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Evaluation of Chitosan/Biphasic Calcium Phosphate Scaffolds for Maxillofacial Bone Tissue Engineering

Aylin Sendemir Urkmez,[*1,7] *Sherrie G. Clark,*[2] *Matthew B. Wheeler,*[3,4] *Michael S. Goldwasser,*[4,5,6] *Russell D. Jamison*[1,6]

Summary: Porous, 3D chitosan/biphasic calcium phosphate (BCP) scaffolds were used to prepare tissue engineering constructs for maxillofacial bone tissue reconstruction. Mesenchymal stem cells (MSC's) were seeded and cultured on clinically relevant sized scaffolds. *In vitro* engineered constructs facilitated the healing of mandibular defects in pigs if accompanied with delivery of basic fibroblast growth factor (bFGF).

Keywords: biomaterials; biphasic calcium phosphate (BCP); bone tissue engineering; chitosan; scaffold

Introduction

One of the primary challenges in clinical application for tissue engineered devices relates to blood supply. The most successful tissue engineering applications so far have been in avascular tissues like cartilage,[1–3] or in thin tissues like skin grafts,[4–6] i.e. in conditions wherein cell survival relies on the diffusion of nutrients and oxygen from the surroundings. Cells that are 150–200 μm from a blood supply can survive by diffusion.[7–10] Tissue engineered devices that are larger than this size must be vascularized before or soon after implantation to facilitate survival of both transplanted and host cells at the interior. Locally enhanced angiogenesis has been shown to correlate with greater transplanted cell survival.[11]

[1] Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, Urbana, IL. 61801, USA
E-mail: sendemir@gmail.com
[2] College of Veterinary Medicine, University of Illinois at Urbana-Champaign, Urbana, IL. 61801, USA
[3] Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL. 61801, USA
[4] Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL. 61801, USA
[5] College of Medicine, University of Illinois at Urbana-Champaign, Urbana, IL. 61801, USA
[6] Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL. 61801, USA
[7] Current Affiliation: Center for Brain Research, Ege University, Bornova, Izmir, Turkey

In order to induce neovascularization after implantation, bone tissue engineering constructs loaded with gelatin microspheres were designed to deliver basic fibroblast growth factor (bFGF), a potent angiogenic factor that promotes endothelial cell migration, proliferation, and capillary differentiation.[12–14] The advantages of chitosan/BCP as bone tissue engineering scaffolds were previously shown.[15] Chitosan/hydroxyapatite scaffolds were also reported to show great potential for tissue engineering applications.[16] Porcine bone marrow derived mesenchymal stem cells (MSCs) were culture expanded and differentiated into osteoblastic phenotype after seeding onto chitosan/BCP scaffolds *in vitro* for two weeks before implantation as tissue engineered constructs. Osteoblastic differentiation of these cells on chitosan/BCP scaffolds was also demonstrated.[17]

The sustained release of biologically active bFGF using gelatin sheets[18–20] and microspheres[21] were shown to be controlled by enzymatic degradation of gelatin *in vivo*. Since the duration of growth factor exposure is critical in stimulating neovascularization,[22,23] use of gelatin microspheres provides beneficial control over designing the release rate and duration of bFGF.

In the current study, the effects of bFGF delivery within tissue engineered constructs were tested in a mandibular defect model in

Yorkshire pigs. Gelatin microspheres were engineered to degrade and release the angiogenic growth factor bFGF within two weeks, since capillary growth precedes bone formation, and needs to be stimulated at the early stages of healing. Tissue engineered constructs were implanted with and without bFGF, and the healing responses were compared histologically to those in empty defects and empty scaffolds.

Materials and Methods

Experimental Design

Twelve skeletally mature female Yorkshire pigs, 6 months of age were used for the study. All animal experiments conformed to the University of Illinois Institutional Animal Care and Use Committee (IACUC) guidelines and approvals, and were supervised by the Division of Animal Resources. Two 10 mm diameter transcortical defects were created on posterior domain of each side of the mandibles. Four experimental groups were defined: Group 1) empty defect as the control group; Group 2) empty scaffolds that contained no cells, but were treated similar to the cellular scaffolds; Group 3) tissue engineered constructs containing porcine MSCs cultured two weeks on scaffolds under osteoblastic conditions; Group 4) tissue engineered constructs same as Group 3 loaded with gelatin microspheres carrying 20 μg bFGF. Each animal received all four experimental groups in randomized order. Four animals were euthanized ($n = 4$ for each experimental group) at intervals of 2, 4, and 8 weeks, and their mandibles including defects and implants were surgically retrieved with a surgical saw.

Scaffold Fabrication

The chitosan/BCP scaffolds were prepared by a freeze-drying technique described elsewhere.[15] Briefly, 2% (w/w) chitosan flakes were dissolved in 0.2 M acetic acid aqueous solvent and vacuum filtered. BCP particles of 45–53 μm size were added 25% (w/w) to the filtered solution and the mixture was frozen and lyophilized. Cylinders of 10 mm diameter and 5 mm height were cut from the lyophilized sponges using a biopsy punch. Resulting cylindrical sponges were rehydrated and sterilized through a series of ethanol/phosphate buffered saline (PBS) solutions (100, 95, 75, 50, 0% ethanol).

Cell Isolation and Culture

Porcine mesenchymal stem cells (MSCs) isolated from bone marrow of 4 week-old Yorkshire pigs were expanded in DMEM media containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic mixture at 37 °C in a 5% CO_2 humidified environment. After 80% confluence, cells were harvested by gentle digestion with 0.05% Trypsin/EDTA, and seeded onto chitosan/BCP scaffolds at a density of 1.6×10^6 cells/mL. After one day of culture, the media were replaced with differentiation media consisting of expansion media supplemented with L-ascorbic acid-2 phosphate, β-glycerolphosphate and dexamethasone for two weeks.[24]

Application of bFGF Loaded Gelatin Microspheres

Gelatin with an isoelectric point (pI) of 5.0 was kindly provided by Nitta Gelatin Corp (Osaka, Japan). Gelatin microspheres were fabricated by Dr. Young Bin Choy by a precision particle fabrication (PPF) technique [25,26] and sterilized by ethylene oxide gas one week before the surgery. The recombinant human bFGF (R&D Systems, Minneapolis, MN) was thawed before the surgery and allowed to absorb into the microspheres for 2 h at room temperature before injection into the tissue engineered constructs. Scaffolds and constructs were placed in the 10 mm transcortical holes on the mandible.

Fluorochrome Labeling

For assesment of bone turnover, the animals were administered triple-color fluorochrome bone labels (alizarin complexone (30 mg/kg; 5 mL/min) subcutaneously three weeks after the surgery.

Histological Evaluation

After retrieval, the defect sites with a margin of bone equal to twice the diameter of the defect was processed for undecalcified histology sections. The scaffolds and surrounding bone were fixed in 10% neutral buffered formalin and dehydrated in an ethanol series. Tissue was embedded in PMMA as described elsewhere.[27] The defect sites were sectioned in the coronal plane in 1 mm transverse sections through the center, polished, etched and stained with 1% toluidine blue at pH 8 in 0.1 M phosphate buffer at 57 °C.[28] Toluidine blue stains woven bone and osteoblast nuclei in dark blue, and lamellar bone in light blue. Histological sections from the middle of the defect area were examined with light microscopy. In order to determine the number of blood vessels, three random 1mm x 1 mm regions of interest (ROI) were chosen within the defect area. Blood vessels were identified by the presence of endothelial cells and luminal structure, and were counted for each ROI, averaged for each sample, and expressed as ''number of blood vessels per square millimeter (#/mm^2)''.

Fluorochrome Evaluation

Laser scanning confocal microscopy of the samples was accomplished using a Leica SP-2 confocal and multiphoton instrumentation. The samples were scanned with blue-green light in xyz mode with a 10x objective. A dye separation algorithm provided by Leica Microsystems was used to separate the fluorescence signals from different bone labels and the scaffolds.

Results and Discussion

No complications were encountered during the course of the study. At two weeks, there was considerable callus formation on the lateral side of the defects, where periosteum was preserved during the surgery. Accompanying the callus, irregular hypertrophic bone formation was observed at the vicinity of the defect, stabilizing the area. There was minimal new bone formation originating from the drill line. Active osteoblasts, characterized by their cuboidal shape were located adjacent to the bone surface. The number of blood vessels in the defect area of the group that received bFGF was significantly higher than that of the other three groups (Figure 1). Between the bone and the construct or scaffold, there was a transition area filled with undifferentiated mesenchymal cells that created a trabecular structure patterned in accordance with the new growing vasculature. In and around the scaffolds, there were neutrophils and some macrophages.

At four weeks, callus and hypertrophic bone increased in size in all groups, but it was largest in the group that received bFGF. The defect area started to be filled

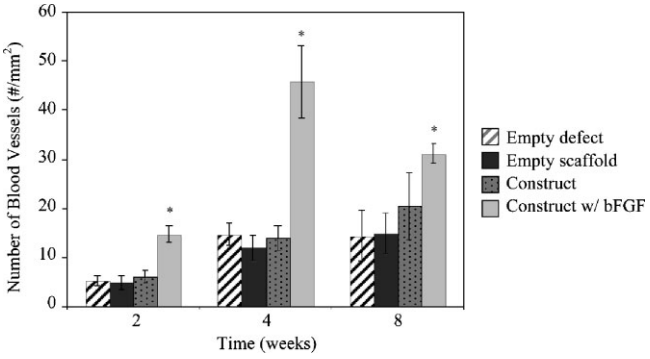

Figure 1.
For all time points evaluated, the number of blood vessels in regions of interest (ROI) was significantly higher in the group that received bFGF. Data are shown as means ± SD (n = 4). *Significant difference, $p < 0.05$.

mainly from the lateral side and the defect edges toward the middle, accompanied with some growth from the medial callus. In the defect area, bone formation occurred through the mineralization of the trabecular structure that was evident in two weeks. Neutrophils were still present, and there was an increase in the number of macrophages and multinucleated inflammatory cells in all groups that were implanted with scaffolds or constructs. The scaffolds were about 50% intact in the middle of the defect, but the transition areas were filled with scaffold remnants being engaged by the macrophages. There was no difference in the inflammatory response between the cellular constructs and the scaffolds. The number of blood vessels increased in all groups, but was still significantly higher in the group that received bFGF (Figure 1). Higher density of blood vessels around the bFGF carrying microspheres were noted (Figure 2).

At eight weeks, bone union was achieved in the lateral side, and the hypertrophic bone was notably reduced in size by osteoclastic remodeling in all groups. The scaffolds were no longer intact in cellular groups; only remnants were seen within the defect area. The number of macrophages and multinucleated cells was reduced. There was a decrease in number of blood vessels in bFGF group, but it was still higher than that of the other groups (Figure 1).

The repair process in bone is governed by the redevelopment of vascular supply. Healing response observed in this study showed that capillary growth preceded new bone formation in the transition zone between the bone and the implant. Undifferentiated mesenchymal cells were immediately organized in a trabecular structure and deposited collagenous matrix. Bone growth was achieved by the subsequent mineralization of this trabecular structure. Orientation of the trabecular structure and the new bone was determined by the invading vasculature.

Existence of a scaffold, whether loaded with syngeneic cells or not, seemed to cause a delay in the filling of the defect area. This delay was overcome only by delivery of bFGF within the constructs. The defect areas were filled mainly with bone growth originated from these hypertrophic sites toward the center, accompanied with minimal growth from the drill line. Callus formation, cambial proliferation and growth of osteoid from the cambium into the defect has been shown as typical bone response to drill hole defects.[29] Hypertrophic bone in irregular shapes similar to that we have observed was also reported for segmental mandibular defects.[30,31]

The inflammation was limited to the defect area, and began to clear as the scaffold materials were degraded. The scaffold

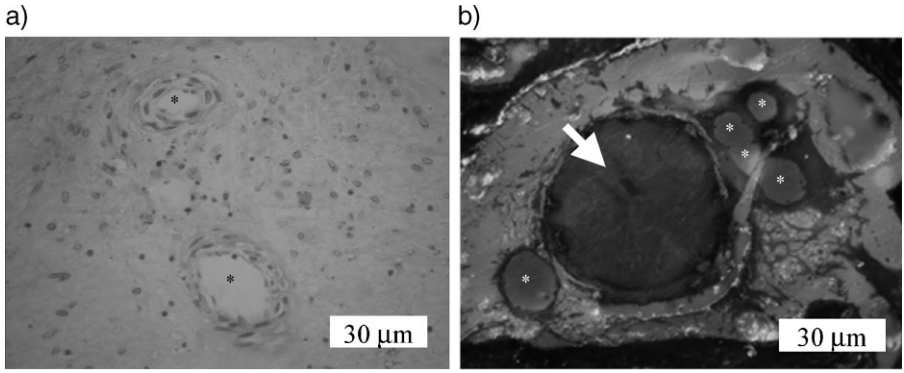

Figure 2.
At four weeks, invasion of blood vessels (*) in the defect area was observed in all groups (a), but they were particularly concentrated around the bFGF releasing microspheres (white arrow) (b).

degradation is attributable to digestion by macrophages and multinucleated cells. The syngeneic MSCs did not induce an additional immune response. The constructs degraded faster compared to empty scaffolds, insinuating contribution of implanted cells to the inflammatory response, but it could have been an effect of acidic cellular by-products poorly eliminated due to limited vasculature.

Within the constructs in bFGF group, small areas of fluorochrome uptake were evident, and corresponded with the dark blue stained mineral-like features in the histographs of the same sample taken 2mm apart (Figure 3). Fluorochromes enter the bloodstream and are incorporated into new mineralized tissue within 2–3 days of application. They bind to bone mineral through chelation of calcium at the surface of newly formed apatite crystal. This result shows active new bone formation within the bFGF-received constructs three weeks after surgery. There was no significant increase in the area of mineralization within the constructs between 4 and 8 weeks. This suggests that the current model requires further optimization for maintaining construct viability through continuing vascular growth and organization.

Mineralization within implanted material without any connection to pre-existing bone is rarely reported. Examples include bioglass particles that contained autogeneic bone[32] and a titanium mesh that was precultured with syngeneic bone marrow cells.[33] The common factor in these examples and the current model is the existence of transplanted cells into the defect area. It is not evident that the mineralization within the constructs was originated from the transplanted cells. It is also possible that these cells or their ECM facilitated the

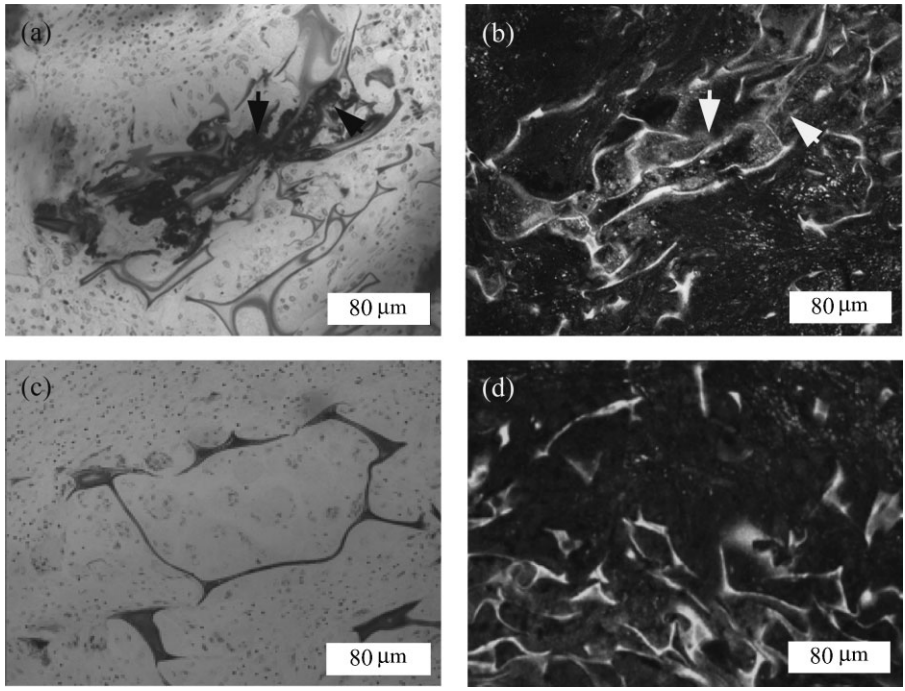

Figure 3.
After 8 weeks, in bFGF group, dark blue stained mineralized areas (black arrows) in the histology slide (a) correspond with the alizarin complexone labeled regions (white arrows) in the confocal image taken 2 mm apart from the same sample (b). In the other two groups that received scaffolds, but no bFGF, remnants of the scaffolds were visible, but no mineralization was observed either in the histological sections (c) or the confocal images (d).

osteoblastic function of the invading host mesenchymal cells. Regions of mineralization within the constructs may act like bone growth centers and could be the critical factor for improving bone regeneration process.

Conclusion

Chitosan/BCP scaffolds facilitated the culture and osteoblastic differentiation of porcine MSCs for production of bone tissue engineering constructs. These constructs assisted the healing of mandibular defects if accompanied with delivery of bFGF via gelatin microspheres. bFGF release from the constructs improved neovascularization in the defect area and therefore enhanced new bone formation. The current tissue engineered system requires further optimization for maintaining construct viability, but it has shown prospect in improving healing response by creating mineralized regions within the constructs after implantation, and success in enhancing neovascularization that remains to be the critical factor in bone regeneration.

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