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DO HUMAN OSTEOBLASTS GROW INTO OPEN-POROUS TITANIUM?

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Abstract

A titanium foam for spine fusion and other applications was tested by cell culture. Its high porosity and surface roughness should enable bone cells to grow through it, resulting in a better fixation of the vertebral body.

The foam was tested by *in vitro* experiments with human osteoblasts under static culture conditions and in a perfused system. By means of cell number, viability, scanning electron microscopy and histological staining, cell proliferation could be observed. The expression of osteogenic genes like collagen-I, alkaline phosphatase and osteocalcin was proven by reverse transcription polymerase chain reaction (RT-PCR) as well as in the case of alkaline phosphatase with biochemical methods.

The conducted experiments showed that human osteoblasts could grow through the interconnected porosity of the metal foam and that they expressed an osteoblast like phenotype. The results suggest that *in vivo* osteoblasts are likely to form a trabecular bone bridge through this titanium foam. Consequently, with this osteoconductive material, there may be a reduced need for autologous bone in spinal fusion procedures.

Keywords: titanium, human osteoblasts, proliferation, perfusion, cell culture, cage, spine fusion, scaffold.

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Introduction

The use of lumbar interbody fusion cage devices has attracted interest over the last few years. Since 1988 when Bagby invented the first cage device (Bagby, 1988) numerous types of implants, made from metal, carbon fibre composites or titanium, have been designed (Blumenthal and Ohnmeiss, 2003; McAffee, 1999; Ray, 1997; Steffen *et al.,* 2000a; Zdeblick and Phillips, 2003). While initial clinical reports looked quite promising (Kuslich *et al.,* 1998; Ray, 1997) more recent studies suggest that complications may occur with the available devices (Elias *et al*., 2000; McAffee *et al*., 1999). It has been observed, that the geometry of many cages leads to the development of local stress peaks which can cause the cage to subside (Beutler and Peppelman, 2003), migrate (McAffe *et al*., 1999) or fail mechanically (Tullberg, 1998). Recent publications emphasize the influence of the cage geometry on the alignment of the fused spine (Gödde *et al*., 2003; Palm *et al*., 2002; Polikeit *et al*., 2003).

Another problem is that to promote interbody fusion and growth of trabecular bone through the cage, they have to be filled with cancellous bone chips (Blumenthal and Ohnmeiss, 2003; McAffee, 1999). The most readily available source for autologous bone graft is still the iliac crest (the golden standard). Unfortunately this is associated with an increase in complications and postoperative morbidity (Banwart *et al*., 1995; Fernyhough *et al*., 1992; Goulet *et al*., 1997). Although interbody cages have reduced the amount of necessary bone graft and likewise the bone graft harvesting techniques may become less invasive (Steffen *et al*., 2000b), it would be unquestionably of great advantage to get by without using bone grafts from the iliac crest.

Alternative approaches combined cages with bone inducing growth factors (Cunningham *et al*., 1999; Kandziora *et al*., 2002) used bioabsorbable materials (Van Dijk *et al*., 2002; Steffen *et al*., 2001) or tissue-engineered bone constructs (Van Gaalen *et al.* 2004). However most of these alternatives are either not fully developed or have been investigated with varying success (Weiner and Welker, 2003; Van Gaalen *et al*., 2004).

Therefore, Synthes Stratec (Oberdorf, Switzerland) developed a novel commercially pure titanium (cp Ti) foam for spine fusion and other applications, which should avoid the above mentioned problems. It has been reported that titanium possesses an excellent biocompatibility since it spontaneously forms a surface oxide layer up to 7 nm thick (Long and Rack, 1998; Schmidt *et al*., 2001). Because of the space holder production process, its

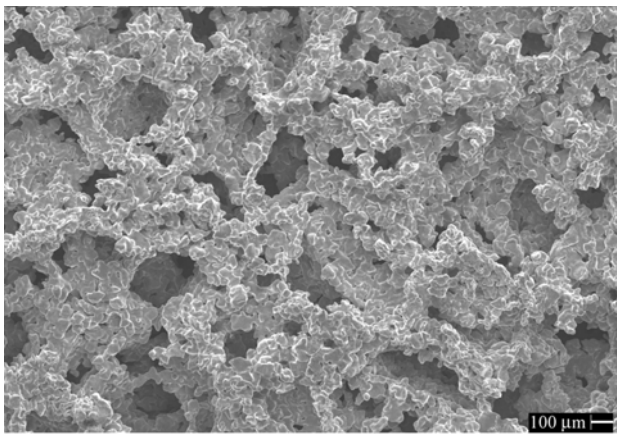

Figure 1. SEM picture of unseeded cp titanium foam (surface level), with a porosity of 65-70% and pores with a diameter of 350 to 550 µm. As visible the surface roughness is quite high, which should promote adhesion and differentiation of osteoblasts.

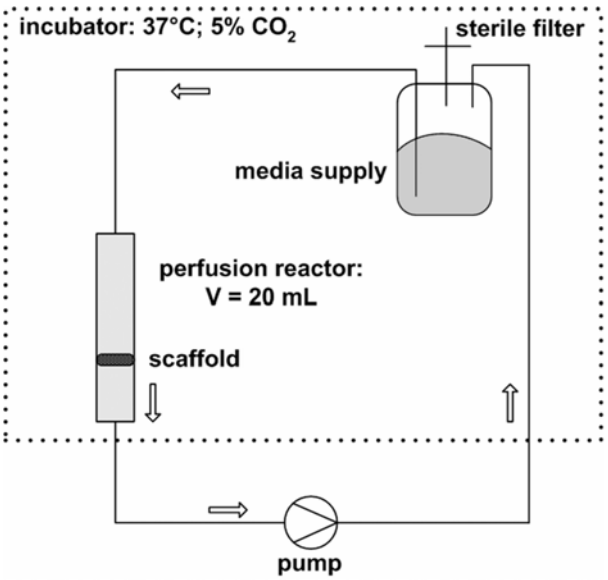

Figure 2. Diagram of the developed perfusion system. A silicon tube, in which the scaffold was clamped, served as a very simplistic reactor system. Media was constantly circulated from the media supply through the perfusion reactor and scaffold. Exchange of air was permitted over a sterile filter of the media supply.

porosity (65-70%) and surface roughness is quite high which promotes the adhesion and differentiation of osteoblasts as well as extra cellular matrix (ECM) formation (Açil *et al*., 2000; Flemming *et al*., 1999; Lincks *et al*., 1998; Schmidt *et al*., 2002). Its high porosity should help the scaffold to interlock with the vertebral body, thereby avoiding the development of local stress peaks, subsidence or migration of the implant. For instance Simmons *et al.* (1999) showed that porous-surfaced implants could improve early implant stability and resistance of mechanical removal.

Before the titanium foam is implanted the vertebral bodies will be abraded so that by means of the angiogenic response osteoblasts should grow through the cage and start the ossification process. Interbody fusion and formation of bridging trabecular bone would be promoted without the use of autologous bone graft. The purpose of this work was to evaluate if osteoblasts *in vitro* could grow through the titanium foam, express the osteoblast like phenotype and form the right ECM.

Materials and Methods

Materials

Trypsin, phosphate-buffered saline (PBS), foetal calf serum (FCS) and high glucose Dulbecco's modified eagle's medium (DMEM) were purchased from Gibco BRL (Grand Island, NY). Proliferation media was DMEM with 10% FCS, 1% antibiotics (penicillin, streptomycin 100 mg ml^{-1} /100 IE ml^{-1}) and 2 mM L-glutamine (Merk). The osteoblastic media was proliferation media with 100nM dexamethasone (Sigma), 50 nM L-ascorbic acid-2-phosphate (Sigma) and 10 mM β-glycerol phosphate (Fluka).

Cell culture

For the seeding experiments, bone tumour cells (SAOS-2) and primary human osteoblasts (Dr. Behrens, University hospital Lübeck, donor: 76 year old German, p3) were used. Both cell lines were cultivated in T-75 culture flasks (Greiner) with proliferation media at 37°C and 5% CO_2. Every two to three days the medium was changed completely. After seven to ten days when cells reached 75% confluence, they were rinsed with PBS, detached with 5 ml trypsin (5min at 37°C and 5%CO_2) and concentrated by centrifugation at 200g. For static cultivation the cells were diluted to $3x10^5$ cells in 100 µl media, seeded onto the titanium foam in a six well plate and incubated for 2 h at 37°C and 5% CO_2. Afterwards 6 ml osteoblastic medium was added. The scaffold was incubated at the same conditions and the media was changed every two to three days. For cultivation under perfusion the cells were diluted to $9x10^5$ cells in 1 ml media, seeded onto the titanium foam in the reactor system and incubated at the same conditions for 2 h. Before the perfusion was started 15 ml osteoblastic media was added, followed by another incubation for 24 h at 37°C and 5% CO_2. Osteocalcin (BGP) was induced with 24 nM calcitriol in osteoblastic media, 24 h before cells were detached.

Cp titanium scaffolds

Cp titanium scaffolds (diameter: 16 mm; height: 5 mm) from Synthes Stratec (Oberdorf, Switzerland) were used. The test pieces (Fig. 1) consisted of cp titanium (purity grade 4) with a porosity of 65-70% and have been produced by powder metallurgy according to the space holder procedure resulting in pores with a diameter of 100 to 700 µm (the majority of them were between 250 and 500 µm in diameter). After a sintering step (1200 °C) the scaffolds were treated with ethanol and ultrasonic to get rid of possible air pockets. The surface roughness Ra was determined to be above 200 µm.

Table 1. Human osteoblast sequence specific oligonucleotide primers, designed on the basis of published sequences and predicted PCR product sizes. Key: BP, base pairs; f, forward primer; r, reverse primer.

Target gene		Primer sequence (5`-3`)	Expected product size (bp)
ALP	f	GGGGTGAAGGCCAATGAGGG	417
	r	GCTCTTCCAGGTGTCAACGAG	
Col-1	f	CATGCCAATCTTTACAAGAGG	469
	r	TTTGAAGCCAGGAAGTCCAG	
BGP	f	CACACTCCTCGCCCTATT	306
	r	CAGCAGAGCGACACCCTAGAC	

Perfusion system

A perfusion reactor was developed which, as was preferred, enabled a constant flow through the scaffold. A silicone tube (internal diameter: 15mm), in which the scaffold was clamped, served as a reactor system (Fig. 2). The total volume of media was 100 ml (for 4 scaffolds) and 2/3 of it was changed every two to three days. By means of a MCP-Process IP65 pump (Ismatec SA, Glattbrug, Switzerland) a constant media flow Q of 0.02 ml min^{-1} was maintained. Assuming flow was distributed uniformly across the titanium foam surface with the diameter D of 16 mm and porosity ϕ of 65-70%, the mean velocity V_m through the pores can be calculated with equation 1.

$$V_m = \frac{Q}{\phi\pi\left(\frac{D}{2}\right)^2} = 0.16\,\mathrm{mm\,min^{-1}} \quad (1)$$

When further a parabolic flow of media with viscosity μ of 0.01 g cm^{-1} s^{-1} through the cylindrical pores with diameter d of 350 µm is assumed, the resulting shear stress of the cells at the wall τ_w can be calculated with equation 2.

$$\tau_w = \frac{8\mu V_m}{d} = 5.7\times10^{-4}\,\mathrm{dyn\,cm^2} \quad (2)$$

These calculations indicate that shear stress experienced by the seeded cells under perfusion is more than four magnitudes lower than the estimated 8-30 dyn cm^{-2} peak stress for osteocytes in bone in bone tissue under interstitial flow (Goldstein *et al*., 2001; Weinbaum *et al*., 1994).

Cell proliferation

Cells on the scaffold were detached repeatedly with a trypsin solution (1% in PBS, 10 min at 37°C) and cell number and viability were counted manually after trypan blue staining in a Neubauer chamber.

Alkaline phosphatase activity (ALP)

Alkaline phosphatase activity (ALP), was assayed as the release of p-nitro phenol which was measured at a wavelength of 410 nm, permitting the calculation of alkaline phosphatase activity in units (1 U = 1 µmol substrate min^{-1} $1x10^6$ $cells^{-1}$).

Expression of osteogenic genes

To determine the expression of mRNA species that correspond to the osteogenic activity of osteoblasts, total RNA was isolated from the detached cells using RNeasy test kits (Qiagen, Basel, CH). Copies of double stranded DNA (cDNA) were synthesized from 1 µg of total RNA using OneStep RT-PCR kit (Qiagen). Human specific oligonucleotide primers (Table 1), designed on the basis of published sequences, were obtained from Microsynth (Balgach, Switzerland). The cDNA was resolved by electrophoresis and viewed under ultraviolet light. The marker specific bands were cut out with a scalpel and cDNA was cleaned up with QIAquick gel extraction kit (Qiagen). Sequencing of received cDNA, corresponding to mRNA encoding human gene products for alkaline phosphatase (ALP), osteocalcin (Bone GLA-protein, GLP) and collagen-1 (Col-1), was done by cycle sequencing (Findlay *et al*., 2004). The products were cleaned up with the DyeEx 2.0 spin kit (Qiagen) and revealed with the ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, USA). The obtained sequences were processed with the software "Sequencher" (Gene Codes Cooperation, Ann Arbour, USA) and homology comparisons took place over the NCBI web page (Web ref. 1).

Histology

The seeded scaffold was fixed in 40% alcohol and drained over a descending alcohol concentration and dyed with toluidine blue.

Scanning electron microscopy (SEM)

The seeded scaffolds were carefully broken in the middle, fixed in 3% aldehyde (in PBS), drained over an ascending acetone concentration and critical point dried (CPD 030, Bal-Tech, Houston, USA). The specimen were mounted onto stubs with silver paint and coated with 8 nm of gold. Scanning microscopical examination was executed with a Zeiss DSM 940 A (Feldbach, CH).

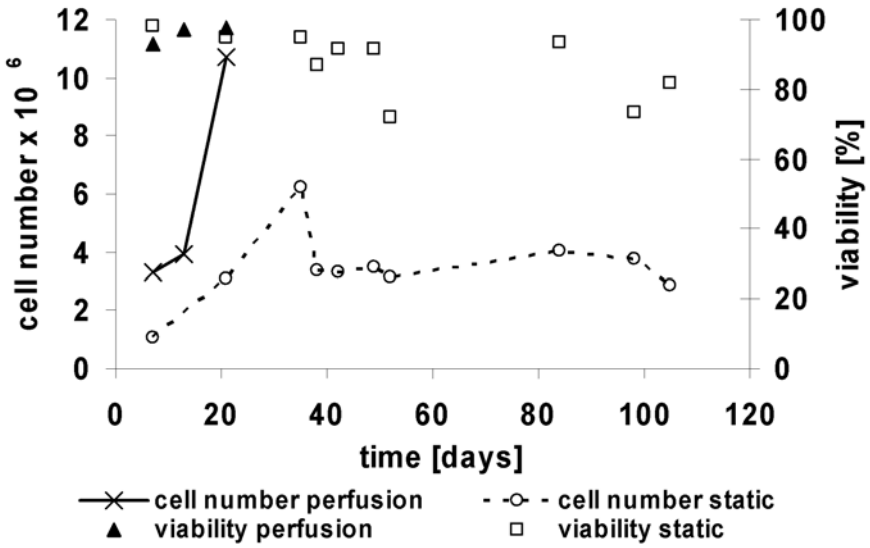

Figure 3. Cell number and viability of SAOS cells which were seeded on cp titanium foams and cultivated under perfusion and static culture conditions. After 5 weeks of static culture conditions the cell number remained constant whereas the viability decreased continually. Under Perfusion a much higher cell number could be reached.

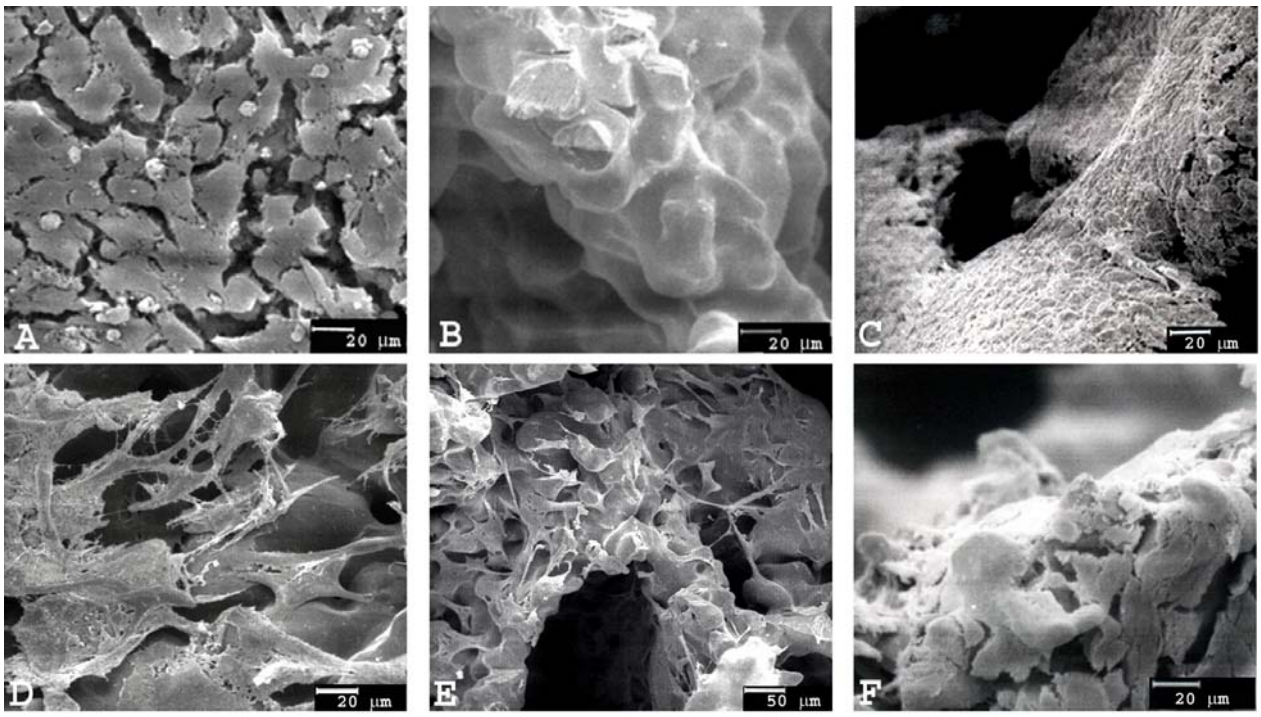

Figure 4. SEM picture of seeded cp titanium foam under static culture conditions after nine weeks (A-B) and under perfusion after three weeks incubation (C-F). Osteoblast under static culture conditions could only be found on the outer seeded surface. However under perfusion the cells could be found through the entire scaffold. Legend: A: Seeded side with SAOS cells; B: Broken scaffold, inside view of middle section. C: SAOS-cells, seeded outer surface side. D: Human osteoblasts, seeded surface side. E: Human osteoblasts, broken scaffold, inside middle view. F: SAOS-cells, unseeded surface side.

Results

When the cp titanium foams were seeded with SAOS cells and cultivated under static conditions, the cell number increased continually to $6.3x10^6$ until it dropped after 38 days to $3.4x10^6$ which was also accompanied with a decrease of cell viability from 98% in the first week to 92% after 6 weeks of incubation (Fig. 3). SEM images (Fig. 4) and histological staining (Fig. 5) showed that the cells were only growing on the outer seeded surface. No cells were observed inside and outside on the unseeded surface of the scaffold. The high porosity (65-70%) and the broad pores (diameter of 350 to 550 µm) should have been sufficient to enable an ample nutrition supply inside the scaffold. The cells on the seeded surface however were growing as a thick and dense cell layer (Fig. 4) so that most of these pores were covered (Fig. 5). Osteoblastic phenotype was strongly indicated by biochemical ALP

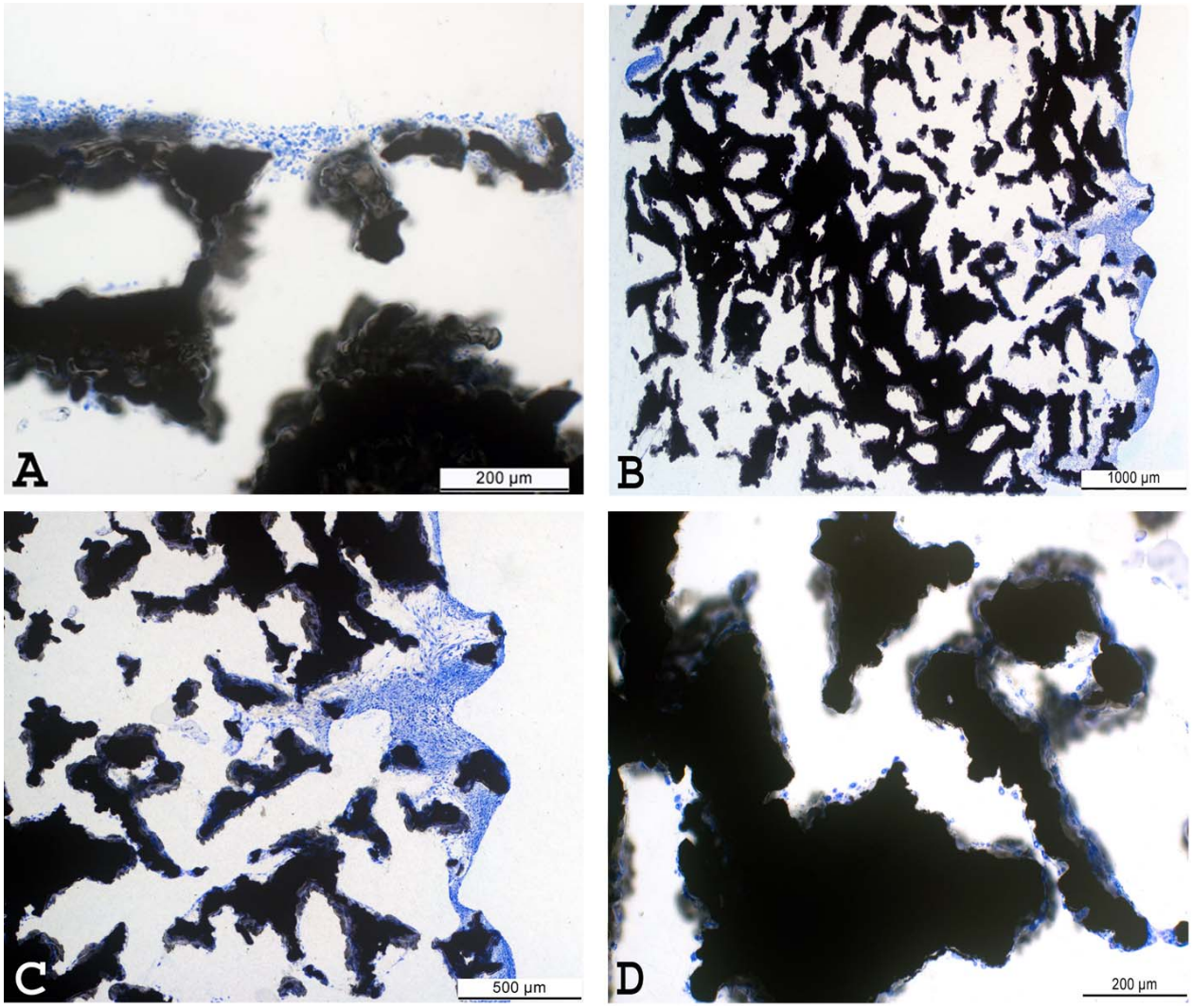

Figure 5. Histological staining with toluidine blue of scaffold with seeded SAOS cells. A: Cultivated under static conditions for 11 weeks. B-D: Perfused for three weeks. Under static culture conditions the cells were only growing on the outer surface (A). However under perfusion the osteoblasts could grow through the entire scaffold (B-D).

activity measurement. ALP activity after seven days of cultivation was 0.02 U 10^{-6} cells and dropped after five weeks to an average of $4x10^{-3}$ U 10^{-6} cells (data not shown).

To enable the cells to grow through the entire scaffold and to imitate better the conditions of bone tissue *in vivo*, a perfusion system was developed. The system should be cheap, easy to handle and easy to construct. Therefore the scaffolds were clamped in a silicone tube, which served as a reactor system. A constant media flow from a media container though the scaffold was maintained by a peristaltic pump (Fig. 2). With this newly developed perfusion system, various seeding experiments (with different flow rates between 0.05 and 0.69 mm min^{-1}) were carried out (data not shown). By means of cell number, viability and SEM the optimal flow rate of 0.15 mm min^{-1}, which is about in the same magnitude of what is recommended in the literature, was determined (Goldstein *et al.*, 2001; Pazzano *et al.*, 2000). After seeding with SAOS cells and cultivating under perfusion, the cell number increased after three weeks to $1.1x10^{7}$cells which was far more than under static cultivation (Fig. 3). The cell viability after three weeks of perfusion was 98%. The cells were now growing through the entire titanium scaffold, which was shown by histological staining and SEM pictures (Figs. 4 and 5). Osteoblast like phenotype was shown by increased ALP activity. After one week of perfusion, the ALP activity was 0.12 U 10^{-6} cells and increased after three weeks to 0.54 U 10^{-6} cells, which was significantly higher than what was observed under static cultivation. The mRNA expression of ALP, collagen-1 (col-1) and osteocalcin (BGP) could be shown by RT-PCR (Fig. 6). Sequencing of marker specific bands was performed and the results were compared with already published mRNA sequences of BGP, col-1 and ALP (Web ref. 1). Identities in the DNA sequences between 99 and 100% were determined.

The seeding experiments were also reproduced with primary human osteoblasts. The cells were growing much slower than SAOS cells. After 5 weeks of perfusion only $1.1x10^{6}$ cells with a viability of 95% were found on the scaffold. It could be shown that they were also growing through the entire scaffold (Fig. 4) and expressed ALP and Col-1(Fig. 6). However after five weeks BGP expression could still not be observed. The slow cell growth

and the missing expression of BGP could be due to the advanced age (76 years) of the donor.

Discussion

The aim of this study was to evaluate a cp titanium scaffold for spine fusion and other applications. Since the scaffold is not filled with any additional bone grafts, osteoblasts have to be able to form trabecular bone through the porous scaffold. Therefore the titanium foams were seeded with SAOS cell and primary osteoblasts. Differentiation of static cultivated osteoblasts was shown by biochemical ALP activity, which after seven days of cultivation was comparable to what had been published under the same conditions (Goldstein *et al*., 2001). However SEM pictures and histological staining (Fig. 4, 5) showed that the cells were only growing on the surface of the scaffold.

One reason for that result could have been that, because of the thick cell layer on the surface, the conditions within the scaffold were not meeting up with the metabolic requirements of the cells – which lead to the noticed decrease of viability after 38 days of incubation. The design of the experiment under static cultivation was also not imitating the conditions of bone tissue *in vivo*, where the osteoblasts are exposed to interstitial fluid flow and shear stress which initiates ECM formation (Fritton *et al*., 2000; Goldstein *et al*., 2001; Hillsley and Frangos, 1994). Therefore a simple perfusion system analogous to that described by Pazzano *et al.* (2000) was developed, in order to improve the nutrient supply within the scaffold and to improve cell differentiation and ECM formation (Fig. 2) (Minuth *et al*., 2000; Pazzano *et al*., 2000).

After cultivation under perfusion the cell number was almost twice as high as under static cultivation. SEM pictures and histological staining showed that SAOS and primary human osteoblasts could now grow through the entire scaffold. The expression of osteogenic genes like ALP, BGP and Col-1 was shown by RT-PCR and sequencing of corresponding bands.

The bone specific ALP plays an important role in normal skeletal mineralization and is therefore an early marker of bone formation (Calvo *et al*., 1996; Weiss *et al*., 1988). Col-1 is a very important component of the bone matrix (90% of the bone consists of Col-1). BGP expression occurs at a late stage of osteoblast differentiation and plays a vital role in bone formation and interaction with hydroxyapatite (Sommer *et al*., 1996). Perfusion and thereby executed fluid shear stimulated the ECM formation which was illustrated by the increased ALP activity which was 27 times higher than under static cultivation.

A further interesting possibility for this osteoconductive material would be to modify the surface, so that certain growth factors (osteogenic protein-1, BMP-2 or IGF-1/ TGF-b1), which are known to stimulate bone formation, could be tied to the biomaterial (Cunningham *et al*., 1999; Kandziora *et al*., 2002; Nanci *et al*., 1998).

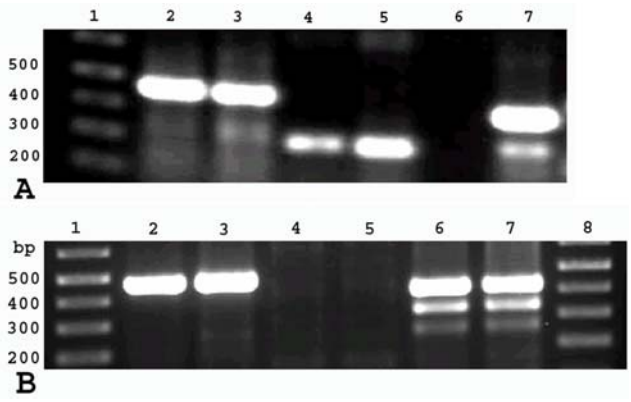

Figure 6. Agarose gel (1%) of RT-PCR products. A: SAOS cells after three weeks perfusion on the titanium foam. Key: 1: 1 kB marker; 2+3: col-I, 4+5: BGP, 6+7: ALP. The mRNA expression of ALP, Col-1 and BGP could be shown. B: primary human osteoblasts after 40 days perfusion on the titanium foam. Key: 1+8: 1 kB-marker, 2+3: col-I, 4+5: BGP, 6+7: ALP. The expression of ALP and Col-1 could be shown.

Conclusion

The results of this study clearly show that human osteoblasts under perfusion are able to grow into the scaffold, form the osteoblast specific phenotype and express bone specific matrix proteins. This newly developed cp titanium foam might reduce the need for autologous bone and is therefore an interesting alternative to existing cages and biomaterials. The next step will be to test the cp titanium foam *in vivo* for spine fusion and other applications in animal experiments. The results of this *in vitro* study are quite promising, therefore there is a high chance that the animal experiments will be also successful.

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