phases. The initial bony ingrowth into the implant surface is subsequently followed by "bone remodeling", an adaptation to the new weight-bearing situation (Morscher, 1987; Søballe, 1993; Thomas and Cook, 1985). Both *in vivo* and *in vitro* studies suggest that osteoblasts independently from mechanical stresses favor ingrowth into rough and porous surfaces while a smooth surface even with biocompatible materials impedes the initial adsorption of osteoblasts (Schmitz, 1991). Copper vapor laser technology for structuring of micromaterials has allowed for the development of a porous surface structure with pore diameters up to 1-5µm. This has permitted the development of defined, reproducible and economical surface topographies (Bergmann *et al*., 1994). In the following *in vitro* study we examine the application of the copper vapor laser system in structuring the implant surface and discuss the influence of pore geometry at the cell culture level.

Materials and Methods

The human fetal osteoblast cell line hFOB 1.19 used was obtained from Dr. Thomas C. Spelsberg, Mayo-Clinic, Rochester, MN, USA. This cell line is transfected with a gene that codes for the temperature sensitive mutant (tsA58), SV 40 T-antigen, together with a gene coding for gentamycin resistance (Harris *et al*., 1995). Osteoblasts were subcultured to a 12/14 cycle. Cells were cultured in DMEM (Dulbecco´s modified essential medium) with 10% FCS (Fetal Calf Serum). The medium was changed every second day until the end of the experimental period on days 12 to 17. The laser-structured materials to be tested were incubated in 24 well plates with 50,000 cells/well at a temperature of 34°C in a humidified incubator containing 5%CO_2. The hFOB 1.19 cell lines were maintained in the DMEM medium until a confluent layer of cells was obtained. At that time, in most cases approximately on day

3, the medium was changed to BGJb medium (Fitton Jackson modification) (Hendrich *et al*., 1996a,b).

After removal of the medium the 24 well plates were rinsed two times with 1 ml PBS (phosphate-buffered saline) to withdraw the non-adherent cells. Additionally 0.5ml Trypsin/EDTA-solution was given to the cells for 5 minutes. The trypsin effect was stopped using 0.5 ml of the culture medium followed by aspiration of the cells for ten times using an Eppendorf pipette. It could be demonstrated randomly by use of the Live-Dead Viability Assay (Molecular Probes, Eugene, OR, USA) that no vital cells remained on the surface. *Cell number* was determined using an electronic cell counter (CASY 1, Mod. TTC, Schärfe System, Reutlingen, Germany). The system works according to the resistance principle combined with an additional pulse area analysis and a signal scanning frequency of 1 MHz. Equipped with a multichannel analyzer with 512000 channels, this system allows an extremely sensitive, reproducible, and nearly error free analysis of the size distribution of a whole cell population within 1 min. For analysis a 100µL aliquot of the cell sample was directly diluted 1:100 in a phosphate-buffered saline (PBS) based electrolyte-solution and gently mixed. Then an exactly adjustable aliquot was automatically measured by the system (Seewöster and Lehmann, 1997).

C*ell viability* was demonstrated using the Live-Dead-Viability assay (Molecular Probes). The main principle of the test is to demonstrate the esterase activity in intact cells and the proof of cell membrane permeability. Intact cells incorporate the polyanionic fluorescent dye calcein-AM and metabolize the dye to a green fluorescing calcein. The red dye ethidium-homodimer penetrates destroyed membranes and binds nucleic acids. According to this theory intact cells reflect green, whereas dead cells reflect red.. The test procedure was performed using 4µM ethidium-homodimer and 2µM Calcein-AM in a PBS-solution, each of them prepared from a fresh stock solution. After 5 days in cell culture the medium was suctioned off. Unspecific esterases of the FCS-medium were removed by rinsing the plates two times with PBS. Subsequently, the cultured cells were covered with 100 µl test solution and incubated for 20 minutes at room temperature. Fluorescence microscopy was performed with a Zeiss axioscope (Zeiss, Oberkochen, Germany) using a blue filter with a wave-length of 450 and 490 nm. Photodocumentation was done at 50-, 100- and 200 times magnification with a Zeiss MC 63 exposure automatic machine and a scotch chrome daylight film 800/3200 P at 800 ASA.

C*ell viability* of the implant adherent osteoblasts, which demonstrates ongoing mitochondrial metabolism, was examined using the MTT-test method. Here the color of the WST-reagent was changed to a red color by mitochondrial dehydrogenase (succinate dehydrogenase) in the citric acid cycle (Formazan) or due to a reaction of NADH at the plasma membrane and the presence of this was determined photometrically at 450nm (Ishiyama *et al*., 1995). This investigation was completed using the WST-1 test (Boehringer Mannheim, Germany). Incubation of the osteoblasts with 500µl DMEM and 50µl WST-1 reagent at 34°C in a humidified incubator containing 5% CO_2 was completed for 30 minutes. Determination of the quantity of formed Formazan was performed using an ELISA reader (SLT, Crailheim, Germany) read at a wavelength of 450nm (Liu *et al*., 1995).

Alkaline phosphatase activity was determined spectrophotometrically in the ELISA reader (SLT, Crailheim, Germany) at a wavelength of 405 nm, related to the amount of p-nitrophenols. The method of Bradford (Bradford, 1976) was used to measure the total protein thereby permitting the calculation of specific alkaline phosphatase activity (U/mg).

Scanning electron microscopy (SEM) examination of the various structured surface areas (100 – 2000 magnification) was done after 1.5 culture days. After removal of the culture media the cells were rinsed for 1min with Sörensen Buffer (pH 7.4). Primary fixation of the cells was carried out in 6.25% glutaraldehyde for 15 h. Rinsing was done 5 times for 5 minutes in Sörensen-Buffer (pH 7.4). After primary fixation, the dehydration procedure was done using 30% ethanol for 15 min, 50% ethanol for 20 min, 75% ethanol for 30 min, 100% ethanol for 45 min and 100% ethanol 5 times for 30 min. Finally the samples were critical-point dried. The specimen were mounted onto stubs with silver paint and coated with 8 nm of gold. Scanning electron microscopical examination of the specimen was executed with a Zeiss DSM 940 with an accelerating voltage of 1 to 30 kV.

Implant: The titanium slices were obtained from Brehm Co., Weisendorf, Germany. Outer surface structuring was produced using a copper vapour laser system with a mean output of 120 - 140W, a maximum pulse-frequency of 6.5 kHz (ATZ-EVUS, Vilseck, Germany) and with nanosecond precision control of the stimulation point using a so called Master-Timing-System. Lasers with a fixed wavelength of either 511 or 578nm were used. The pore size was measured on the cross sections of the specimens using backscattered scanning electron microscopy. The influence of variations of the parameter pulse frequency (fp), pulse power (Pp), focal distance (F), focus position (z) and number of pulses on the drill hole geometry was tested (Mayerhofer, 1997; Bergmann *et al*., 1996). The beard around the drill-holes was grinded using grind paper with a roughness of 1200 and 2400. Subsequently, the specimen were cleaned in an ultrasound bath and sterilized according to the FDA 100 criteria (Food and Drug Administration, Rockville, MD, USA) including gamma-sterilization at 28 kV. Surface characteristics of the rough-blasted implants (Al_2O_3 beads 500 – 710µm) were measured using a fine needle perthometer (Fa. Mahr, Göttingen, Germany). Additionally, the surface characteristics of the laser-structured implants and the rough-blasted implants were analyzed using 3 D analysis by laser electron microscopy (Leica Microsystems, Heidelberg, Germany).

50 round, one-sided, laser structured titanium slices (cpTi) were manufactured with an outer diameter of 15.5mm (surface area = 188.6mm^2), a central hole (area=0.8mm^2) and an open pore surface area with a defined pore-diameter, -depth, -geometry and -interpore spacing (Table 1).

Each titanium slice had a thickness of 2mm. The interpore distance was determined from the midpoint pore-to-pore distance. The ground area of each titanium slice

Titanium	Pore-depth in µm	Pore-diameter in µm	Pore-to-pore distance in µm
500µm	500	500	1500
400µm	400	400	1200
300µm	300	300	900
200µm	200	200	400
100µm	100	100	200
50µm	50	50	100
25µm	25	25	50
Polystyrene	Polystyrene as negative control		

Table 1. Porous geometry on the titanium discs

excluding the central hole was 187.9mm^2.

Mathematical analysis was performed to determine the increase in porous surface area available for cell growth for the various pore sizes. This was based on the pore geometry (Total surface area = structured surface area + area of the pore wall). The 200µm pore has a cylindrical form (increase in surface area factor, magnification factor, (MF), 1.78), whereas all other pores had either a conical or elliptical form. The increase in surface area available for growth was smallest in the case of a conical geometry (MF, 0.31), whereas with an elliptical shape there was an MF of 0.71. The shape of both the 25 and 50µm pores can only be considered approximately as either elliptical or conical and was assigned an MF of 0.5 (Table 2).

Surface / pore-geometry	*25 µm*	*50 µm*	*100 µm*	*200 µm*	*300 µm*	*400 µm*	*500 µm*
Number of pores / titanium disc	75160	18790	4697	1174	231	130	84
Additional surface area dependent on the porous geometry in mm^2							
cylindrical (MF 1.78)	147.5	147.5	147.5	147.5	65.3	65.3	65.9
conical (MF 0.31)	45.7	45.7	45.7	-	20.25	20.25	20.34
elliptical (MF 0.75)	110.6	110.6	110.6	-	48.97	48.97	49.42
mean (MF 0.5)	73.75	73.75	73.75	-	32.65	32.65	32.95

MF = Magnification factor

Table 2. Surface area and surface magnification of the titanium discs.

For each porous surface 2 to 3 independent readings were obtained for each of the defined parameters on alternate culture days (day 2, 4 ,6, 8, etc.). Mean values were calculated relative to the polystyrene standard value. Cell number, viability and alkaline phosphatase activity were calculated in relation to the polystyrene levels. Due to the small number of values a confirmatory statistic test was not indicated, whereas a certain difference was evident.

Results

The morphological behavior of osteoblasts at the cell-implant interface is the first qualitative parameter of implant biocompatibility. Osteoblasts on the 25µm, 50µm, 200µm, 300µm, 400µm and 500µm porous surfaces were predominantly of an elongated, favorable cell formation. In Figure 1 we show osteoblast alignment at a pore. Such migration occurs possibly either due to a mechanical or a electrochemical effect. Cell numbers obtained on the laser-structured surface varied with surface topography (Figure 2). Table 3 shows the corresponding datasheet.

The highest cellular proliferation was obtained on titanium slices with a pore geometry of 50µm, 300µm, 400µm and 500µm. These surfaces demonstrated a comparable or slightly better increase in cell growth than polystyrene controls. Cell number on the 50µm porous surface was lower

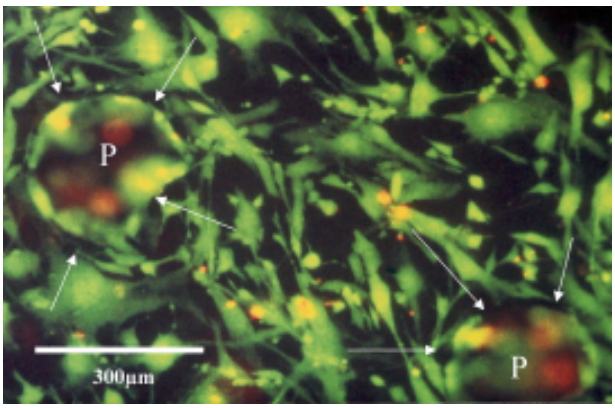

Figure 1. Cellular growth on a 300 µm implant surface, day 4 in culture (P = pore).

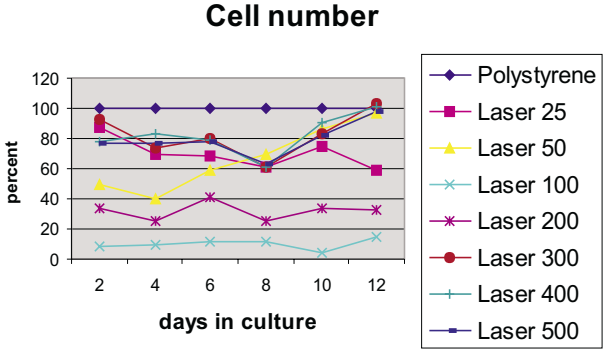

Figure 2. Influence of pore geometry on cell number (expressed as a % of the polystyrene control).

Type of surface	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Polystyrene	100	100	100	100	100	100
Laser 25	87(77-92)	70(37-86)	68(61-76)	61(4-83)	75(69-84)	59(27-68)
Laser 50	49(29-59)	40(36-48)	59(49-65)	70(62-75)	86(82-93)	97(87-107)
Laser 100	9(6-13)	9(0-16)	12(9-14)	12(6-18)	5(4-6)	14(10-21)
Laser 200	34(19-48)	25(22-30)	41(27-60)	25(12-41)	34(16-45)	32(7-49)
Laser 300	93(91-95)	73(68-77)	80(79-82)	61(58-62)	83(69-98)	103(97-105)
Laser 400	78(74-81)	83(77-90)	79(77-80)	61(58-65)	91(90-93)	102(97-106)
Laser 500	77(71-82)	76(69-84)	78(77-79)	63(62-64)	82(66-93)	98(92-106)

mean (min – max) in %

Table 3. Datasheet corresponding to Figure 2.

at earlier time points and thereafter continuously increased. Cell number on the 500µm porous surface was minimally lower than those on the 300µm and 400µm surfaces. Cell number on the 25µm surface did not reach the level of the 50µm, 300µm, 400µm or 500µm pores, although cell numbers were higher at early time points. The implant series with a 100µm porous structure demonstrated the poorest cellular development. Although titanium slices with a 200µm pore structure supported more cell development when compared with 100µm surface, this proved to be significantly less optimal than titanium slices with 50µm, 300µm, 400µm and 500µm pores.

Results of cell viability determinations correlate with the results of the cellular counts. However, relative activity of cell viability is influenced by the amount of cells. Figure 3 and Table 4 show the relative amount of cell viability in relation to the number of cells.

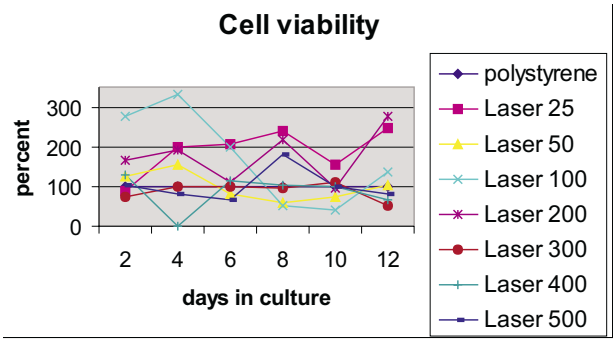

Figure 3. Relative cellular vitality (expressed as a % of the polystyrene control).

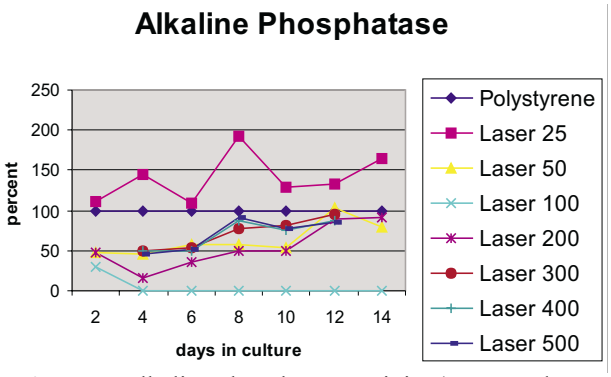

Figure 4. Alkaline phosphatase activity (expressed as a % of the polystyrene control).

Type of surface	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Polystyrene	100	100	100	100	100	100
Laser 25	87	198	206	241	156	247
Laser 50	124	155	81	59	74	103
Laser 100	278	333	200	50	40	135
Laser 200	165	192	110	216	97	275
Laser 300	75	101	100	95	112	52
Laser 400	130	-	114	105	98	66
Laser 500	105	82	65	179	101	80

mean (min – max) in %, - = no value

Table 4. Datasheet corresponding to Figure 3.

Type of surface	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Polystyrol	100	100	100	100	100	100	100
Laser 25	111(104-113)	145(135-155)	110(106-115)	192(152-223)	129(116-143)	133(131-135)	166(159-172)
Laser 50	48(31-61)	47(35-58)	57(48-70)	57(46-65)	53(50-57)	103(90-120)	79(72-89)
Laser 100	30(30)	0	0	0	0	0	0
Laser 200	48(30-61)	15(0-23)	35(27-48)	50(42-58)	51(46-57)	90(55-115)	91(60-117)
Laser 300	-	50(47-54)	54(45-62)	77(71-83)	82(77-87)	95(95-96)	-
Laser 400	-	50(49-52)	50(47-54)	87(84-90)	76(76-77)	88(84-92)	-
Laser 500	-	45(34-56)	51(45-57)	91(89-93)	77(76-78)	85(81-89)	-

mean (min – max) in %, - = no value

Table 5. Datasheet corresponding to Figure 4.

Osteoblasts on the 25µm surfaces demonstrated the highest relative metabolic activity, followed by those on the 200µm, 300µm, 400µm and 500µm surfaces, respectively. However, cells on the 200µm titanium discs showed the highest activity at day 12. Initially, cells on the titanium slices with a pore diameter of 100µm showed a relatively high cell viability (day 2 to 6), but in view of the small number of cells these results need careful interpretation. Titanium series with a surface pore diameter of 50µm, 300µm, 400µm and 500µm behaved almost identically and viability values reached levels between those of the 25µm and the 100µm pore geometry between day 8 and 12. Furthermore, cell viability on the different porous surfaces was better than on the polystyrene surface on the different test days. The 25µm and 200µm surfaces showed the most favorable relative cell viability.

Osteoblastic alkaline phosphatase activity is a recognized parameter of cellular differentiation on the implant surface. We observed that hFOB 1.19 cells on 50µm porous titanium surface show an optimal alkaline phosphatase activity after 12 days whereas those on the 25µm porous titanium slices reach maximum activity on day 8 (Figure 4, Table 5). These results were better than those of the polystyrene standard on every day. Cells on the 100µm porous titanium slices persistently show extremely low alkaline phosphatase activity as well as low maximums for the duration of the experiment. Cells on implants with a pore geometry of 50µm, 300µm, 400µm and 500µm develop identically and reach a maximal activity between days 8 and 12. Alkaline phosphatase activity of cells on 50µm laser-structured surface exceeded that of cells on polystyrene on culture day 12. Cellular alkaline phosphatase activity on the 200µm surface was lower than that on 50µm, 300µm, 400µm and 500µm surfaces.

Scanning electron microscopy visually confirmed the development of cellular adhesions on the different surfaces. As expected from our cell culture analysis, 100µm surfaces demonstrated almost no capacity for cellular adhesion. Pore "bridging" growth was not demonstrable on 200µm, 300µm, 400µm and 500µm porous surfaces (Figure 5). However, scanning electron microscopy demonstrated that on 25µm and 50µm porous surfaces cell growth occurred both with an ingrowth into the pore depth as well as with at least partial bridging of the pores (Figure 6).

Discussion

The operative technique, the bone - implant interface (implant material, surface geometry, corrosive resistance), the individual immune response, the biomechanics of weight bearing, infection and rare material allergies influence the normal course of integration that occurs at the interface of the implant surface and the bony tissues. As the osteoblast is one of the primary cells involved in osseointegration it is possible to examine by means of cell culture many of the individual biological reactions that occur at the bone-implant interface – stem cell recruitment, cell proliferation, cell differentiation in addition to the extracellular matrix (Clokie and Warshawsky, 1995; Merklein *et al.*, 1998; Morrison *et al.*, 1995). The various culture systems documented in current literature may be classified as follows; primary culture systems (e.g., bone marrow stem cells, intramembraneous bone or trabecular long bone), non-transformed cloned cell lines (e.g. MC3T3-E1), osteosarcoma cell lines (UMR-106, ROS 17/2.8, MG63) and immortalized cell lines (e.g. RCT, HOBIT) from which the

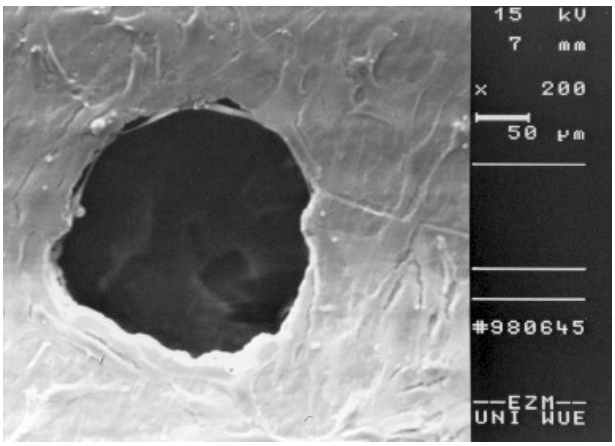

Figure 5. Cellular behavior in culture with the 200 µm pores without pore bridging.

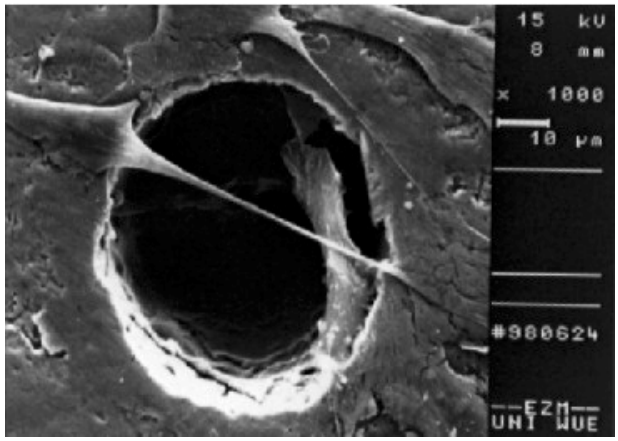

Figure 6. Pore bridging and ingrowth in the pore depth in the presence of the 50µm porous geometry.

hFOB1.19 is derived. The advantage of cell line systems over primary cell culture systems is their reproducibility; the disadvantage, however, is that it is not a natural biosystem. The hFOB 1.19 cell line combines the standardization capability with an osteoblast specific development cycle and therefore the possibility of observation over a time course without the effect of tumor cell specific deregulation.

The osteoblastic response changes significantly with very small porous surface differences (Clark *et al*., 1990, Martin *et al*., 1995). On different coated surfaces there is variability in cell specific expression of alkaline phosphatase, fibronectin and collagen (Anselme *et al*., 1994; Windeler *et al*., 1991). Bowers and Keller showed that by increasing the surface roughness osteoblastic adhesion increased (Bowers *et al*., 1992; Keller *et al*., 1994). In contrast, there are several studies on rat osteoblasts (Meyer *et al*., 1996), from either the MG 63 osteoblast cell line (Martin *et al*., 1995) or the ROS 17/2.8 osteoscarcoma cell line (Alliot-Licht *et al*., 1991) or on human osteoblasts (Desantis *et al*., 1996) where a smooth surface proved better for osteoblast adherence. A positive effect of the surface microstructure (pore diameter of 3-8µm) on both osteoblast fixation and number has also been demonstrated (Keller *et al*., 1994; Qu *et al*., 1996). A possible explanation for the increased cell growth is that a porous microstructure presents a stimulus to an orientated cell development. In the case of the osteoblast cell lines this effect was demonstrable approximately 20 minutes after incubating the cells on the structured titanium surfaces (Qu *et al*., 1996). It is certain that the cellular growth of human osteoblasts is superior on titanium surfaces compared with cobalt surfaces (Sinha *et al*., 1994). By contrast, rat osteoblast growth is better on a cobalt chromium base than on a titanium one (Puleo *et al*., 1991). It remains uncertain whether the observed differences in cellular growth are influenced by the implant surface roughness, the porous geometry or other specific surface aspects (e.g., oxide layer).

Schwartz described a difference in the cellular response with respect to variations in surface roughness (Schwartz *et al*., 1997). He postulated that the reduced cell growth observed on smooth surfaces was due to a reduction in surface area and the consequently increased cell death. Porous surfaces have by contrast a larger culture surface and thereby a lower cell density. On the smooth surface the proliferation was significantly higher in the early phases due to the faster growth. At later time points, as expected, the porous surfaces yield better results (Nöth *et al*., 1999). Our own cell culture results point to the fact that an increase in surface area is not the deciding regulating factor of cell growth at the bone-implant interface. Osteoblasts in culture with the 200µm porous surface, which had the greatest surface area, had the second lowest level of cell growth. However relative cell viability and alkaline phosphatase expression reached better results. 25 µm pores, with medium surface magnification, demonstrated the highest values for relative cell viability and alkaline phosphatase activity.

The second part of this study revealed a time dependent effect on cell viability. According to the observations of Noeth cells in culture with either polystyrene and smooth titanium had an initial high activity. However, porous titanium surface lead to a higher activity than a smooth one from the 10th day in culture (Nöth *et al*., 1999). In addition, it has been shown that rough cpTi is more favourable than stainless steel or CoCrMo in terms of effect on cellular activity (Merklein *et al*., 1998). In our study we reaffirmed the time dependency of viability in osteoblasts at the cell-implant interface. The 25µm pore geometry showed the highest activity throughout the study, reaching this at the earliest time point.

Various studies have shown that the synthesis of the extra-cellular matrix and subsequent mineralization are substantially increased on a rough surface material. In contrast however, osteocalcin levels and alkaline phosphatase activity as an indicator of osteoblastic specific activity have been demonstrated to be highest on a smooth surface (Stanford *et al*., 1994). Lohmann investigated the influence of the rough surface of titanium implants on osteoblast differentiation (Lohmann *et al*., 1999). He demonstrated that this effect was mediated through PLA2 (phospholipase A2), which regulates the Prostaglandin E2 metabolism. CpTi results in a higher level of alkaline phosphatase activity than either stainless steel (SS) or CoCrMb (Merklein *et al*., 1998), whereas polished titanium has a higher specific alkaline phosphatase activity than both the

sand treated and sandtreated-warm handled surface. We have demonstrated the positive influence of a 25µm porous titanium surface when compared with all other surfaces. For our cell line the alkaline phosphatase activity was not only an indicator of increased cell number and cell viability but also provided proof of osteoblast specific activity; osteoblastic alkaline phosphatase being synthesized by all cell types from the pre-osteoblast to the secondary osteoblast (Bruder *et al*., 1997). The surface-related effect of increased alkaline phosphatase expression and collagen synthesis may be a better indicator of osseointegration. However, the comparison with other cell culture systems is limited as for example fibroblasts and osteoblasts have different sensitivities to material surface modification (Cheung and Haak, 1989; Naji and Harmaond, 1990).

With regard to reports on bony ingrowth, the favorable surface pore sizes reported are in the range from 10 to 500µm, although in some studies a border level of 75µm has been identified (Flately *et al*., 1983; Klawitter and Hulbert, 1971). Homsy (1973) described a 300µm pore size as the most favorable and Pilliar (1987) proposed that a minimum pore size of 50µm be used. Li *et al*. (1997) suggested that for maximal bony ingrowth a porous surface with pore diameter of 140µm should be used. Schmitz. (1991) demonstrated that using excimer–laser pulsed materials a pore diameter of 40 – 50µm with a pore depth of 100µm proves maximal cell answer. Porous diameters of 200 – 400µm have long been preferred and it is suggested that such diameters produce optimal cell migration, adhesion and cellular proliferation (Goshima *et al*., 1991; Nakahara *et al*., 1992).

It is obvious from cell culture in vitro studies that an increase in the surface area of the porous surface alone is not responsible for an increase in cellular proliferation, cell vitality or cell synthesis capability. The 500µm porous surface for example, with its lower surface area produces an identical cellular response to those surfaces with pore diameter of 50µm, 300µm and 400µm. An interaction with the surface material itself may explain this observed effect. In our study we confirm the importance of the implant microstructure; however, we demonstrate unexpected findings such as that the 100µm pore surface is extremely unfavorable for cell growth in culture. This result was not predicted as by many authors 100µm is quoted as the border level for pore diameter when considering bony ingrowth in mineralized bone. It is possible that oxidative modifications or variations in oxide thickness may play a role in this response. The heat-effect of laser-treatment may influence the oxide layer (Kilpadi *et al*., 2000). However, the estimation of the oxide layer modification in the pores is very problematic (Ong and Lucas, 1998).

Scanning electron microscopy offers an explanation for the difference in the cellular parameters described above. Cells cultured with the 50µm porous titanium surface demonstrated a bridging over the pores whereas with a pore diameter of 200µm or more no cellular bridging was observed.

Although a number of statements can be made about the cell-implant interface from currently available cell culture systems we must consider that in interpreting our results we have not included factors such as biomechanical influences, inflammatory parameters, neovascularisation, platelet interaction, the influence of the stem cells or other cells in our system. Method specific limitations such as the influence of FCS in the medium or the specific NaCl solution used have been previously standardized. However, a density dependent regulation factor on cell growth is possibly relevant. The application of this "interface hypothesis" , based on our cellular results, is already on the way being assessed in a comparable animal model.

Conclusion

The analysis of cell culture results demonstrated that 25 µm and 200µm porous geometries showed results similar to those obtained with the polystyrene control; there was no hint of toxic effects. However, the 100 µm porous geometry showed an impressive negative influence on the calculated parameters. The reason for this effect is unclear. The series with 50µm, 300µm, 400µm and 500µm showed a similar, intermediate behavior in the cell culture in respect of the calculated parameters, however, worse than the 25 and 200µm porous geometry. In conclusion, the 25µm and 200µm porous geometry seems to have the most positive effect on growth and activity on the human osteoblastic cell line hFOB 1.19.

Acknowledgements

We would like to thank Dr. A. Lang and Dr. M. Hartmann from AZT-EVUS Co. as well as Dr. U. Holzwarth, Fa. P. Brehm Co., Weisendorf, for their assistance in the production of the laser-structured implants used. Furthermore we would like to thank Dr. Anne Lyons for translating the manuscript.

Funding was obtained from the Bavarian Ministry for Culture in the form of a research grant FORBIOMAT.

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Discussion with Reviewers

A. Wennerberg: When preparing a titanium surface with laser, the oxide thickness and structure will be altered, how has this been controlled? It is possible that the differences in oxide structure contributed to the results?

Authors: Yes, we agree completely, that oxide thickness is important. We expect a rather thick oxide. However, the measurement of the oxide thickness at a certain time point was not done. According to the literature the oxide thickness should be around 2-6 nm. Because of the thermal laser treatment the oxide thickness was probably increased (Kasemo and Lausmaa, 1983, Larsson *et al*., 1994, Lausmaa *et al*., 1990)

R.G. Richards: The authors mention that ALP activity indicated increased cell number and vitality and provided proof of osteoblastic specific activity? The last point is questioned since fibroblasts are also known to have ALP activity. Please comment.

Authors: Control measurements in our laboratory with BALB/3T3 fibroblasts demonstrated that alkaline phosphatase activity was less than 5% of the alkaline phosphatase activity of the osteoblast.

R.S. Tuan: It should be noted that MTT is in fact generally used as a means for determining cell number, given that mitochondrial activity is relatively constant among cells. If that were the case, the MTT data will resemble somewhat the cell data, provided the latter is done correctly. At the least, assuming the cell numbers are correct, then the MTT data should be normalized to cell number, if the authors indeed wish to use the MTT activity as a parameter for „cell viability". A better way would be to measure metabolic leucine incorporation, for example for protein synthetic activity. This issue is very problematic.

Authors: The MTT data are now normalized to cell number. The WST-test correlates with the electronic count. In the meantime the WST-1 Test is used in different cells. To our knowledge the WST test was used to check the GT3TKB cellsystem (Liu *et al*., 1995), the fetal rat liver parenchymal cell (Gressner *et al*., 1996), the HI-60 cells (Takenouchi and Munekata, 1995), Rat C6 glioma cells (Iwaki *et al*., 1995), and BKH-21 cells (Yano *et al*., 1994). We have performed a check for correlation between cell number and WST in the exponential growing phase, which resulted in a Pearson value of 0.994 and a one-sided significance of 0.003. WST seems to be a little bit more sensitive than XTT. However, it is correct, that it was much more easier to use the WST-Test (less cytotoxic, less mistakes, shorter incubation time) (Ishiyama *et al*., 1995). Metabolic leucine incorporation was not measured.

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