Image-Based Extracorporeal Tissue Engineering of Individualized Bone Constructs

Ulrich Meyer, MD, DDS, PhD[1]/Andre Büchter, DDS[2]/Ariane Hohoff, DDS[3]/Elke Stoffels, PhD[4]/
Thomas Szuwart, PhD (Biol)[5]/Christoph Runte, DDS, PhD[6]/Dieter Dirksen, PhD (Phys)[7]/
Hans-Peter Wiesmann, PhD (Phys)[2]

Purpose: *Computer-aided technologies have been recently employed for use in extracorporeal bone tissue engineering strategies. In this pilot animal experimental study, the intention was to test whether autologous osteoblast-like cells cultured in vitro on individualized scaffolds can be used to support bone regeneration in a clinical environment.* ***Materials and Methods:*** *For this purpose, mandibular bone defects were surgically introduced into the mandibles of minipigs and the scaffold of the defect site was modeled by computer-aided design/computer-aided manufacturing technique. Autologous bone cells from porcine calvaria were harvested from minipigs and grown in culture. Cells were seeded on scaffolds generated by rapid prototyping of polylactic acid/polyglycolic acid copolymers. The defects were then reconstructed by implanting the tissue constructs.* ***Results:*** *The intraoperative sites as well as the postoperative computerized tomographic scans demonstrated an accurate fit in the defect sites. The implanted scaffold constructs enriched with osteoblast-like cells were well tolerated and appeared to support bone formation, as revealed by histologic and immunohistochemical analyses.* ***Discussion:*** *These results indicated that in vitro expanded osteoblast-like cells spread on a resorbable individualized scaffold can be capable of promoting the repair of bony defects in vivo.* ***Conclusion:*** *These results warrant further attempts to combine computer modeling and tissue engineering for use in bone reconstructive surgery.* INT J ORAL MAXILLOFAC IMPLANTS 2005;20:882–890

Key words: *animal model, bioengineering, bone, osteoblasts*

Bone repair is an issue of intensive investigation in maxillofacial surgery. The replacement of lost bone structures or augmentation of insufficient bone volume is a recent challenge in preimplantologic strategies. Current approaches in bone reconstructive surgery exploit biomaterials, autografts, or allografts, although restrictions on all of these strategies exist. One important advantage of tissue grafts over biomaterials is that they contain living cells and tissue-inducing substances conferring biologic plasticity. Research is in progress to develop cell-containing hybrid materials that remain biointeractive after implantation. Within the last decade, the emerging field of tissue engineering has reached a level of clinical applicability that now offers promising alternatives for maxillofacial bone reconstruction.[1]

For extracorporeal tissue engineering, a complete 3-dimensional tissue consisting of autologous or donor cells within a scaffold is grown in vitro and implanted once it has reached "maturity."[2] The engineered bone scaffold should in principle resemble in an idealized fashion the 3-dimensional gross-, micro-, and nanomorphology of the bone to be replaced. When the anatomic structure is of major concern for

[1]Deputy Director, Clinic for Maxillofacial and Plastic Facial Surgery, University of Düsseldorf, Germany.
[2]Assistant Professor, Department of Cranio-Maxillofacial Surgery, University of Münster, Germany.
[3]Assistant Professor, Department of Orthodontics, University of Münster, Germany.
[4]Senior Researcher, Department of Cranio-Maxillofacial Surgery, University of Münster, Germany.
[5]Institute of Anatomy, University of Münster, Germany.
[6]Assistant Professor, Department of Prosthodontics, University of Münster, Germany.
[7]Assistant Professor, Laboratory of Biophysics, University of Münster, Germany.

Correspondence to: Dr Ulrich Meyer, Clinic for Maxillofacial and Plastic Facial Surgery, University of Düsseldorf, Moorenstrasse 5, D-40001 Dusseldorf, Germany. Fax: + 49 211 811 8877. E-mail: ulrich.meyer@med.uni-dusseldorf.de

the functional outcome, individualized tissue constructs, according to the external geometry, are desirable. Alsberg and associates[1] and Goldstein[3] have given a recent overview of the state of bone tissue engineering. Bone as a load-bearing organ is very susceptible towards stress transduction. A perfect congruency between the defect site and the artificial substrate is, therefore, important. Almost all tissue engineering concepts require the use of some form of biocompatible scaffold, which serves as a template for cell proliferation and ultimately for tissue formation. Optimized biocompatible and resorbable materials[2] organized into a complex morphology are essential components of successful tissue engineering. Bone scaffolds, like virtually all other scaffolds used in tissue engineering, are intended to degrade slowly and be replaced by newly formed regenerative tissue when implanted at appropriate sites.

Modern computer-aided technologies, medical imaging, and manufacturing have assisted in these advances and created new possibilities in the development of bone tissue substitutes (for review, see Sun and Lal[4]). This approach combines cell biologic methods with image-based techniques. Driven by computer-aided design (CAD)/computer-aided manufacturing (CAM) systems, individualized bone scaffolds can now be built outside the body. The use of noninvasive imaging techniques such as computerized tomography (CT) and magnetic resonance imaging in combination with minor surgical procedures for the explantation and isolation of bone cells allows extracorporeal tissue construction with only minimal donor morbidity. Imaging techniques and data processing are employed to monitor the clinical success of bone reconstruction procedures.

Engineering of bonelike tissues in vitro has to take into account the complexity of and scale required for clinical use.[2] One critical aspect of such a tissue engineering approach is the fate of the extracorporeally propagated cells in terms of their viability and differentiation during culture and after implantation. Although numerous clinical studies have documented the long-term healing process of bone defects after restoration with hybrid bone substitutes, only a few studies have focused on the cell-driven mode of bone regeneration in the early and later postoperative stages of craniofacial defect reconstruction.[5]

The purpose of the present study was to construct an individualized external shaped hybrid bone substitute in vitro and assess its clinical applicability in mandibular defect regeneration. Emphasis was placed on control of the external shape and size of the tissue construct as well as on the features of defect regeneration in a clinical setting.

MATERIALS AND METHODS

Materials

The experimental study was performed on 6 mature male Göttinger minipigs that were 14 to 16 months of age, with an average body weight of 35 kg. Minipigs were chosen to ensure a mandibular size comparable to the human mandible for bone substitute fabrication. This animal model has been successfully used in previous studies for bone reconstruction procedures. All surgery was performed under sterile conditions in an animal operating theater with an intramuscular injection of ketamine (10 mg/kg), atropine (0.06 mg/kg), and azaperone (0.03 mL/kg) to induce anesthesia. The study was approved by the Animal Ethics Committee of the University of Münster under the reference number G 67/2002. All animals underwent the same operative procedure.

Cell Culture

Osteoblast-like cells were derived from cranial periosteum harvested from the minipigs. The periosteum was removed from the calvaria under general anesthesia (Fig 1a). It was cut into 3- to 6-mm pieces and transferred to culture dishes with the osteogenic layer facing downward. Osteoprogenitor cells migrated from the tissue explants. Cells were cultured for 5 weeks in High Growth Enhancement Medium (ICN Biomedicals, Eschwege, Germany) supplemented with 10% fetal calf serum, 250 (μg/mL amphotericin B, 10,000 IU/mL penicillin, 10 mg/mL streptomycin, 200 mmol/L L-glutamine (Biochrom KG Seromed, Berlin, Germany), 10 mmol/L β-glycerophosphate, and 25 μg/mL ascorbic acid (Sigma-Aldrich, St Louis, MO) at 37°C and 5% CO_2 in humidified air. The medium was replaced twice a week. The differentiation state of cells was continuously monitored before seeding and during growth in the scaffold by immunocytochemical methods in an established fashion[6] to control their osteoblast-like features.[2]

For construction of the hybrid complex, only cells from the first passage were used. Primary culture cells were harvested by incubation in collagenase-containing Tyrode's solution (Biochrom KG Seromed), collected, and centrifuged to create pellets. Resuspended cells were seeded in a bioreactor on the prefabricated polylactic acid/polyglycolic acid (PLA/PGA) constructs. Cell culture conditions were similar to those used for the periosteum outgrowth culture. Cell viability was assessed at different times during cultivation. Three days after seeding, the hybrid materials were washed 3 times with phosphate buffer solution (PBS) and prepared for implantation. To assess the extracorporeal long-term out-

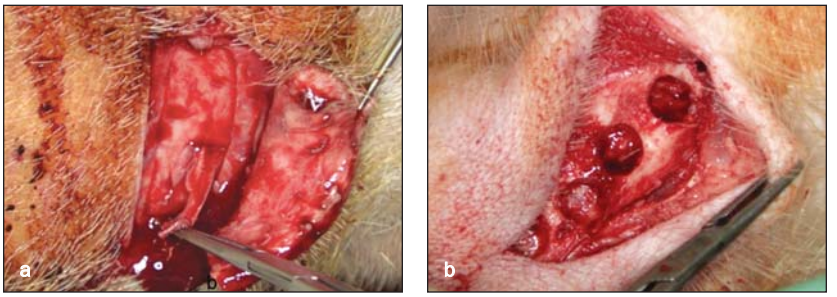

Fig 1 *(a)* Clinical view of the harvest of periosteum from a porcine calvarium. *(b)* Defects were created in the mandibular bodies of the minipigs.

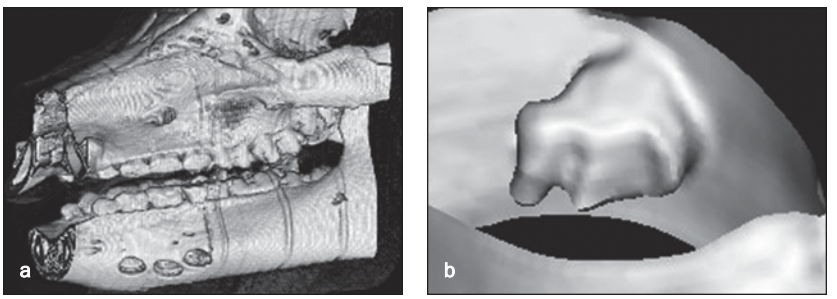

Fig 2 *(a)* Three-dimensional view of a mandibular defect site. *(b)* Transformation of the digital DICOM data into STL format.

come of cells in the scaffold itself, control hybrid materials (not used for the in vivo implantation) were additionally cultured for up to 3 weeks in the bioreactor and evaluated by immunohistochemistry.[6]

Scaffold Construction

Four weeks after removal of the periosteum, 4 mandibular defects were created in the mandible of each minipig. The mandible was exposed through a submandibular approach under general anesthesia. Three defects with diameters of 1 cm were created by using a bur to drill subperiosteally in the mandibular body (Fig 1b). After wound closure, CT scans of the mandible were performed (Philips, Eindhoven, The Netherlands) (Fig 2a). The scanning process was performed in 2-mm slices with 1-mm overlap technique. The data were stored on an optic disc using the DICOM (Digital Imaging and Communications in Medicine) format. If a 3-dimensional visualization was desired, the DICOM data sets were transferred to a G3 workstation (Apple, Cupertino, CA) for image reformation and viewing. All hard- and software applications of the system were coordinated with the Macintosh operating system version 8.5 (Apple). NIH-Image 1.61 (US National Institutes of Health, Bethesda, MD) was used for DICOM import, data analysis, and volume-rendering visualization. Based on the DICOM data set, a surface reconstruction of the volume of each defect was performed using a special software tool developed at the University of Münster's Laboratory of Biophysics. With this program, written in the computer language PV-Wave (Visual Numerics, Houston, TX), the single CT slices were analyzed and the contours of the defect area were detected. These contours were then combined into a 3-dimensional surface through a dense net of triangles. The scaffold design was finished by transforming this triangle net into a NURBS (non-uniform rational b-splines) surface which was done using a 3-D modeling program (Rhinoceros; Robert McNeel & Associates, Seattle, WA). Unlike triangle nets, NURBS allow a smooth tooling of the surface for final adaptations. Finally, the data set was transferred to a stereolithography (STL) format (Fig 2b), which was then used to create a model made of synthetic resin via rapid prototyping (stereolithography), after which the PLA/PGA construct was formed.

A digital image representation of the external scaffold was generated, and the defect site was manufactured as an external form by rapid prototyping (H&H Engineering and Rapid Prototyping, Lemgo, Germany) (Fig 3a). The internal structure (material composition, pore size) was controlled by a defined polymerization of PLA/PGA beads (50% PLA, 48% PGA, 2% carbonic acid [H_2CO_3]) of various sizes. The pore size of the inner part of the scaffold was approximately 200 (μm in diameter to allow the ingrowth of vessels and enhance nutrition and oxygen supply by diffusion.

Tissue Engineering

For the engineering of the viable bone tissue substitutes, autologous bone cells were spread on the scaffold and cultured in a bioreactor for 3 days. The tissue constructs were then implanted into the mandibular defects 1 week after defect creation through the submandibular approach used previously. The periosteum was carefully sutured, and the wound was

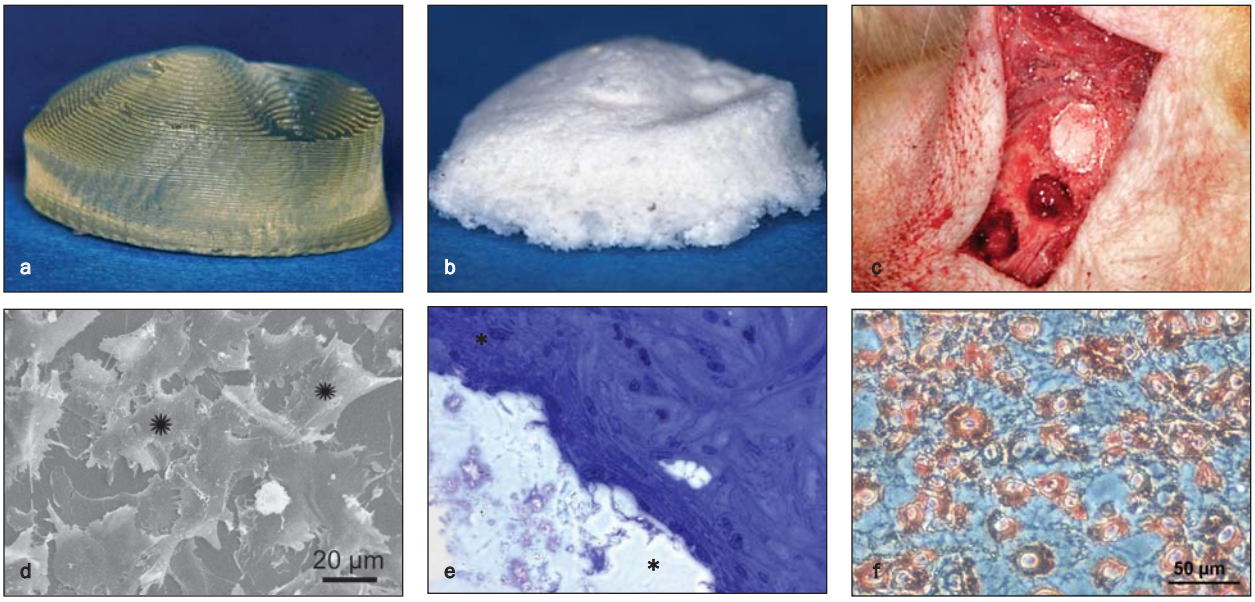

Fig 3 *(a)* Stereolithographic model of the defect site. *(b)* Individual scaffold fabrication by polymerization of PLA/PGA beads of various sizes. *(c)* Surgical implantation of the scaffold in the defect site. *(d)* Scanning electron microscopy shows polygonal osteoblasts attached to the PLA/PGA surface after 3 days of culture (* indicates osteoblastic cells, bar indicates original magnification). *(e)* Histological appearance of the hybrid material examined after 21 days of culture in the bioreactor (hematoxylin-eosin [H&E]; original magnification ×60). Multiple cell layers are located between the PLA/PGA material (* indicates the location of the PLA/PGA material removed the embedding process). *(f)* Immunohistochemical detection of osteonectin synthesized by osteoblasts located at the surface of PLA/PGA (levamisole/hematoxylin; original magnification ×40).

closed by multilayered sutures at the end of the operation. For the first several days, the animals were inspected continuously for signs of wound dehiscence or infection. Later, their general health was assessed weekly. The minipigs were fed a normal diet. At 3 days and 30 days, the animals were sacrificed with an overdose of an excitation-free narcotic solution (T61, Hoechst, Germany) administered intravenously. Following euthanasia, a CT scan was performed, and mandibular block specimens containing the bone substitutes with surrounding tissue were removed from all the animals. The block samples were sectioned by a saw to remove unnecessary portions of bone and soft tissue and finally prepared for histologic analysis.

Histology and Immunohistochemistry

For histologic and immunohistochemical analysis, the extracorporeally engineered constructs as well as the mandibular specimens were fixed in 4% phosphate-buffered paraformaldehyde solution (pH 7.2) for 4 hours at room temperature. Samples were decalcified in ethylenediaminetetraacetate acid (EDTA) and embedded in paraffin. Consecutive sections, 5 (μm in thickness, were mounted on slides coated with poly-L-lysine. Sections were deparaffinized with xylene and subsequently passed through a series of decreasing concentrations of alcohol into deionized water. After rinsing in tris-buffered saline (TBS), sections were stained with hematoxylin-eosin for conventional histology or alternatively incubated with primary antibodies in moisture chambers for 16 hours at 4°C for immunohistochemistry.

All primary antibodies were diluted in Dako Antibody Diluent (Dakopatts, Glostrup, Denmark). The monoclonal antibodies anti-osteocalcin from clone OC4-30 and anti-osteonectin from clone OSN4-2, both diluted 1:500, were obtained from Takara (Shiga, Japan). After incubation with primary antibody, the sections were rinsed 3 times in TBS. For immunohistochemical staining, the Dako EnVision technique (Dakopatts) was applied. Briefly, sections were incubated with polymeric conjugates of soluble dextran polymers labeled with alkaline phosphatase enzymes. Bound antibodies were detected using the Fast Red staining method with levamisole. The chromogenic reaction was stopped after 25 minutes, and the sections were counterstained with Mayer's hematoxylin. Positive controls using anti-actin as a primary antibody and negative controls with non-immune serum were also performed.

RESULTS

Scaffold Fabrication

Rapid prototyping of mandibular body defects allowed the scaffold fabrication of individualized

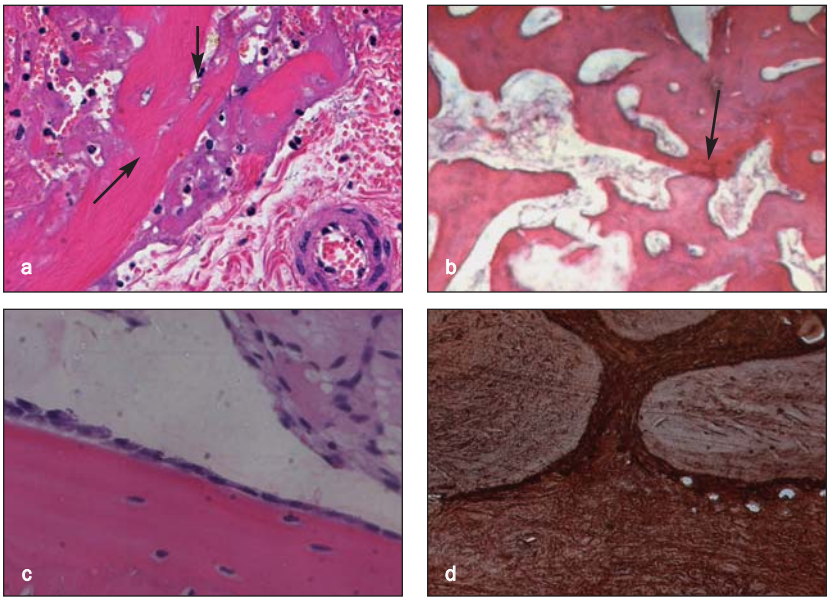

Fig 4 *(a)* Histology of bone regeneration 3 days postimplantation (arrows indicate regions of mineralized matrix; H&E; original magnification ×10). *(b)* Overview of the defect site 30 days after surgery). The increase in the amount of newly formed bone is obvious (arrows indicate regions of mineralized matrix; H&E; original magnification ×10). *(c)* No interfacial layer could be observed between the lining cells at the surface of mineralized matrix and the PLA/PGA particles (H&E; original magnification ×40). *(d)* Synthesis of osteocalcin is indicative of a viable and differentiated stage of osteoblast-like cells (levamisole/hematoxylin; original magnification ×40).

bone substitutes (Fig 3b). Fabrication of defined porous PLA/PGA (50/50) copolymers resulted in a scaffold morphology with a spongiosal internal part. The shape of the copolymers allowed an individual site-specific insertion of the implants during surgery (Fig 3c). Four days were needed to fabricate the scaffold after defect creation. This interval is important for the clinical situation, where bone remodeling may alter the defect geometry after the surgical event.

Scaffold Culture

Osteoblast-like cells commenced to attach approximately 4 hours after seeding and actively colonized the surface of the PLA/PGA matrix within 3 days of culture. Scanning electron microscopy demonstrated the attachment of osteoblast-like cells at the PLA/PGA surface (Fig 3d). Careful review of the histologic specimens obtained after 3 and 14 days in culture demonstrated evidence of osteoblast-like polygonal cells at the surface of the material. Microscopically, it was observed that the cells proliferated in the bulk material for at least up to 14 days. Frequently the cells filled the space of the interconnecting pores between the PLA/PGA particles (Fig 3e). At higher magnification, the cells displayed firm attachment toward the material surface without signs of interfacial layer formation. Cells remained in their differentiated stage during the entire culture time. Most cells were cuboidal in appearance both at the material surface as well as in the macropores within the bulk of the material. Immunohistochemistry revealed the synthesis of bone-specific proteins in the PLA/PGA scaffold. Cells synthesized bone-specific marker proteins, as seen by the presence of newly deposited osteocalcin in the pericellular matrix (Fig 3f). Signs of apoptotic cell death were not apparent as judged by conventional histology and immunohistology or as evaluated by a modified terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) method.

Histologic Examination

Osteoblasts seeded on the PLA/PGA restorative material were viable under cell culture conditions. Furthermore, after implantation, the hybrid material displayed histologic signs comparable to those found in the bioreactor. Viable and differentiated cells were observed throughout the implanted material. Cells were in direct contact with the surface of the underlying PLA/PGA material (Fig 4a). Comparison of the histology between the early (3 days postimplantation) and later time points (4 weeks postimplantation) revealed evidence of a progressive degradation of the scaffold material during the 1-month postimplantation period, as shown by an increase in the amount of newly formed bone. Original bone contacted the hybrid material in some areas at the margins of the defect site. At 4 weeks, lamellar and spongiosal bone transversed the entire width of the defect and were integrated with the scaffolds in all defect sites examined (Fig 4b). Cells lined the surface of the mineralized matrix (Fig 4c) and synthesized osteocalcin (Fig 4d) and osteonectin, which was indicative of their osteoblastoid phenotype. A small gap was seen in some areas between the original bone and the regenerative tissue (Fig 5a), which indicated the beginning of bone formation from the inner part of the defect site. Mature bone cells appeared to be attached to the remnants of the PLA/PGA particles (Fig 5b). No interfacial layer was observed around the particles at later stages of scaf-

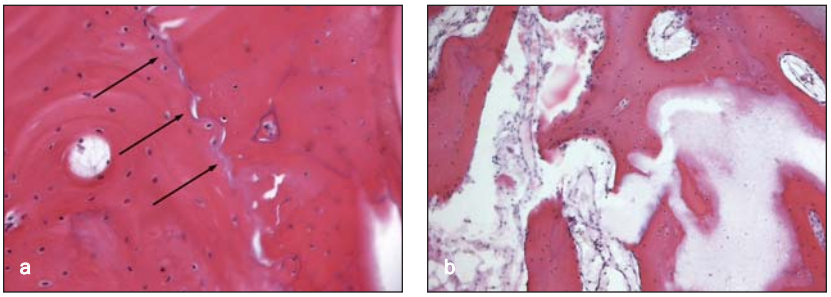

Fig 5 *(a)* New lamellar bone *(arrows)* was observed in some areas of the defect site separated from the original bone (H&E; original magnification ×40). *(b)* After 4 weeks, PLA/PGA remnants were at late stages of degradation (H&E, original magnification ×40).

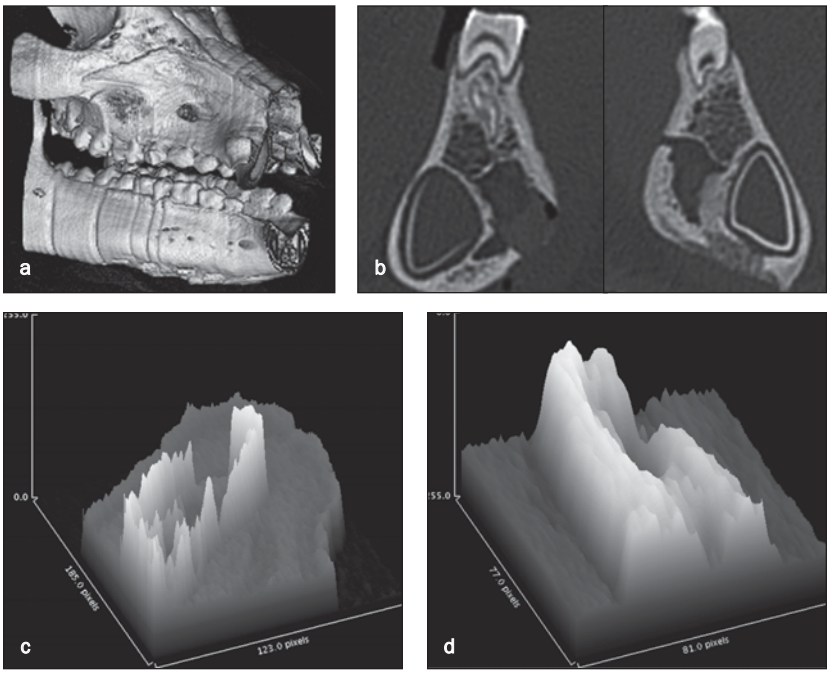

Fig 6 *(a)* Three-dimensional CT image of the right mandibular body 4 weeks after surgery. *(b)* Vertical CT slices of the regenerative area 3 days *(left)* and 4 weeks *(right)* postimplantation. *(c and d)* Comparison of bone densities *(c)* 3 days and *(d)* 4 weeks following implantation. Densities in the defect area are displayed by image analysis in an area-density plot. A reduced density gradient is detectable from peripheral to central areas of the defect site at both observation times.

fold integration. Most of the implantation sites were filled by new bone tissue, with only some small amounts of PLA/PGA particles left in the defect area. After 4 weeks, signs of vascularization were observed, as judged by the presence of endothelial cells and accompanying mesenchymal cells. Occasionally, a few multinucleated cells associated with erosion pits were seen at the scaffold surface.

Radiographic Analysis

The CT scans obtained after 3 days and 4 weeks (Fig 6a) postimplantation demonstrated a subsequent filling of the defect by an osseous-like material. The density of the implanted tissue substitutes at day 3 was lower than in areas of original cortical or spongiosal bone, but slightly higher than in the adjacent soft tissue (Fig 6b). The density of the regeneration area after 4 weeks (Fig 6b) was significantly higher than that of the early postimplantation period, indicating a progressive osteoid formation during tissue repair. A reduced density gradient was detectable from the peripheral to central areas of the defect site at both observation times (Figs 6c and 6d).

DISCUSSION

The present study demonstrated that autologous osteoblast-like cells can be cultured extracorporeally and seeded on prefabricated individualized PLA/PGA scaffolds without obvious loss of viability and differentiation. Following implantation of the hybrid material into mandibular defect sites, the cells remained viable for at least 30 days. Thus, in vitro expanded osteoblast-like cells spread on a resorbable individualized scaffold are capable of promoting the repair of bony defects in vivo.

Critical requirements for the success of tissue engineering are the biocompatibility of the material, the design of the external and internal structures of the scaffold, the time period between defect creation and reconstruction, and the choice of cultured cells.

The strategy to develop individualized hybrid substitutes consisted of the following 4 basic steps: *(1)* First, periosteum was harvested by a minor surgical procedure, and osteoblast-like cells therefrom were isolated and multiplied in culture. *(2)* The size and shape of the defect was defined by image processing and digitization of a CT scan. *(3)* The scaffold was created by polymerization of PLA/PGA beads of various sizes within the external scaffold designed by rapid prototyping. *(4)* Finally, the construct was occupied by autologous bone cells in a bioreactor for later implantation at the defect site.

It has long been known that craniofacial bone has a vast capacity for regeneration when using osteoblast-like cells.[5] Extracorporeal bone tissue engineering requires living osteoblast-like cells, and in a few cases that is all that may be needed. Fortunately, it is possible to maintain and propagate osteoblast-like cell types outside the human body for prolonged periods.[2] It has been shown that autologous cells are capable of forming new bone in animal models when a number of different vehicles are exploited.[7–10] There are no legal concerns with the use of autologous cells, and there should be no problems resulting from immune rejection. The present study confirms that autologous osteoblast-like cells are easily harvested by a minor surgical procedure and can be successfully propagated in culture, making them a reliable cell source for tissue engineering in clinical settings.

The method described allowed fabrication of biocompatible and resorbable cell-containing scaffolds with controlled external geometry in a reasonable time frame for use in mandibular surgery. In particular, the combined application of CAD/CAM technology and cell-based tissue engineering permits the tailoring of external scaffold geometries for bone tissue requirement. The bone scaffolds fabricated in this study seemed largely to resemble the external morphology of the bone to be replaced. The internal architecture, based on porogen leaching, allowed the placing, orientation, spacing, and maintenance of autologous osteoblasts and their synthesized extracellular products in the construct, as revealed by immunohistochemical investigations.

The beneficial biocompatibility may be attributed in part to the PLA/PGA matrices copolymerized in the presence of a carbonate buffer system. Numerous investigators have reported on the well-known favorable biocompatibility of PLA/PGA and found no impairment of cell function when using these polymers as a restorative material.[11–13] Ishaug and colleagues showed that the alkaline phosphatase activity expressed by osteoblasts cultured on PLA was comparable to that on tissue culture polystyrene.[14] Otto and associates indicated that the PLA constructs promoted the differentiation of bone cells.[15] In contrast to the beneficial biocompatibility of bulk materials, degradable materials present problems of their own. One major problem of PLA/PGA is the shift in pH related to hydrolysis. Lowering of the pH by degradation threatens the viability of surrounding cells. The pH stability of the PLA/PGA–carbonate scaffold used seemed to contribute to the favorable results seen in the present study. The reduced density gradient from peripheral to central areas of the defect site at both observation times indicates that bone repair was more pronounced at the defect borders. This might be based on the fact that in these areas both cell populations, the host cells and the transplanted cells, account for the repair process. Additionally, nutrition of transplanted cells is better at superficial transplant sites.

For use in tissue engineering, scaffolds are commonly fabricated from degradable materials[16,17] and are shaped intraoperatively to fit into the defect site. The material composition, pore size, and chemistry were based on previous studies in extracorporeal bone tissue engineering studies.[2] A limitation of the presented study is the conventional fabrication of the internal structure despite the use of CAD/CAM technique. In contrast to conventional methods, the internal design of scaffolds can also be achieved individually on the basis of digital data formats.[18,19] Many different processing techniques have been developed to design internal scaffold structure, including fiber bonding, solvent casting, particulate leaching, membrane lamination, melt molding, temperature-induced phase separation, gas foaming, and 3-dimensional printing.[20] A wide range of internal scaffold characteristics, such as porosity and pore size, have been modeled with such fabrication techniques. Most of the computer-driven techniques for scaffold fabrication have been evaluated on a material-specific level or controlled for their biologic behavior in cell cultures.[21] However, techniques for computer-controlled modeling processes, which allow researchers to fabricate individualized cell/scaffold complexes for use in extracorporeal tissue engineering and, eventually, clinical settings, are seldom investigated in craniofacial reconstruction. Using the strategy presented, it has been shown that it is possible to fabricate a cellular hybrid substitute with a desired external geometry.

Various studies have demonstrated the applicability of individualized bone substitutes.[22] Modeling of the external and internal structure on the basis of digital data was performed by various software tools.[23] Whereas most studies used nonviable materials or were performed only on theoretical assump-

tions, the application of a cell-driven, image-based extracorporeal tissue engineering approach in a clinical setting has seldom been performed. Various studies addressing assessment of the clinical outcome of bone tissue engineering used cells in combination with or without a stable scaffold. The cells were either seeded on a substitute, injected into the defect, or cultured on a suitable biomaterial prior to implantation. Although most authors have described the healing of bone defects by implantation of cellular hybrid materials in long-term experiments,[24–28] it is unclear whether this finding can be attributed to the biomaterial alone or its cellular components. The addition of bone marrow–derived cells to various materials has been shown to enhance the performance of a wide variety of implanted bone substitutes.[24] Most studies have used young animals or graft sites that are surrounded by normal healthy tissues or wound sites that almost surely ossificate alone.[25,26] It has been suggested from these studies that bone healing seems to be more rapid, complete, and reproducible when a cell-based therapy is employed.[27,28] However, some experimental studies have revealed that bone healing is accelerated when only extracts from cultured human osteosarcoma cells are injected,[29] which indicates that the addition of nonviable cellular components to a matrix also supports bone regeneration.

CONCLUSION

This preliminary study indicates that autologous osteoblast-like cells can be easily harvested and kept viable while proliferating in a bioreactor for further use in bone tissue engineering. The information presented confirms that it is feasible to harvest small amounts of autologous periosteum with minimal morbidity, expand the cells therein, and then seed them on individualized scaffolds in bioreactors[30] to repair mandibular bone defects.[5] The combined application of CAD/CAM systems and tissue engineering techniques allows a cell-based biomimetic approach. The advances in image-based design and bioreactor cell cultivation techniques make it possible to engineer patient-specific hybrid bone constructs that may improve bone defect reconstruction.

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