TISSUE ENGINEERING
Volume 13, Number 7, 2007

DOI: 10.1089/ten.2006.0387

Axial Prevascularization of Porous Matrices Using an Arteriovenous Loop Promotes Survival and Differentiation of Transplanted Autologous Osteoblasts

A. ARKUDAS, M.D.,[1,*] J.P. BEIER, M.D.,[1,*] K. HEIDNER, M.D.,[1] J. TJIAWI, M.D.,[1] E. POLYKANDRIOTIS, M.D.,[1] S. SROUR, D.M.D.,[2] M. STURZL, Ph.D.,[3] R.E. HORCH, M.D.,[1] and U. KNESER, M.D.[1]

ABSTRACT

Generation of axially vascularized bioartificial bone might be performed using matrix neovascularization in connection with osteoblast injection. We sought to evaluate whether prevascularization of porous hard matrices using an arteriovenous (AV) loop promotes survival of transplanted osteoblasts. A processed bovine cancellous bone matrix was inserted into the AV loop. Six weeks later, 5×10^6 carboxyfluorescein diacetate–stained osteoblasts were injected into the matrix (group A, $n=34$). Osteoblast-seeded matrices without prevascularization were implanted subcutaneously as controls (group B, $n=32$). Specimens were subjected to histologic, morphometric, and molecular-biological analysis after 1, 4, 8, and 16 weeks. Upon cell injection, matrices were completely vascularized. An intense foreign body reaction was observed in matrices from both groups. Group A was significantly superior to group B in terms of osteoblast survival at any time point. Expression of bone-specific genes was detected in the AV loop group but not in the subcutaneous control. Bone formation was only detectable in 1 long-term animal of group A. This study demonstrates for the first time that axial prevascularization increases the survival of implanted osteoblasts in porous matrices. Matrices with optimized biocompatibility might eventually facilitate generation of axially vascularized bone tissue after injection of osteogenic cells in the AV loop model.

INTRODUCTION

THE RECONSTRUCTION OF EXTENDED BONE DEFECTS remains a major challenge in reconstructive surgery. Autologous bone grafting is still the gold standard for osteogenic bone replacement.[1] However, autologous grafting is associated with several shortcomings and complications, including limited quantities of bone for harvest, often resulting in significant donor-site morbidity. In some cases, only free microsurgical tissue transfer such as fibula, scapula, iliac crest, and others can accomplish adequate reconstruction.[2–4]

A multitude of different biomaterials have been developed for bone replacement. Processed human cancellous bone matrices have been successfully used for reconstruction of human bone defects.[5] Adhesion, proliferation, and differentiation of osteogenic cells within processed bovine cancellous bone (PBCB) matrices, as well as secretion of calcified extracellular matrix, have been demonstrated in previous studies by our group.[6,7] Nutrition and oxygen supply via diffusion proves to be the main limitation after transfer of *in vitro* engineered cellular constructs, because diffusion is only sufficient within a maximum range of 200 μm in the matrix.[8,9] For this reason, vascularization is a core requirement for the survival of cells in the center of large constructs.[10] In traditional tissue engineering approaches, the matrix was inserted into the recipient animal simultaneously

[1]Department of Plastic and Hand Surgery, [2]Department of Oral and Maxillofacial Surgery, and [3]Department of Molecular and Experimental Surgery, University of Erlangen Medical Center, Erlangen, Germany.
*These authors contributed equally to this work.

with cell implantation. In 1979, Erol and Spira reported the formation of a new capillary bed around an implanted AV shunt loop using artery or vein grafts in a rat model.[11] Morrison *et al*. augmented this model and implanted the arteriovenous (AV) loop into polycarbonate isolation chambers filled with polymer matrices.[12,13] As a vascular carrier, the superiority of the AV loop to the vascular bundle in terms of vascular density and capacity for generation of new tissue has been demonstrated.[14] The axial type of vascularization, which is also referred to as the intrinsic type of vascularization, allows the vascularization and transfer of biomaterials independent of local conditions at the recipient site. We reported recently about the axial vascularization of a custom-made PBCB matrix using an AV loop.[15] After 4 and 8 weeks, the matrices displayed a significant degree of vascularization. With new concepts based on axial vascularization, the time point of cell injection becomes crucial. So far, there has been one study simultaneously incorporating the AV loop model with cell implantation.[16] The induction of vascularization in biomaterials before cell injection (the prevascularization of scaffolds) may help to increase the initial survival and engraftment of transplanted cells and may consecutively optimize bone formation in bioartificial osteogenic bone tissues.

The aim of this study was to evaluate whether prevascularization of porous hard matrices using an AV loop promotes the survival of transplanted osteoblasts, targeting on the creation of custom made bioartificial, axially vascularized bone flaps.

MATERIALS AND METHODS

Experimental design

Syngenic male Lewis rats were used as donors and recipients. Sixty-six recipients were divided into 2 groups (groups A and B). In 34 animals from group A, an AV loop was constructed microsurgically between the left femoral artery and vein using a contralateral vein graft. A clinically approved PBCB matrix was inserted into the AV loop. Processing protocols and properties of the matrix have been described previously.[6] The matrix and the loop were placed into a Teflon isolation chamber. Six weeks after implantation of the AV loop and the matrix, 5×10^6 carboxyfluorescein diacetate (CFDA)-stained fibrin gel–immobilized osteoblasts were injected into the matrix of each animal. In group B, the nonprevascularized osteoblast-seeded matrix (5×10^6 CFDA-stained fibrin gel–immobilized osteoblasts/matrix) was placed subcutaneously on the backs of 32 animals. Eight matrices per group and time point were explanted after 1, 4, 8, and 16 weeks and subjected to histologic, morphometric, and reverse transcriptase polymerase chain reaction (RT-PCR) analysis (Fig. 1). Two animals from group A underwent corrosion cast 6 weeks after implantation of the AV loop to verify the vascularization of the matrix before cell injection.

Design of matrix and isolation chamber

The matrix and design of the isolation chamber have been described previously.[15] Briefly, the matrix was comprised of a clinically approved PBCB (Tutobone, Tutogen Medical, Neunkirchen, Germany) with a matrix pore size of 400 to 1000 μm and a porosity of 65% to 80% that completely filled the cylindrical Teflon chamber, with an inner diameter of 10 mm and a height of 6 mm (provided by P. Greil, Department of Materials Science, Glass and Ceramics, University of Erlangen). As a modification for this study, canals for future injection of gel-immobilized osteoblasts were included in the matrix design. Medical plastic placeholders were stuck into these canals to prevent the ingrowth of tissue until osteoblast injection (Fig. 2).

Animals

Syngenic male Lewis rats (Charles River Laboratories, Sulzfeld, Germany) served as donors and recipients. German regulations for the care and use of laboratory animals were observed at all times. The animal care committee of the University of Erlangen and the government of Mittelfranken, Germany, approved all experiments. The animals were housed in the veterinary care facility of the University of Erlangen Medical Center and submitted to a 12-h dark/light cycle with free access to standard chow (Altromin, Hamburg, Germany) and water.

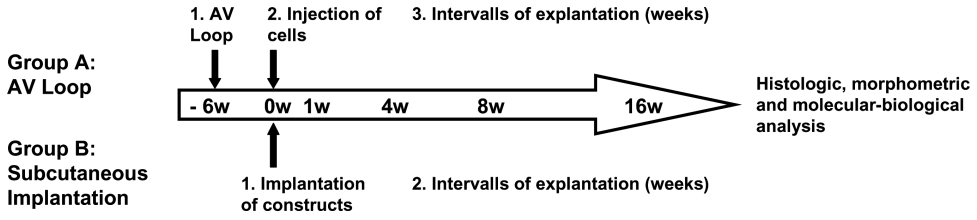

FIG. 1. Groups and study design. In animals from group A, an arteriovenous (AV) loop was constructed, and the processed bovine cancellous bone matrix and the loop were placed into an isolation chamber. Six weeks after implantation, carboxyfluorescein diacetate–stained gel-immobilized osteoblasts were injected into the matrix of each animal. In group B, the osteoblast-seeded matrix was placed subcutaneously in the back. The matrices were explanted after 1, 4, 8, and 16 weeks.

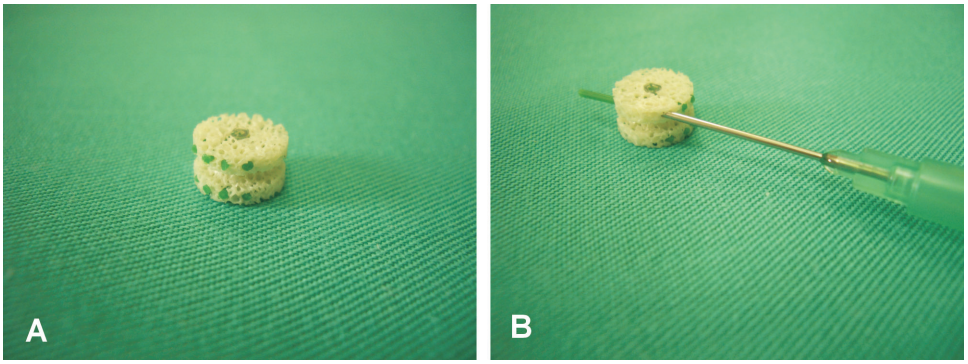

FIG. 2. Processed bovine cancellous bone matrix with canals for future injection of gel-immobilized osteoblasts included in the matrix design. The canals were filled with medical plastic placeholders to prevent the ingrowth of tissue until the osteoblast injection (**A**). During the second operation (injection of osteoblasts), placeholders are removed, and cells are injected using the Duploject (two-way) syringe system with a blunt needle to avoid injury of the loop (**B**). Color images available online at www.liebertpub.com/ten.

Isolation and expansion of primary rat osteoblasts

After sacrificing the rats using carbon dioxide (CO_2) asphyxiation, femora and tibiae were dissected and transferred into Dulbecco's modified Eagle medium (DMEM) containing penicillin/streptomycin 100 U/mL (Life Technologies, Paisley, UK). Samples were cleaned of soft tissue and cartilage and minced into 2-mm pieces. Twenty mL of collagenase type II solution (2 mg/mL collagenase type 2 (Biochrom, Berlin, Germany)) in DMEM were added. Bone pieces were digested for 30 min at 37°C under continuous mechanical agitation. The supernatant from the first digestion step was discarded. Digestion was then repeated 3 times. The supernatants were pooled and purified using centrifugation (10 min, 1200 r.p.m., 4°C). Cell pellets were resuspended in basal medium (BM) (DMEM containing penicillin/streptomycin 100 U/mL, and 10% fetal calf serum (Biochrom) from selected lots). Cells were plated at a density of 5000 cells/cm^2 in 225-cm^2 culture flasks (BD Falcon, Heidelberg, Germany) and cultured in an incubator with a humidified, 5% CO_2 atmosphere at 37 °C. Culture medium (BM) was changed every third day. Cells were passaged at 90% confluency using trypsin/ethylenediaminetetraacetic acid (Viralex, Gibco, Karlsruhe, Germany) according to standard protocols. Second-passage cells were used for transplantation.

CFDA labeling

Initial osteoblast survival and engraftment were evaluated 1, 4, and 8 weeks after implantation using CFDA staining. Passage 2 osteoblasts were detached as described above and incubated for 15 min with 10 mm freshly prepared CFDA (Vybrant CFDA SE Cell Tracer-Kit, Molecular Probes, Eugene, OR) reconstituted in dimethyl sulfoxide and phosphate buffered saline (PBS) according to the manufacturer's recommendations. After several washing and incubation steps, cells were immobilized in fibrin gel (group A), or cell-seeded constructs (group B) were prepared as described below. After cell immobilization in a fibrin-gel containing 3 mg/mL fibrinogen, 10 IU/mL thrombin, and 1000 IU/mL aprotinin, cells were injected in the matrix using the DUPLOJECT-system (Baxter, Vienna, Austria).

Cell-seeding technique (group B)

Before implantation, the PBCB discs were rehydrated by incubation in 5 mL BM containing 1000 U/mL aprotinin (Trasylol, Bayer, Leverkusen, Germany) and 100 IE/mL thrombin (TissuCol, Baxter) at 4°C for 12 h. Five$\times 10^6$ second-passage primary osteoblasts were resuspended in 50 mL BM containing 1000 U/mL aprotinin and 10 mg/mL fibrinogen (Sigma, Irvine, UK). Disks were dried shortly and then seeded with the osteoblast–fibrinogen solution. The seeded constructs were placed into the incubator for 30 min. After polymerization of the fibrin gel, disks were kept in cell culture medium in 6-well culture dishes (BD Falcon) and placed on ice until implantation.

Surgical procedures

The same microsurgeon performed all operations under an operative microscope (Karl Zeiss, Jena, Germany). Operations were performed under general anesthesia with Isoflurane (Baxter, Unterschleißheim, Germany). In group A, the left femoral vessels were exposed using a longitudinal skin incision from the groin to the knee. The artery and vein were dissected from the pelvic artery in the groin to the bifurcation of the femoral artery in the knee. A 20-mm vein graft was harvested from the right femoral vessels. This vein graft was interposed between the recipient left femoral vein and artery using microsurgical techniques with 11–0 sutures (Ethicon, Norderstedt, Germany). The AV loop was placed in the circular groove of the custom-made PBCB matrix. The matrix and the loop were then placed in the chamber, the lid was closed, and the chamber was fixed in the groin using Prolene 3–0 (Ethicon) sutures. Hemostasis was assured, and the wound was closed using Vicryl 5–0

(Ethicon). In group B, the matrix was placed without an isolation chamber subcutaneously in the back. All animals received 0.2 mL benzylpenicillin-benzathine (Tardomycel, Bayer), buprenorphine (0.3 mg/kg rat weight, Temgesic, Essex Chemie AG, Luzern, Switzerland), and heparin (80 IU/kg Liquemin, Ratiopharm, Germany) postoperatively.

Injection of gel-immobilized osteoblasts (group A)

Six weeks after implantation of the matrices and the AV loop in animals of group A, the constructs were exposed using a longitudinal skin incision from the groin to the knee. The lid was then opened, and the matrix with the loop was carefully taken out of the chamber. The placeholders were removed and the canals were injected with gel-immobilized osteoblasts. After injection, the lid was closed, and the matrix was placed back into the chamber. Patency of the AV loop was assessed before skin closure.

India ink injection and explantation of the matrices

As described previously, India ink perfusion[15] was performed by cannulating the aorta: flushing the aorta with heparin solution and India ink solution (50% v/v India ink, Rohrer, Germany in 5% gelatin (Roth, Karlsruhe, Germany) in 4% mannitol (Neolab, Heidelberg, Germany) at 37 °C). The rat was then placed at –20 °C for approximately 30 min; specimens were explanted *in toto* and fixed in 3.5% formalin solution for histological processing.

Polychrome fluorescent sequential labeling

Bone formation in the constructs was evaluated using fluorescent sequential labeling techniques, as described previously.[17] Animals were injected with xylenol orange (Merck, Darmstadt, Germany, 90 mg/kg body weight), calcein green (Merck, 10 mg/kg body weight), and oxytetracylcine (Intervet, Tönisvorst, Germany, 25 mg/kg body weight) subcutaneously at 3, 2, and 1 week before explantation, respectively. Unstained, nondecalcified, plastic-embedded cross-sections were evaluated using a fluorescence microscope with appropriate filter settings (Zeiss, Oberkochen, Germany).

Histological and statistical analysis

Specimens were formalin fixed, decalcified, and paraffin embedded or processed for hard tissue histology according to standard protocols. Three-μm cross sections were obtained from 2 standardized planes. Both planes were oriented rectangularly to the longitudinal axis of the AV loop. One plain was located 1 mm proximal, the other 1 mm distal to the transverse midline. Cross-sections were stained using hematoxylin and eosin and von Kossa stains according to standard protocols. CFDA-labeled osteoblasts were quantified in 2 planes per implant. Two independent, blinded observers evaluated the images. All cross-sections were divided into 12 equal sectors (6 at the periphery and 6 at the central part of the section (Fig. 3)), and all images of each sector were acquired using a Leica microscope and digital camera under 100× magnification. The images were rendered bimodal (standardized threshold) (WinQ, Leica Microsystems, Bensheim, Germany). The mean value of CFDA-positive osteoblasts per field was calculated for each group and each time. Results are given as means ± standard deviations. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). A two-tailed unpaired Student *t*-test was applied for statistical analysis. The critical level of statistical significance chosen was $p < 0.05$.

Immunohistology

Rat endothelial cells were detected immunohistochemically 4 and 8 weeks after implantation using the lectin *Bandeiraea simplicifolia* agglutinin. Paraffinated sections were treated in a xylol/ethanol sequence, and after rinsing in PBS solution, endogenous peroxidase activity was blocked in 30% hydrogen peroxide for 10 min. Blocking was completed with avidin and biotin 15 min each (Vector Laboratories, Burlingame, CA) as well as 5% normal goat serum for 1 h. Biotinylated lectin (*Bandeiraea simplicifolia*, Sigma) was applied 1:100 overnight at 4°C. Solutions were diluted in PBS. Between incubation steps, slides were rinsed in PBS solution. For detection, Streptavidin AB Complex/horseradish peroxide (Dako GmbH, Hamburg, Germany), was applied for 30 min, followed by development with DAB + Chromogen (Dako GmbH). Sections from heart muscle served as positive controls and omission of the lectin as negative control.

Corrosion cast technique

Two animals of group A were sacrificed 6 weeks after matrix implantation (at the time of cell injection) for corrosion casting to verify adequate vascularization of the constructs before cell injection. Corrosion casting was performed

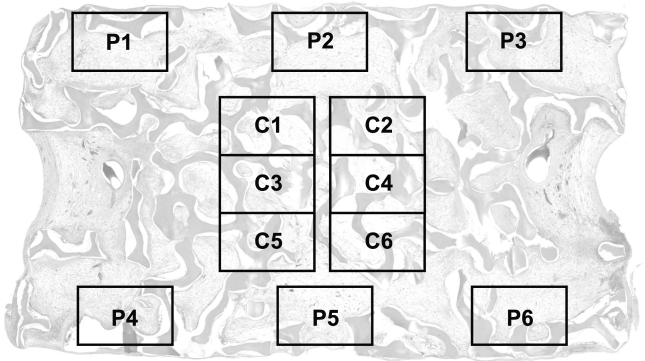

FIG. 3. Schematic representation of morphological evaluation. All cross-sections were divided into 12 equal sectors (6 at the periphery and 6 at the central part of the matrix), and all images of each sector were acquired under 100× magnification. The mean value of osteoblasts per field was calculated for each group and each time.

as described previously.[15] Briefly, cannulation of the aorta was followed by perfusion of the caudal vascular system with different solutions, ending up with a mixture of 4:1 prepolymerized oligomer:methylmethacrylate monomer (Aldrich Chemie, Steinheim, Germany) and benzoyl oxide as a catalyst (Mercox, Ladd Research Industries, Burlington, VT) using a method described by Lametschwandtner *et al.*[18]

The rat body was put for at least 6 h in a warm waterbath (50°C). The construct was then left for 12 h in 7.5% sodium hydroxide at 60°C. The cycle was repeated 3 times. Decalcification was achieved by leaving the PBCB matrix in 2.5% hydrochloric acid solution at room temperature for 12 h. For removal of the calcium deposits, the vascular cast was placed into a 5% formic acid solution for 15 min. Final drying was performed uisng lyophilization.

Ribonucleic acid isolation, complementary deoxyribonucleic acid synthesis and PCR

Homogenization was performed after explantation in RNAlater (Qiagen, Heidelberg, Germany) using an Ultra-Turrax homogenizer (IKA, Staufen, Germany). Total ribonucleic acid (RNA) was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was treated with DNase I (Qiagen, Hilden), and purity, concentration, and integrity were determined according to A_{260} and A_{280} ($A_{260}/A_{280} = 1.7–2.0$) measurements and electrophoresis on 0.9% formaldehyde agarose gel. Aliquots of 5 μg of total RNA per explant were reverse-transcribed using oligo(dT)-primed first-strand complementary deoxyribonucleic acid (cDNA) synthesis with the ProSTAR First-Strand RT-PCR Kit (Stratagene, La Jolla, CA), according to the manufacturer's recommendations. From all study groups, a no-RT control was preserved. All cDNA probes confirmed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and were analyzed for expression of the following genes: alkaline phosphatase (outward primers: forward 5′-CGCCTATCAGCTAATGCACA-3′, reverse 5′-GCTGTGAAGGGCTTCTTGTC-3′, product size 624 bp; inward primers: forward 5′-CGATGTGATCATGGGTGGT-3′, reverse 5′-CGTGGGAGTGATCAGCAGTA-3′, product size 500 bp), osteopontin (outward primers: forward 5′-GAGGAGAAGGCGCATTACAG-3′, reverse 5′-GACTGCTCAGTGCTCTCGTG-3′, product size 647; inward primers: forward 5′-AAGCCTGACCCATCTCAGAA-3′, reverse 5′-GCAACTGGGATGACCTTGAT-3′, product size 596 bp), bone sialoprotein (outward primers: forward 5′-CGGCCACGCTACTTTCTTTA-3′, reverse 5′-ACTCAACCGTGCTGCTCTTT-3′, product size 670; inward primers: forward 5′-ATGGAGATGGCGATAGTTCG-3′, reverse 5′-GTTCCTTCTGCACCTGCTTC-3′ product size 439), collagen I (outward primers: forward 5′-GTCCTTCTGGTGAACGTGGT-3′, reverse 5′-CCCTTAGGACCTGGAAGACC-3′, product size 712 bp; inward primers: forward 5′-GTCCCAAAGGTTCTCCTGGT-3′, reverse 5′-GGAAACCTCTCTCGCCTCTT-3′, product size 492 bp), osteocalcin (outward primers: forward 5′-CATGAGGACCCTCTCTCTGC-3′, reverse 5′-GTCCGCTAGCTCGTCACAAT-3′ product size 733 bp; inward primers: forward 5′-GCATTCTGCCTCTCTGACCT-3′, reverse 5′-ACCCAAGTCCATTGTTGAGG-3′, product size 414) and GAPDH (forward primer 5′-AACGACCCCTTCATTGACC-3′, reverse primer 5′-TGAAGACGCCAGTAGACTCC-3′).

PCR was performed as a nested 25-cycle touchdown PCR (except for GAPDH, which was performed as a 1-step PCR) using TaqDNA polymerase with Q-Solution (Qiagen, Hilden, Germany), 5 μL of RT products as templates for the first 25 cycles, and 1 μL of that PCR product for the second (nested) PCR in a PTC-200 DNA Engine Peltier Thermal Cycler (MJ Research, Incline Village, NV). Electrophoresis was carried out in 1.5% agarose gels containing ethidium bromide. Products were visualized and photographed under ultraviolet light.

RESULTS

Surgery and animals

All animals tolerated the surgical procedure well. There were no major postoperative complications such as infection, hematomas, or wound dehiscence. No extrusion of the implants occurred over the observation period. Patency of the previously generated AV loop was assessed during the secondary procedure of cell injection. In more than 90% of the animals, patent AV loops were observed. Animals with obliterated AV loops were excluded from the study and replaced with newly operated animals. Thus, a total number of 37 rats had to be operated on, resulting in 34 animals with patent AV loops (group A). There were no complications in animals in group B (subcutaneous implantation of matrices).

Injection of gel-immobilized osteoblasts

The injection of fibrin gel-immobilized primary osteoblasts in prevascularized hard matrices proved to be technically feasible. After the groin was opened, there were newly formed capsules around the chambers containing the matrix with the AV loop. After removal of the placeholders, the fibrin gel–immobilized osteoblasts were easily injected into the canals of the matrix using the Duploject syringe system, as schematically shown in Fig. 2. The gel immobilization provided reproducible and homogenous cell distribution within the canals of the construct and helped to minimize cell loss during injection. However, ingrowth of fibrotic tissue had occurred since prevascularization, facilitating cell injection exclusively in the previously blocked canals.

Histology and corrosion casting

India ink injection and corrosion casting demonstrated a dense network of newly formed vessels originating from

the AV loop and completely interfusing the PBCB matrix (Fig. 4). Over time, a steady increase of tissue fibrosis and foreign body reaction with presence of multinucleated giant cells was detectable in both groups. However, the intensity of foreign body reaction and the amount of tissue fibrosis were more pronounced in matrices from group A (Fig. 5).

In group A, gel-immobilized osteoblasts were visible at 1 week after cell injection. Bone formation was seen in 1 long-term (16 weeks) animal of group A (AV loop) (Fig. 6), whereas the other matrices contained only fibrotic vascularized tissue (Fig. 7). Nuclei within this newly formed bone tissue displayed the typical cobblestone aspect of osteoblasts.

Using lectin staining procedures, we were able to demonstrate endothelial cells in the vessels of the implant 4 and 8 weeks after implantation of the cells (Fig. 8).

Fluorescence sequential labeling failed to detect bone formation in implants from both groups (data not shown).

CFDA labeling and morphometric analysis

At weeks 1, 4, and 8 after osteoblast injection, CFDA-positive cells were evident in high numbers in the peripheral and central regions of the matrix in group A (AV loop) (Fig. 9), as detected by evaluation of serial sections. In contrast, sections of the subcutaneously implanted matrices with injected osteoblasts contained a significantly lower number of viable osteoblasts at each time point. In group A, most of the detected osteoblasts were evident in the central parts of the specimens and fewer in the peripheral regions.

In detail, after 1 week, we found significant differences in terms of osteoblast survival between groups A and B. In the AV loop group (group A), a significantly greater number of CFDA-positive cells was observed (282 ± 49.3 cells per field of view (FOV) vs 2 ± 1.69 in the control group (group B) ($p < 0.05$). Similar results could be obtained after 4 weeks, with 19 ± 2.58 CFDA-positive cells in group A versus 2 ± 0.29 CFDA-positive cells in group B ($p < 0.05$). Also, after 8 weeks, significantly more CFDA-positive cells were detected in group A (11 ± 3.57) than in group B ($0 \pm$

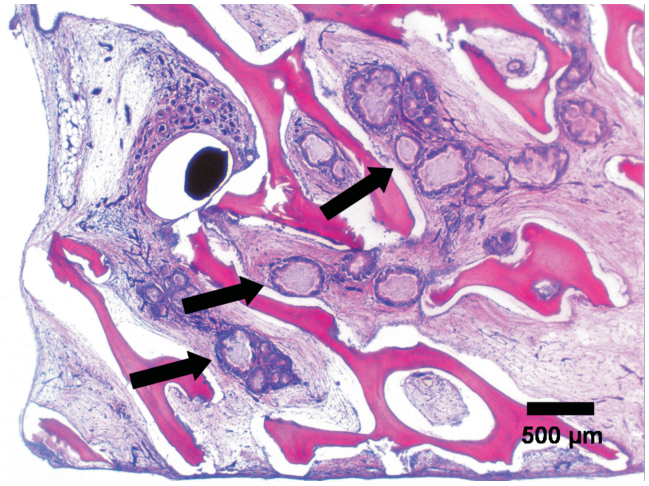

FIG. 5. Hematoxylin and eosin staining of specimen of group A at 16 weeks showing a significant foreign body reaction to processed bovine cancellous bone matrix (arrows indicate typical multinucleated giant cells) (magnification ×25). Color images available online at www.liebertpub.com/ten.

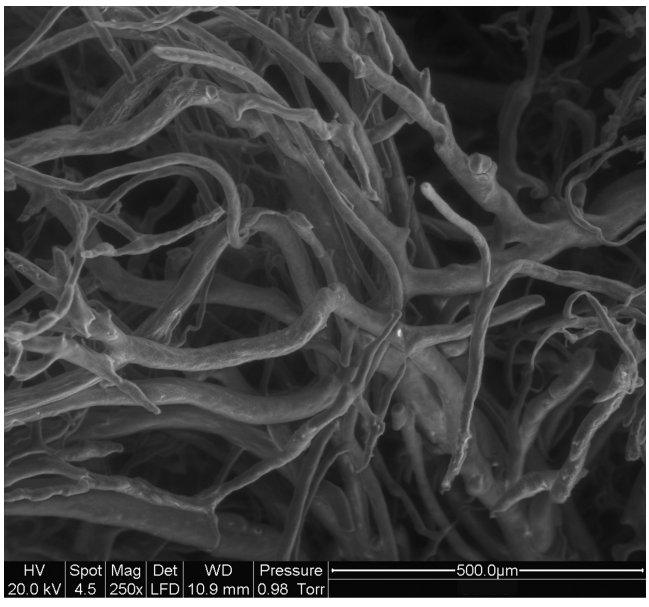

FIG. 4. Vascular corrosion cast of a group A (arteriovenous (AV) loop) construct after 6 weeks demonstrating dense neovascularization originating from the AV loop (scanning electron microscopy ×250).

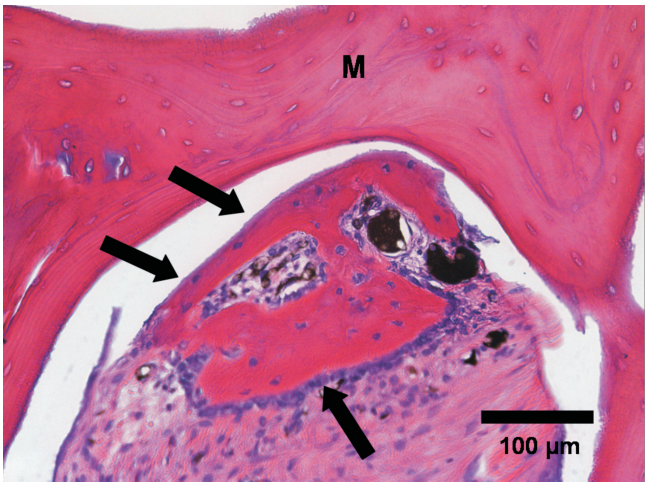

FIG. 6. Specimen of group A (ateriovenous loop) 16 weeks after cell injection showing the formation of new bone (arrow) containing osteoblast-like cells, in the direct neighborhood of processed bovine cancellous bone matrix (M) (hematoxylin and eosin staining, magnification ×200). Color images available online at www.liebertpub.com/ten.

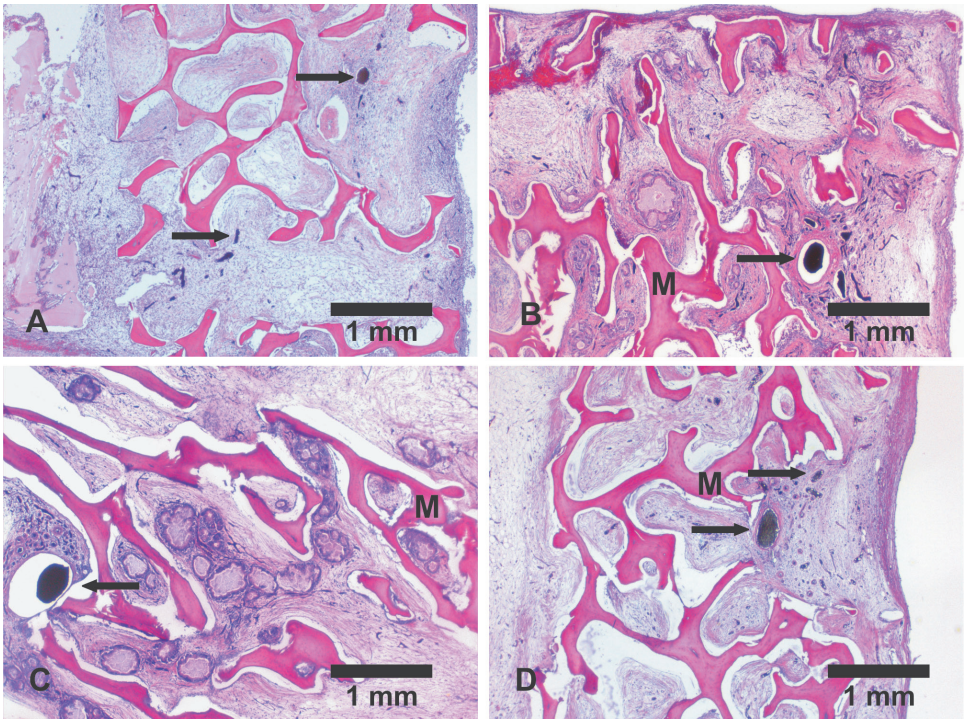

FIG. 7. Significant degree of vascularization in the matrices of group A as demonstrated using India ink perfusion (arrows indicating perfused vessels originating from the arteriovenous loop), followed by hematoxylin and eosin staining. There is also a significant increase in tissue fibrosis and foreign body reaction over time ((**A**) 1 week, (**B**) 4 weeks, (**C**) 8 weeks, (**D**) 16 weeks×25 magnification; M, processed bovine cancellous bone matrix). Color images available online at www.liebertpub.com/ten.

0.41; $p < 0.05$). The results are displayed graphically in Figure 10.

RT-PCR analysis

RT-PCR of analysis of explants in group A (16 weeks after cell injection) showed messenger RNA (mRNA) expression of all genes examined: collagen I, osteocalcin, osteopontin, bone sialoprotein, and alkaline phosphatase. In group B, no expression of any of these genes was observed at this time point, whereas GAPDH mRNA was expressed at comparable levels (Fig. 11).

DISCUSSION

Induction of vascularization is a core element for transfer of tissue-engineering models from an *in vitro* to an *in vivo* environment. Diffusion limits oxygen and nutrition supply to a maximum range of 200 µm into a given matrix.[8,9] Thus,

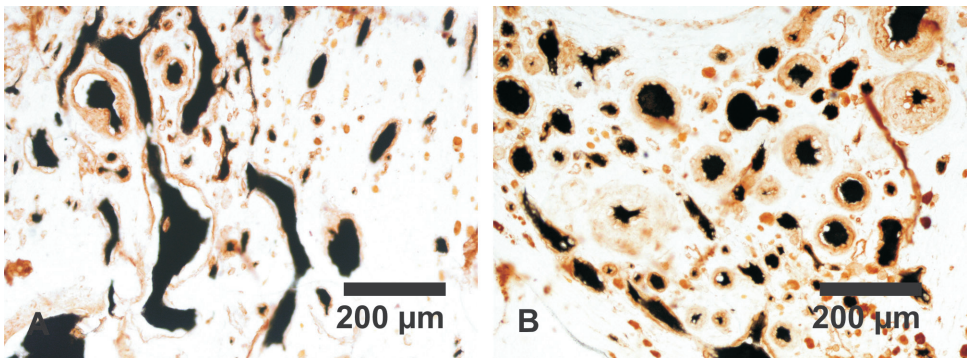

FIG. 8. Lectin staining 4 (**A**) and 8 (**B**) weeks after implantation. Endothelial cells were evident in all India ink–filled vessels, as demonstrated by positive lectin staining (stained brown, magnification×200). Color images available online at www.liebertpub.com/ten.

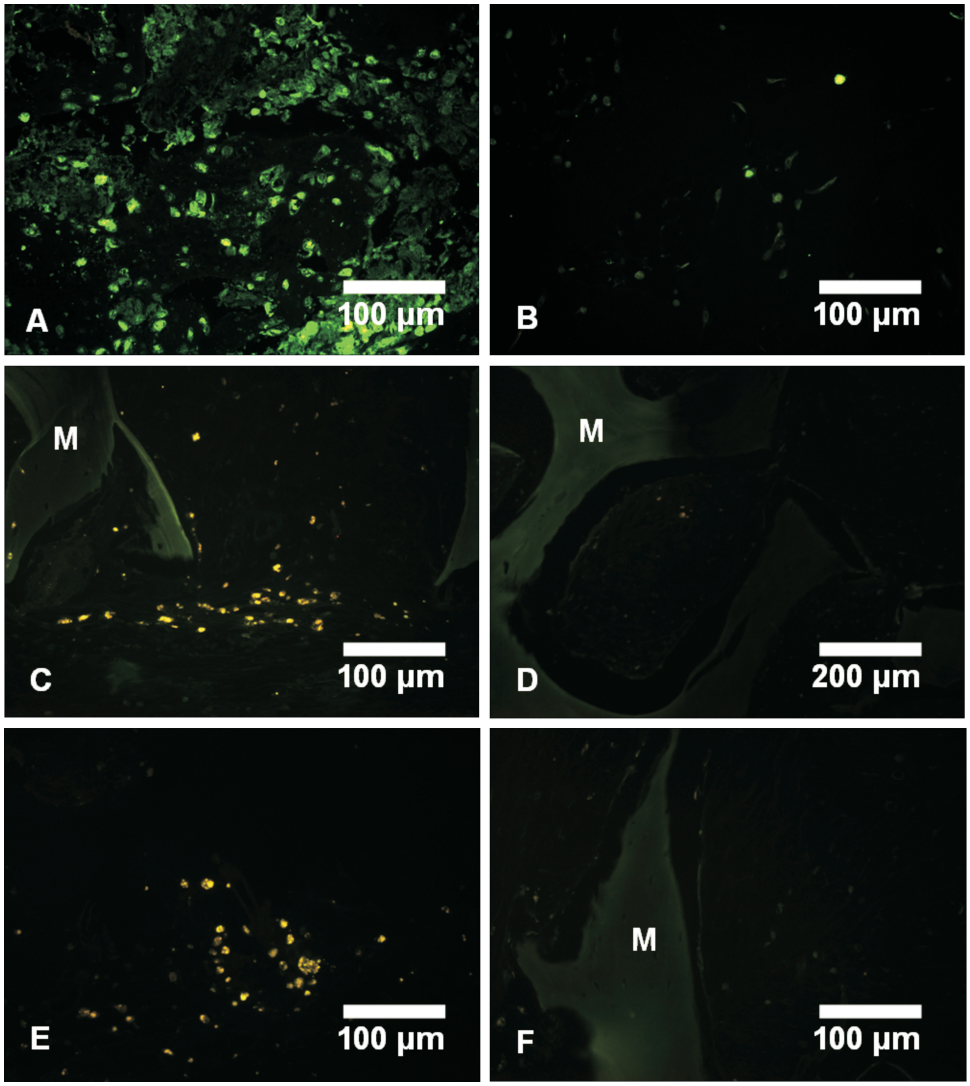

FIG. 9. Carboxyfluorescein diacetate (CFDA)-labeling experiment. CFDA-positive cells were evident in high numbers in the peripheral and central regions of the matrix in group A (arteriovenous loop) 1 (**A**), 4 (**C**), and 8 (**E**) weeks after implantation (fluorescent microscopy×200). The matrices in group B (subcutaneously implanted cell-seeded matrices) contained only a few viable osteoblasts after 1 (**B**), 4 (**D**), and 8 (**F**) weeks (fluorescent microscopy×200; M, processed bovine cancellous bone matrix). Color images available online at www.liebertpub.com/ten.

suboptimal initial vascularization often limits survival of cells in the center of large constructs.[10] The majority of currently applied tissue-engineering approaches rely on the so-called ''extrinsic'' mode of neovascularization. In this setting, the neovascular bed originates from the periphery of the construct, which should be implanted into a site of high vascularization potential. Subcutaneous,[19] intramuscular,[20] and intraperitoneal[10] implantation has also been reported, facing the above-mentioned obstacles. Although generation of bone tissue is feasible using these techniques, the newly formed tissue is vascularized in a random pattern, making a transfer to distant implantation sites impossible without destruction of the vascular network. Reconstructive surgeons therefore aim to generate so-called ''axially vascularized'' tissues that could be transferred to the defect site using microsurgical techniques of vascular anastomosis.[21]

Since first mention of the AV loop by Erol and Spira in 1979[11] and further developments by Morrison and coworkers,[12–14] the superiority of the AV loop as a vascular carrier for an axial type of vascularization has been clearly demonstrated. In previous studies, we showed for the first time successful axial vascularization of mechanically stabile solid matrices (custom-made PBCB) using an AV loop.[15] After 4 and 8 weeks, the PBCB matrices displayed a significant degree of vascularization. In another study, we were recently able to show that a combination of axial prevascularization of a fibrin matrix using an AV loop and secondary cell injection promotes survival of injected myoblasts.[22] In

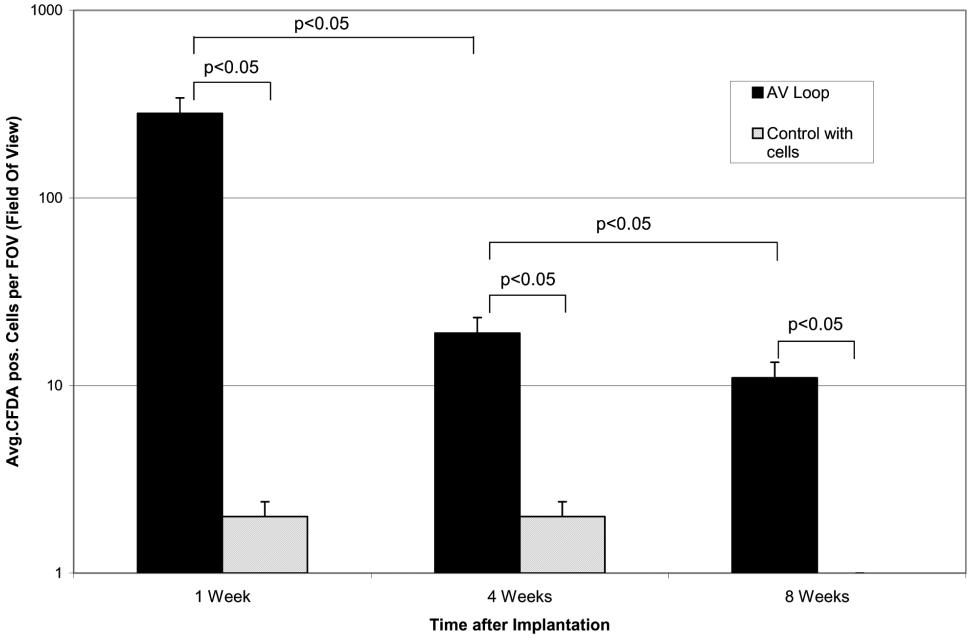

FIG. 10. Morphometric analysis of cell engraftment. There were a significantly higher number of carboxyfluorescein diacetate (CFDA)-positive cells in the arteriovenous (AV) loop group (group A), than in the control group (group B) ($p < 0.05$) was observed at any time point. Also, there were significantly fewer CFDA-positive cells in group A at 8 weeks than in group A at 4 weeks and in group A at 4 weeks than in group A at 1 week.

contrast, the only other study published on the combination of AV loop vascularization and cell implantation was performed with a simultaneous approach, leading to a high degree of initial cell death and scar and fatty tissue formation.[16] However, before the present experiments, we performed cell injection studies with a small group of AV loop chambers in which the osteoblasts were added without prevascularization. As a result, we were not able to detect CFDA-labeled cells in significant numbers, suggesting no relevant survival of the cells after the first week after transplantation, probably due to a lack of vascularization of the matrix. These observations were as expected and in accordance with several previous studies showing cell death or apoptosis due to a lack of oxygen and nutrient supply[17] (data not shown). Hence, our approach combined the experience of successful axial vascularization of matrices using an AV loop with the advantages of secondary cell injection. To the best of our knowledge, the study presented here is the first one to explore the potential of fibrin gel–immobilized osteoblast injection into prevascularized solid matrices.

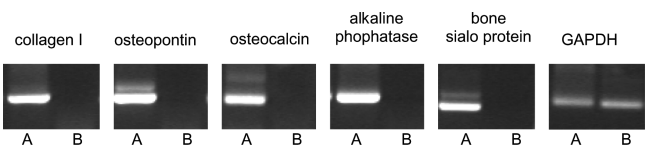

FIG. 11. Reverse transcriptase polymerase chain reaction analysis showed messenger ribonucleic acid (mRNA) expression of bone genes in group A (arteriovenous loop) after 16 weeks. There was no mRNA expression of these genes detectable in group B (normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression).

Based on our experience from previous experiments, we used a 6-week prevascularization interval for the time point of cell injection. As reported previously,[15,23,24] by week 6, the maturation process of the neovascular network is underway. Morphological characteristics of a mature vascular bed, including formation of a capillary loop connecting the arterial and venous side of the microcirculation; organization of the vessels into a hierarchy with vessels of different orders from small arteries into precapillary arterioles, capillaries, and postcapillary venules draining into numerous venules; and reduction of the number of the neovessels through the combined processes of regression and persistence, could be confirmed using scanning electron microscopy of corrosion casts.

One, 4, and 8 weeks after injection, we observed a significantly higher survival of the transplanted autologous osteoblasts in group A than of non-vascularized, subcutaneously implanted matrices in group B. One might speculate that further enhancement of vascularization in the PBCB matrix might be achieved by incorporating vascular growth factors such as vascular endothelial growth factor[25] or basic fibroblast growth factor.[26] Because the ability of these factors among others to promote neovascularization has been demonstrated, the period between AV loop construction and secondary cell injection might one day be shortened, further

improving the clinical feasibility of this approach. In the current study, the presence of endothelial cells in samples of the AV loop group was demonstrated using lectin stainings, as described above (Fig. 8). This finding underscores that a morphologically intact vascular network originated from the vascular axis over time. It might be possible that cross-talk between transplanted osteoblasts and host endothelial cells supports bone formation within the construct. However, further investigations with the scope of osteoblast–endothelial cell interaction would be necessary to elucidate these effects in detail in the AV loop setting.

Fibrin gel has been widely used as matrix for immobilization of osteogenic cells. Tholpady *et al*. reported successful transplantation of osteoprogenitor cells for repair of an critical-sized osseous facial defect.[27] In previous studies, our group applied fibrin gel–immobilized primary osteoblasts in calcium phosphate bone cement.[19] In previous studies, PBCB provided a suitable environment for adhesion and differentiation of fibrin gel–immobilized primary osteoblasts *in vitro*.[6] Furthermore, when implanted into critical-sized calvarial defects, these matrices exhibited good biointegration, highly osteoconductive properties, and enhanced bone healing.[17] PBCB was chosen as a material in these studies because it is already approved for clinical use and displays suitable mechanical properties for osteosynthetic fixation in bone defects.

The gene expression results showing mRNA expression of all examined genes (i.e. collagen I, osteocalcin, osteopontin, bone sialoprotein, and alkaline phosphatase) in prevascularized matrices (group A) suggest that osteoblasts within the prevascularized biomaterial display differentiated cell function to a certain degree. This is in contrast to group B matrices (without prevascularization), in which housekeeping gene (GAPDH) expression could be detected, whereas bone gene expression was completely absent. Despite proven long-term engraftment, as evidenced using CFDA-labeling and a physiological gene expression pattern of transplanted cells in this study, we were able to detect bone formation within only one of the matrices from group A after 16 weeks. The sensitivity of mRNA analysis using RT-PCR is much higher than that of histological methods based on detection of calcification and/or morphological features of bone formation. Therefore, different sensitivities might explain the discrepancy between the 2 findings of positive osteogenic mRNA detection and negative histological staining.

A significant foreign body reaction was visible in matrices of both groups. This stands in contrast to our previous observations, in which no foreign body reaction to the PBCB matrix was noted.[15] An explanation might be the prolonged time of exposure to the host organism in the current study, covering up to 6 (before cell injection) plus 16 weeks (after cell injection), compared with a maximum period of 16 weeks between PBCB implantation and explantation in previous experiments. In addition, a different production lot of the biological PBCB matrix was used in the current study. Thus, differences in the biological properties between different lots might also account for the difference in terms of foreign body reaction.

In addition, within the current study, we observed a difference between the extent of foreign body reaction in group A (AV loop) and group B (subcutaneously). This difference might also be due to the prolonged time of exposure of the PBCB matrix to the host's circulation in group A, because prevascularization combined with secondary cell injection added 6 weeks of exposure until the date of explantation to that of PBCB matrices in group B. This foreign body reaction with consecutive tissue fibrosis might eventually have negatively affected bone formation of the transplanted osteoblasts in both groups.

Taking the histological and gene expression findings together, ingrowth of fibrotic tissue into the PBCB matrix is taking place at the same time as injected osteoblasts begin to proliferate within the predefined canals. Thus, injected osteoblasts lost the race against scar tissue formation in the PBCB matrix in nearly all of the constructs of both groups. Matrices with improved biocompatibility that induce less foreign body reaction and tissue fibrosis are therefore essential for induction of bone formation using secondary cell injection. Furthermore, osteoinductive factors that have been introduced into the field of bone tissue engineering[28–30] might be applicable for induction of bone formation in axially vascularized tissues.

CONCLUSION

The injection of gel-immobilized osteoblasts into prevascularized hard matrices is technically feasible. We were able to demonstrate successful vascularization of solid porous matrices using an AV loop. After injection into prevascularized porous matrices, osteoblasts show a significant higher engraftment and display a differentiated gene expression pattern than nonvascularized matrices. However, transplantation of osteogenic cells only induced bone formation in one of the matrices, most likely because of inappropriate matrix properties and consecutive ingrowth of scar tissue. The insertion of an AV loop into innovative biomaterials with improved biocompatibility in combination with secondary osteoblast injection may eventually allow the generation of axially vascularized bone tissues for complex reconstructions with minimal donor site morbidity.

ACKNOWLEDGMENTS

This study was supported by research grants from the University of Erlangen (ELAN Program) and from Tutogen Medical Inc. The authors thank Professor Greil and Mr. Reinhard for production of the Teflon chambers. This work contains parts of K. Heidner's doctoral thesis. The authors thank T. Fey for preparation of the scanning electron micro-

scope specimens. The authors also thank Ms. Lucia Grabinger and Mr. Simon Euler for their technical assistance.

REFERENCES

1. Gazdag, A.R., Lane, J.M., Glaser, D., and Forster, R.A. Alternatives to Autogenous bone graft: efficacy and indications. J Am Acad Orthop Surg **3,** 1, 1995.
2. Ozaki, T., Hillmann, A., Wuisman, P., and Winkelmann, W. Reconstruction of tibia by ipsilateral vascularized fibula and allograft. 12 cases with malignant bone tumors. Acta Orthop Scand **68,** 298, 1997.
3. Banis, J.C., Jr., and Swartz, W.M. Refinements in free flaps for head and neck reconstruction. Clin Plast Surg **17,** 673, 1990.
4. Shea, K.G., Coleman, D.A., Scott, S.M., Coleman, S.S., and Christianson, M. Microvascularized free fibular grafts for reconstruction of skeletal defects after tumor resection. J Pediatr Orthop **17,** 424, 1997.
5. Keith, J.D., Jr. Localized ridge augmentation with a block allograft followed by secondary implant placement: a case report. Int J Periodontics Restorative Dent **24,** 11, 2004.
6. Stangenberg, L., Schaefer, D.J., Buettner, O., Ohnolz, J., Mobest, D., Horch, R.E., Stark, G.B., and Kneser, U. Differentiation of osteoblasts in three-dimensional culture in processed cancellous bone matrix: quantitative analysis of gene expression based on real-time reverse transcription-polymerase chain reaction. Tissue Eng **11,** 855, 2005.
7. Trentz, O.A., Hoerstrup, S.P., Sun, L.K., Bestmann, L., Platz, A., and Trentz, O.L. Osteoblasts response to allogenic and xenogenic solvent dehydrated cancellous bone *in vitro*. Biomaterials **24,** 3417, 2003.
8. Goldstein, A.S., Juarez, T.M., Helmke, C.D., Gustin, M.C., and Mikos, A.G. Effect of convection on osteoblastic cell growth and function in biodegradable polymer foam scaffolds. Biomaterials **22,** 1279, 2001.
9. Folkman, J., and Hochberg, M. Self-regulation of growth in three dimensions. J Exp Med **138,** 745, 1973.
10. Kneser, U., Kaufmann, P.M., Fiegel, H.C., Pollok, J.M., Kluth, D., Herbst, H., and Rogiers, X. Long-term differentiated function of heterotopically transplanted hepatocytes on three-dimensional polymer matrices. J Biomed Mater Res **47,** 494, 1999.
11. Erol, O.O., and Spira, M. New capillary bed formation with a surgically constructed arteriovenous fistula. Surg Forum **30,** 530, 1979.
12. Hofer, S.O., Knight, K.M., Cooper-White, J.J., O'Connor, A.J., Perera, J.M., Romeo-Meeuw, R., Penington, A.J., Knight, K.R., Morrison, W.A., and Messina, A. Increasing the volume of vascularized tissue formation in engineered constructs: an experimental study in rats. Plast Reconstr Surg **111,** 1186, 2003.
13. Cassell, O.C., Morrison, W.A., Messina, A., Penington, A.J., Thompson, E.W., Stevens, G.W., Perera, J.M., Kleinman, H.K., Hurley, J.V., Romeo, R., and Knight, K.R. The influence of extracellular matrix on the generation of vascularized, engineered, transplantable tissue. Ann N Y Acad Sci **944,** 429, 2001.
14. Tanaka, Y., Sung, K.C., Tsutsumi, A., Ohba, S., Ueda, K., and Morrison, W.A. Tissue engineering skin flaps: which vascular carrier, arteriovenous shunt loop or arteriovenous bundle, has more potential for angiogenesis and tissue generation? Plast Reconstr Surg **112,** 1636, 2003.
15. Kneser, U., Polykandriotis, E., Ohnolz, J., Heidner, K., Grabinger, L., Euler, S., Aman, K.U., Hess, A., Brune, K., Greil, P., Sturzl, M., and Horch, R.E. Engineering of vascularized transplantable bone tissues: induction of axial vascularization in an osteconductive matrix using an arteriovenous loop. Tissue Eng **12,** 1721, 2006.
16. Messina, A., Bortolotto, S.K., Cassell, O.C., Kelly, J., Abberton, K.M., and Morrison, W.A. Generation of a vascularized organoid using skeletal muscle as the inductive source. Faseb J **19,** 1570, 2005.
17. Kneser, U., Stangenberg, L., Ohnolz, J., Buettner, O., Stern-Straeter, J., Mobest, D., Horch, R.E., Stark, G.B., and Schaefer, D.J. Evaluation of processed bovine cancellous bone matrix seeded with syngenic osteoblasts in a critical size calvarial defect rat model. J Cell Mol Med **10,** 695, 2006.
18. Lametschwandtner, A., Lametschwandtner, U., and Weiger, T. Scanning electron microscopy of vascular corrosion casts—technique and applications. Scan Electron Microsc 663, 1984.
19. Kneser, U., Voogd, A., Ohnolz, J., Buettner, O., Stangenberg, L., Zhang, Y.H., Stark, G.B., and Schaefer, D.J. Fibrin gel-immobilized primary osteoblasts in calcium phosphate bone cement: *in vivo* evaluation with regard to application as injectable biological bone substitute. Cells Tissues Organs **179,** 158, 2005.
20. Beier, J.P., Kneser, U., Stern-Strater, J., Stark, G.B., and Bach, A.D. Y chromosome detection of three-dimensional tissue-engineered skeletal muscle constructs in a syngeneic rat animal model. Cell Transplant **13,** 45, 2004.
21. Kneser, U., Schaefer, D.J., Polykandriotis, E., and Horch, R.E. Tissue engineering of bone: the reconstructive surgeon's point of view. J Cell Mol Med. **10,** 7, 2006.
22. Bach, A.D., Arkudas, A., Tjiawi, J., Polykandriotis, E., Kneser, U., Horch, R.E., and Beier, J.P. A new approach to tissue engineering of vascularized skeletal muscle. J Cell Mol Med **10,** 716, 2006.
23. Polykandriotis, E., Arkudas, A., Euler, S., Beier, J.P., Horch, R.E., and Kneser, U. [Prevascularisation strategies in tissue engineering]. Handchir Mikrochir Plast Chir **38,** 217, 2006.
24. Kelly, J.L., Findlay, M.W., Knight, K.R., Penington, A., Thompson, E.W., Messina, A., and Morrison, W.A. Contact with existing adipose tissue is inductive for adipogenesis in matrigel. Tissue Eng **12,** 2041, 2006.
25. Byrne, A.M., Bouchier-Hayes, D.J., and Harmey, J.H. Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF). J Cell Mol Med **9,** 777, 2005.
26. Tanaka, Y., Tajima, S., Tsutsumi, A., Akamatsu, J., and Ohba, S. New matrix flap prefabrication by arteriovenous shunting and artificial skin dermis in rats. II. Effect of interpositional vein and artery grafts and bFGF on new tissue generation. J Jpn Plast Reconstr Surg **16,** 679, 1996.
27. Tholpady, S.S., Schlosser, R., Spotnitz, W., Ogle, R.C., and Lindsey, W.H. Repair of an osseous facial critical-size defect using augmented fibrin sealant. Laryngoscope **109,** 1585, 1999.
28. Endres, M., Hutmacher, D.W., Salgado, A.J., Kaps, C., Ringe, J., Reis, R.L., Sittinger, M., Brandwood, A., and Schantz, J.T. Osteogenic induction of human bone marrow-derived

mesenchymal progenitor cells in novel synthetic polymer-hydrogel matrices. Tissue Eng **9,** 689, 2003.
29. Schantz, J.T., Hutmacher, D.W., Ng, K.W., Khor, H.L., Lim, M.T., and Teoh, S.H. Evaluation of a tissue-engineered membrane-cell construct for guided bone regeneration. Int J Oral Maxillofac Implants **17,** 161, 2002.
30. Hutmacher, D.W., Schantz, T., Zein, I., Ng, K.W., Teoh, S.H., and Tan, K.C. Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modeling. J Biomed Mater Res **55,** 203, 2001.

Address reprint requests to:
U. Kneser, M.D.
Department of Plastic and Hand Surgery
University of Erlangen Medical Center
Krankenhausstrasse 12
D-91054 Erlangen
Germany

E-mail: ulrich.kneser@chir.imed.uni-erlangen.de