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Exploratory study on the effect of osteoactivin on bone formation in the rat critical-size calvarial defect model

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Background and Objective: Osteoactivin is a novel glycoprotein shown to exhibit an important role in regulating osteoblast differentiation and function. The aim of the present study was to evaluate the potential of osteoactivin to support bone regeneration using an established defect model.

Material and Methods: Critical-size, 8-mm-diameter through-and-through calvarial osteotomy defects were created in 60 adult male Sprague-Dawley rats. Test animals received 0.1 mL of osteoactivin in phosphate-buffered saline (50 μg/mL) soak-loaded onto an absorbable collagen sponge. Controls received 0.1 mL of phosphate-buffered saline soak-loaded onto the absorbable collagen sponge or no further intervention (sham-surgery). The animals were euthanized 2 and 4 wk after treatment and histometric analyses were performed.

Results: The absorbable collagen sponge control (mean ± standard deviation: 40.9 ± 26.9%) showed borderline significant greater bone fill compared with sham-surgery (22.9 ± 15.8%; $p = 0.10$) and osteoactivin (20.2 ± 11.8%; $p = 0.07$) treatments at 2 wk. In contrast, osteoactivin (84.7 ± 15.8%) showed significantly greater bone fill than sham-surgery (28.4 ± 9.6%; $p < 0.001$) and absorbable collagen sponge (41.8 ± 22.1%; $p < 0.001$) at 4 wk. No animals receiving sham-surgery or absorbable collagen sponge exhibited complete bone fill at 4 wk while 70% of the animals receiving osteoactivin showed complete bone fill.

Conclusion: Osteoactivin demonstrates a significant potential to support bone regeneration/formation. Studies using discriminating large animal models are necessary to explore clinical application for periodontal and craniofacial indications.

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Several biomaterials, biologic factors and technologies from various sources have been proposed to support local bone formation for oral rehabilitation, including autograft bone, devices for guided bone regeneration, bone derivatives or bone substitutes and, as of late, matrix, growth and differentiation factors (1). Frequently, combinations of biomaterials and biologic factors have been proposed to accomplish adequate reconstruction of large craniofacial and alveolar osseous defects. Biomaterials provide a framework/matrix/scaffold for osteogenic cell migration, proliferation and bone formation. The capacity of such biomaterials to regenerate bone may be greatly enhanced by the addition of biologic factors that directly may enhance/accelerate osteogenesis.

Factors including bone morphogenetic proteins (BMPs), platelet-derived growth factor, and transforming growth factor-β have been used for such bone-regenerative concepts encompassing periodontal and oral/maxillofacial indications (1,2).

Osteoactivin is a novel glycoprotein that was first characterized to be up-regulated in naturally mutant osteopetrotic rats (3). Osteoactivin is constitutively expressed in osteoblast cultures, with its secretion progressively increasing as the cells differentiate, and reaching the highest level of secretion during the final stage of osteoblast development (matrix mineralization) (3). Osteoactivin expression is correlated with the expression of other markers of osteoblast development, including alkaline phosphatase and osteocalcin (3). Furthermore, such markers of osteoblast differentiation are inhibited by anti-osteoactivin Igs, implying a critical function for osteoactivin during the differentiation of osteoblasts (4). Such a role is important in regenerating a bone defect, where it is optimal to maximize the number of bone cells capable of secreting a collagenous matrix. Furthermore, osteoactivin expression in a fracture repair model increased with time, reaching a maximum at 2 wk after fracture (5). Osteoactivin may also influence aspects of bone development other than differentiation. Cell migration and matrix remodeling are recognized as key events in tissue repair and restructuring. The RGD sequence in osteoactivin is known to bind integrins, an important component in the mechanisms of cellular attachment and migration. This is supported by the finding that osteoactivin expression induces the migration of human glioma cells *in vitro* and *in vivo* (6). Recently, osteoactivin has been characterized as a downstream mediator of BMP-2-induced osteoblast differentiation and function (7). Osteoactivin has also been shown to be highly expressed by osteoclasts *in vitro*, suggesting that it may regulate osteoclast formation and activity (8). Other cells may also be regulated by osteoactivin under physiological and pathological conditions. To date, no studies have assessed the potential of local delivery of osteoactivin as a therapeutic agent in support of local bone formation with possible applications to craniofacial bone regeneration/augmentation. The aim of the present study was to evaluate the potential of osteoactivin to support bone regeneration in an established defect model.

Material and methods

Animals

Sixty adult male Sprague-Dawley rats, approximately 175 g in weight, were used according to a protocol approved for this study by the Institutional Animal Care and Use Committee, Temple University, Philadelphia, PA, USA. Animals were housed individually in plastic cages in a monitored environment (21°C; 12-light/12-dark cycle). Drinking water and a standard laboratory rat pellet food were supplied *ad libitum*. Animals were monitored for signs of infection and discomfort throughout the study.

Surgery

The animals received buprenorphine HCl (0.02–0.03 mg/kg intramuscularly) presurgery and were anesthetized using isoflurane inhalation anesthesia (E-Z Anesthesia; Euthanex Corp., Palmer, PA, USA; 4–5% induction/2–3% maintenance). Before surgery, the animal's head was shaved, washed with a disinfectant and stabilized by a nose cone apparatus (Euthanex). A midline incision was performed from the nasofrontal area to the external occipital protuberance along the mid-sagittal suture. The skin and underlying tissues including the temporal muscle were reflected bilaterally to expose the calvarial experimental area.

One experienced surgeon (J.P.B.) carried out the surgical procedure on all surgeries. A critical-size, 8-mm diameter, through-and-through calvarial osteotomy defect was created in each animal. Defects were outlined using a slow-speed dental hand-piece using an 8-mm outer diameter trephine bur (Ace Surgical Supply Co., Inc., Brockton, MA, USA) under constant irrigation with sterile saline. Trephined bone was removed from the surgical field to create the through-and-through osteotomy. Absorbable collagen sponges (CollaCote®; Sulzer Dental, Inc., Carlsbad, CA, USA) were trimmed to fit the defects and were soak-loaded with test or control solutions before implantation (see below). Following implantation, the skin and underlying tissues were adapted to cover the exposed calvarium and the wound margins were everted and closed using autoclips (Autoclip® Wound Closing System; Stoelting Co., Wood Dale, IL, USA). A bacitracin/neomycin/polymyxin antibiotic ointment (Vetropolycin® Ophthalmic Ointment; Pharmaderm, Melville, NY, USA) was applied to the eyes and the animals were monitored until they had recovered from the anesthesia.

Experimental groups

Animals in groups of 10 were randomized to control and test conditions using 2- and 4-wk healing intervals as follows: (i) sham-surgery controls; (ii) absorbable collagen sponge controls (treated with absorbable collagen sponge soak-loaded with 0.1 mL of phosphate-buffered saline); and (iii) osteoactivin tests [treated with 0.1 mL of osteoactivin (Anaspec, Inc., Fremont, CA, USA) diluted in phosphate-buffered saline to a concentration of 50 μg/mL and soak-loaded onto absorbable collagen sponge]. The animals were euthanized, 2- and 4-wk after treatment, using CO_2 asphyxiation, and histologic/histometric analyses were performed. Cranial bone, including the defect, was removed in entirety, rinsed in tap water and immediately placed in a 10% buffered formalin solution for 3–5 d. Three animals from each 2-wk group were reserved for evaluations unrelated to the present study. Therefore, the 2- and 4-wk groups included seven and 10 animals, respectively.

Histotechnical preparation

The cranial block specimens were decalcified in formic acid and sodium citrate for 24 h. The specimens were

then rinsed in tap water, dehydrated with ascending concentrations of ethyl alcohol, cleared in xylene and infiltrated with paraffin. Serial sections (7 μm) parallel to the midsagittal suture were cut from the center of each defect using a microtome (RM2155; Leica Microsystems GmbH, Nussloch, Germany) and stained using Massons trichrome stain.

Histologic and histometric analyses

The most central section from each defect site was identified and used for histologic and histometric analyses. One masked calibrated examiner (J.P.B.) analyzed the sections for new bone formation, residual absorbable collagen sponge, residual fibrin clot elements, soft tissue elements and inflammatory reactions using incandescent and polarized light microscopy (BX 60; Olympus America, Inc., Melville, NY, USA), a microscope digital camera system (DP10; Olympus America), and a computer-based image-analysis system including a custom macro (Image-Pro Plus™; Media Cybernetic, Silver Spring, MD, USA). Two linear measurements were recorded for each section: defect width (the distance between the margins of the original bone defect); and bone fill (the length of newly formed bone along an axis bridging the gap between the defect margins).

Statistical analysis

Summary statistics (mean ± standard deviation) were calculated based on measurements from individual animals for the various groups. Additionally, bone fill was categorized according to percentage bone fill of the defect width:

1 None (< 25% of the defect width filled with bone);
2 Limited (≥ 25% to < 50% of the defect width filled with bone);
3 Partial (≥ 50% to < 75% of the defect width filled with bone);
4 Complete (≥ 75% of the defect width filled with bone).

The Wilcoxon Sign Rank Test was used to compare the experimental groups at 2 and 4 wk. The level of significance was set at 5%.

Results

There were no noteworthy clinical differences during the surgical procedures and implantation of biomaterials. Healing was generally uneventful over the 2- and 4-wk experimental periods. One animal of the osteoactivin 2-wk group died preterm.

Representative photomicrographs from the osteoactivin experimental and control groups at 4 wk are shown in Fig. 1. The sham-surgery control exhibited bone formation restricted to the edges of the osteotomy. A somewhat dense fibrous tissue was observed at the center of the defect. The absorbable collagen sponge group showed limited bone formation, including some residual absorbable collagen sponge at the center of the defect. The osteoactivin group exhibited complete or almost complete closure of the defect with very limited signs of the absorbable collagen sponge carrier.

The absorbable collagen sponge control showed approximately twice the bone fill compared with the sham-surgery and osteoactivin groups at 2 wk, with the differences between groups being borderline significant ($p = 0.10$ and $p = 0.07$, respectively; Table 1). In contrast, at 4 wk, the osteoactivin group showed significantly greater bone fill compared with the sham-surgery ($p < 0.001$) and absorbable collagen sponge ($p < 0.001$) controls. No significant increase in bone fill was observed for the sham-surgery and absorbable collagen sponge controls, whereas animals that received osteoactivin showed a significant threefold increase in bone formation ($p < 0.001$). At 2 wk, only one animal from the absorbable collagen sponge group exhibited complete bone fill (Table 2). At 4 wk, no animals exhibited complete fill in the sham-surgery and absorbable collagen sponge controls, whereas 70% of the animals receiving osteoactivin showed complete bone fill.

Discussion

Osteoactivin is a novel glycoprotein that is highly expressed and secreted by osteogenic cells. Osteoactivin has been associated with bone formation and it has been found to regulate osteoblast and osteoclast activity (3–5,7,8). To the best of our knowledge this is the first study to evaluate the regenerative potential of osteoactivin using an established craniofacial defect model. The osteoactivin group showed significantly greater bone fill compared with the controls, indicating that osteoactivin may have a possible utility in craniofacial bone regeneration/augmentation. Osteoactivin showed two- and threefold increases in bone formation when compared with the absorbable collagen sponge and sham-surgery controls, respectively. Interestingly, this effect was observed at 4 wk after implantation with no noticeable effects at 2 wk.

The finding that osteoactivin did not display a distinguishing osteogenic potential at 2 wk, but at 4 wk, bone-fill estimates approximating that of

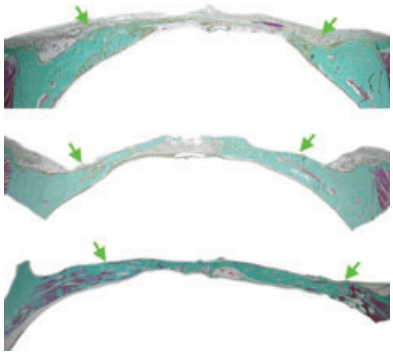

Fig. 1. Representative photomicrographs of the rat calvarial, critical-size (8-mm), through-and-through osteotomy defects receiving sham-surgery (top), absorbable collagen sponge control (center) and osteoactivin (bottom) following a 4-wk healing interval. The arrows delineate the lateral borders of the defects (Masson's trichrome stain).

Table 1. Defect bone fill, stratified according to experimental group and observation interval

Experimental group	Defect bone fill (%)	
	2 wk	4 wk
Sham-surgery	22.9 ± 7.7 Aa	28.4 ± 9.6 Aa
Absorbable collagen sponge	40.9 ± 26.9 Aa	41.8 ± 22.1 Aa
Osteoactivin	20.2 ± 11.8 Aa	84.7 ± 15.8 Bb

Values are given as mean ± standard deviation.
Between-group comparisons: values followed by the same capital letter did not differ significantly ($p > 0.05$).
Within-group comparisons: values followed by the same lower case letter did not differ significantly ($p > 0.05$).

Table 2. Distribution of defect bone fill according to experimental group and observation interval

Observation interval	Defect bone fill (%)			
	None (< 25%)	Limited (≥ 25% to < 50%)	Partial (≥ 50% to < 75%)	Complete (≥ 75%)
2 wk				
Sham-surgery	57.1	42.9	0.0	0.0
Absorbable collagen sponge	28.6	28.6	28.6	14.3
Osteoactivin	83.3	16.7	0.0	0.0
4 wk				
Sham-surgery	30.0	70.0	0.0	0.0
Absorbable collagen sponge	30.0	20.0	50.0	0.0
Osteoactivin	0.0	0.0	30.0	70.0

sham-surgery were found, is intriguing. *In vitro*, osteoblasts have been shown to go through three distinct stages during bone formation: cell proliferation (0–7 d); nodule formation, collagen deposition and matrix maturation (7–14 d); and osteoblast differentiation and matrix mineralization (14–21 d). This may, at least in part, explain the lower bone-fill estimates observed at 2 wk and the significant increase at 4 wk. An alternative explanation to this finding could be that osteoactivin stimulates the proliferation of osteoblast progenitor cells, potentially delaying osteoblast differentiation and consequently bone matrix formation and mineralization. In perspective, the results herein motivate further study of the cellular and molecular temporal and spatial events occurring soon after the application of osteoactivin, to discern factors critical for the enhanced osteogenesis.

The potential of matrix/growth/differentiation factors, biomaterials and devices used to enhance/induce osteogenic bone formation have been tested using similar methodologies. BMP-2, BMP-4 and BMP-7 have been shown to exhibit a relevant potential to induce/support bone formation in a series of studies (9–11). BMP-2, alone or in combination with vascular endothelial growth factor, induced increased bone formation compared with controls (12). In contrast, platelet-rich plasma preparations (13,14), vascular endothelial growth factor (12) and a commercially available enamel matrix derivative (14,15) all failed to enhance bone formation in this gateway preclinical model. Immediate comparisons between these and the present study may not be meaningful or relevant as a result of differences in the study design, defect size, observation intervals, carrier technologies and the screening nature of the model. However, it should be considered that both the BMPs and osteoactivin exhibited bone formation, qualifying properly scaled continued evaluation in discriminating large animal models and eventually, if successful, in clinical settings.

Craniofacial critical-size defects have included mainly calvarial and mandibular defects using murine, porcine and canine platforms (16–23). These experimentally created defects do not regenerate spontaneously within the experimental lifetime of the animals and allow the evaluation of implanted biologics, biomaterials and devices that may induce/enhance tissue regeneration as well as associated adverse reactions (16–19). Osteoactivin exhibits great bone-formation potential using this rodent calvarial critical-size defect model. The rat craniotomy defect is a well-characterized model with a limited native healing potential because of its limited blood supply and relatively sparse bone marrow. Future studies should focus on gaining a better understanding of the biological events leading to bone formation in response to this protein. Additionally, discriminating large animal models should be used to further confirm the potential of osteoactivin to support craniofacial bone augmentation/osseointegration.

Carrier technologies can be sourced from various human and commonly bovine tissues as well as representing any of a large group of synthetically derived ceramic or polymeric biomaterials (24,25). When growth factors are delivered alone, however, they have a rapid clearance *in vivo* and are thus unable to maintain therapeutic concentrations over time (24). An osteoconductive scaffold may serve as a carrier to allow the progressive/sustained release of growth factors. Our approach was to utilize a commonly used collagen matrix as a vehicle for osteoactivin. Type I collagen matrices appear to be the natural and preferred candidates for the delivery of bone-inducing biologics, type I collagen being a major component of bone. The absorbable collagen sponge has been studied exhaustively and shown to support the sustained release of a variety of bone growth factors, including BMPs (24,26–30). Consistent with our hypothesis, the osteoactivin/absorbable collagen sponge combination

produced greater bone formation than either sham-surgery or absorbable collagen sponge alone when implanted into the standard calvarial defect.

In conclusion, although the precise mechanism(s) of action of osteoactivin remain largely unknown, this study provides insight into the functional potential of osteoactivin on bone regeneration, with osteoactivin producing significant bone fill in the rat critical-size calvarial through-and-through defect. Information from this and from subsequent large animal studies may ultimately lead to the development of new therapeutic strategies to enhance bone formation in cases where this approach would be beneficial, including alveolar augmentation for placement of dental/oral implants, periodontal disease, fracture repair and bone-formation disorders.

Disclosure statement

The authors declare no conflict of interest. This was an investigator-funded study.

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