Guided Bone Regeneration Using Injectable Vascular Endothelial Growth Factor Delivery Gel

Darnell Kaigler,*† Eduardo A. Silva,‡ and David J. Mooney‡

Background: Vascularization underlies the success of guided bone regeneration (GBR) procedures. This study evaluates the regenerative potential of GBR in combination with vascular endothelial growth factor (VEGF) delivery via an injectable hydrogel system.

Methods: Critical-sized defects were created in rat calvariae, and GBR procedures were performed with a collagen membrane alone (control), or plus bolus delivery of VEGF, or plus application of VEGF-releasing hydrogels (VEGF–Alg). Four and 8 weeks after treatment, defect sites were evaluated with microcomputed tomographic and histomorphometric analyses for blood vessel and bone formation.

Results: At 4 weeks, relative to the control condition, the bolus addition of VEGF did not affect blood vessel density within the defect site, yet the application of VEGF–Alg significantly ($P <0.05$) increased blood vessel density. Although there was no difference in bone regeneration at 4 weeks, at 8 weeks there was a significant ($P <0.05$) increase in bone regeneration in the VEGF–Alg-treated defects.

Conclusions: These data demonstrate that the application of VEGF–Alg enhanced early angiogenesis, whereas at a later time point, it enhanced bone regeneration. Controlled delivery approaches of angiogenic growth factors used adjunctively with GBR may be a promising strategy for enhancing outcomes of GBR. *J Periodontol 2013;84:230-238.*

KEY WORDS

Angiogenesis-inducing agents; bioengineering; bone and bones; endothelial growth factors; hydrogel; regenerative medicine.

* Department of Periodontics and Oral Medicine, University of Michigan, Ann Arbor, MI.
† Department of Biomedical Engineering, University of Michigan.
‡ School of Engineering and Applied Sciences and Wyss Institute for Biological Inspired Engineering, Harvard University, Cambridge, MA.

Management of bone deficiencies may comprise a significant part of treatment for individuals undergoing dental prosthetic rehabilitation, particularly those being restored with dental implant therapy. Current strategies used to treat these deficiencies often involve various alveolar bone grafting procedures.[1-4] In most instances, these approaches use autogenous, allogenic, or alloplastic bone grafts for bone regeneration.[5,6] Regardless of the material used, it is well documented that the development of a supporting vasculature concomitant to graft maturation is a key determinant to optimize bone regeneration at grafted sites.[7,8]

Since it was first introduced, guided bone regeneration (GBR) has become a routine treatment modality for the regeneration of alveolar bone.[9] The fundamental concept underlying GBR is to exclude specific cell types from the regenerative site while enabling the incorporation of others.[10] Barrier membranes are used to provide this cell-exclusive feature, and commonly, a bone graft material is also used for space maintenance. Nonetheless, regardless of the clinical protocol and materials used in GBR, it is understood that central to its success is the angiogenic response, establishing the vasculature to ultimately support the regenerated tissue.[11,12] Thus, angiogenesis is ultimately a critical determinant of bone regeneration.

To this end, there are several studies that have aimed to address this issue by adjunctively using a commercially available growth factor, platelet-derived growth factor (PDGF), in combination with bone grafting procedures for enhancement of bone regeneration.[13-17] Although PDGF acts primarily through its chemotactic and proliferative properties, it has demonstrated the ability to enhance angiogenesis as well. Although regenerative success has been shown with this approach, there have been no controlled studies to determine whether bolus delivery of a growth factor in this manner makes a difference.[18,19] Coupled with the relatively short half-life of growth factors and that during and after surgery much of the growth factor undoubtedly is diluted out by the patient's own blood, a relatively small fraction of the initial volume is what probably actually remains in the defect site.

As such, an approach to address this limitation could be to deliver an angiogenic growth factor in a controlled, sustained manner to the regenerative site. We have demonstrated previously the ability to develop hydrogels that incorporate growth factors that can be predictably released during a sustained period of time in a localized manner.[20,21] As mentioned, PDGF has been used clinically for treatment of osseous defects, but it has not been used specifically to promote angiogenesis. Vascular endothelial growth factor (VEGF) is a more potent angiogenic growth factor and thus may serve as a good candidate for delivery as part of GBR procedures.[22-24] In the context of therapeutic angiogenesis, we have demonstrated that localized, sustained delivery of VEGF using hydrogels is efficacious in a number of different model systems.[25-29] This study addresses the hypothesis that GBR, combined with controlled sustained delivery of VEGF, can enhance the angiogenic response and, hence, bone regeneration in a critical-sized osseous defect (a defect in which complete regeneration will not occur unless an intervention is performed).[30] The rationale underlying this approach is to turn a passive cell-exclusion procedure into a more active regenerative process.

MATERIALS AND METHODS

VEGF-Releasing Alginate Hydrogel Fabrication

An alginate delivery system was formulated using a bimolecular molecular weight (MW) alginate composed of two polymer components, low and high MW alginate polymer solutions, as described previously.[29] Briefly, ultrapure alginates§ were used as the high MW (≈250 kDa) component. Low MW alginate (≈50 kDa) was obtained by gamma-irradiating high MW alginate at a gamma dose of 3.0 mrad.‖ The injectable delivery alginate system was formulated by combining the two different MW polymers at a ratio of 7.5:2.5. Both alginate polymers were diluted to 1% weight/volume in ddH_2O, and 1% of the sugar residues in the polymer chains were oxidized with sodium periodate.¶ To gel the alginates' solutions, the sterile oxidized alginate stock was reconstituted to a 2% weight/volume solution in basal Eagle's basal medium (EBM) phenol red–free# overnight and subsequently was mixed with recombinant human $VEGF_{165}$ (0.06 µg/µL alginate gel)** and a 0.21 g/mL calcium sulfate slurry (25:1 alginate/calcium solution) via a syringe connector. The mixture was allowed to gel in the syringes for 30 minutes and then was maintained at 4°C before animal applications. For the in vitro mechanical analysis, the gels were stored at 37°C in EBM for 24 hours before testing.

Characterization of Hydrogels

The capability of alginate hydrogels to be used as an injectable delivery system was evaluated by characterizing the rheologic properties of pregelled solutions, as previously described.[29] Briefly, the viscosities of alginate solutions (pregelled) were monitored by using a controlled-stress rheometer†† at a constant temperature of 25°C. Before the quantification, all samples were presheared at a high shear rate, followed by rest for 5 minutes. While increasing the shear stress from 0.008 to 10 Pa, the resulting strains were measured, and the corresponding viscosity (η) was calculated (0.0553 Pa/second). Oscillatory shear experiments were conducted on the alginate samples using a cone-plate system. The rheometer was operated in the frequency sweep mode from 0.20 to 62.75 rad/second^{-1} at a strain of 0.005. During testing, storage modulus (G') was recorded as a function of frequency.

The degradation of hydrogels formed from a combination of high and low MW polymers that were partially oxidized (1%) was evaluated by dry weight loss as a function of degradation time. The degradation behavior was further examined by quantifying changes in the elastic moduli (E) through time in vitro. The E of the gels was measured by compressing at a constant deformation rate of 1 mm/second with a mechanical tester‡‡ at 25°C, as described in detail by Kong et al.[31]

Critical-Sized Calvarial Defect Model and GBR

Treatment of experimental animals was in accordance with the University of Michigan animal care guidelines, and all National Institutes of Health

animal handling procedures were observed. Critical-sized defects in 30 Fischer rats (175 to 200 g)[§§] were created, with one defect being created in each rat, as described previously.[32] Briefly, a midlongitudinal incision was made on the dorsal surface of the head and skin, and the periosteum was reflected to expose the calvaria. A trephine bur was next used to create an 8-mm circular defect in the cranium, and care was taken to remove the full thickness (1.5 to 2 mm) of the calvarial bone. After creation of the defect, resorbable collagen membranes[‖] were immediately placed over the defects alone as a GBR procedure (control), in combination with a 50 ng/mL VEGF saline solution added on top of the membrane (VEGF), or with topical application of the VEGF hydrogel on top of the membrane (VEGF–Alg).

Microcomputed Tomographic Analyses

Four and 8 weeks after GBR, regenerated tissue within the defect site was evaluated with micro-computed tomographic (μCT) and histomorpho-metric analyses. Three-dimensional reconstructions of μCT images of the non-decalcified calvariae were used to determine bone mineral density (BMD) and bone volume fraction (BVF) as described previously.[32] Briefly, software[¶¶] was used to generate three-dimensional reconstructions of the calvaria using images scanned in two-dimensional μCT.[##] For analysis of mineralized bone, a threshold value of 1,100 was used. Using three-dimensional images of the specimens, the total volume (TV; cubic milli-meters), bone volume (BV; cubic millimeters), and BMD of tissue within the defect site were measured directly, and the fractional bone volume (BV/TV; percentage) was calculated from the BV and TV measures.

Blood Vessel Formation/Bone Histomorphometry

Four and 8 weeks after GBR, the regenerated tissue was harvested, and neovascularization was assessed through histologic analysis. Samples from hematox-ylin and eosin-stained tissues in each condition were analyzed for blood vessel and bone formation man-ually and digitally as described previously.[33] Briefly, total blood vessels were counted in each histologic section based on the presence of endothelial cells lining well-defined capillary lumens containing red blood cells. The vessel number was then divided by the total area of the section to report the blood vessel density as vessels per squared millimeter. For bone histomorphometry, identification of bone was based on morphology of eosin-stained tissue and the cells lining (osteoblasts) and within (osteocytes) this tissue. This area was divided by the total area of the section and was reported as the percentage of the bone area/tissue area (BA/TA; percentage).

Statistical Analyses

The statistical analyses were performed using soft-ware.[***] For between-group comparisons, the me-dian and interquartile ranges (IQRs) for the blood vessel counts and bone measures were reported, unless otherwise noted. For within-group compari-sons (with time), differences in the sample means were reported with standard errors of the means. Descriptive analyses were performed initially, and for between-group comparisons, a non-parametric (Kruskal-Wallis) test was used to test for statistical significance between groups, which was defined as $P < 0.05$. For within-group comparisons between the two time points, a Student t test was performed to test for significance.

RESULTS

Alginate Material Properties

The degradation rate of the alginate hydrogels formed from partially oxidized alginate was rapid, with significant mass loss within 7 days ($\approx 50\%$) and almost complete mass loss by 40 days (Fig. 1A). Changes in the E with time were quantified in vitro and showed that the binary gels displayed a gradual reduction in E during the first 2 weeks, supporting their degradation in physiologic medium (Fig. 1B). During examination of the rheologic properties of pregelled solutions formed from a combination of low and high MW alginates (binary gels), they ex-hibited an intermediate G' compared to gels formed from the high or low MW polymers alone (Fig. 1C). All three gel types demonstrated a modest de-pendence of G' on the shear frequency, as expected for viscoelastic gels. The in vivo localization of VEGF released from these hydrogels has been established previously.[29,34,35]

Effects of VEGF on Neovascularization in GBR Sites

At 4 weeks, defect sites from all three conditions exhibited an abundant vascular response indicative of granulation tissue formation within a wound healing site. Although numerous capillaries were present in all conditions, blood vessel densities in the sites treated with VEGF–Alg (Fig. 2A) quali-tatively appeared higher than densities in regen-erated tissues of the sites treated with bolus delivery of VEGF (VEGF) or only GBR (control). Quantifica-tion of vessel densities at this earlier time point re-vealed that blood vessel densities were highest in the VEGF–Alg group (median = 37.1 vessels/mm²; IQR = 12.6), and this higher density was statistically
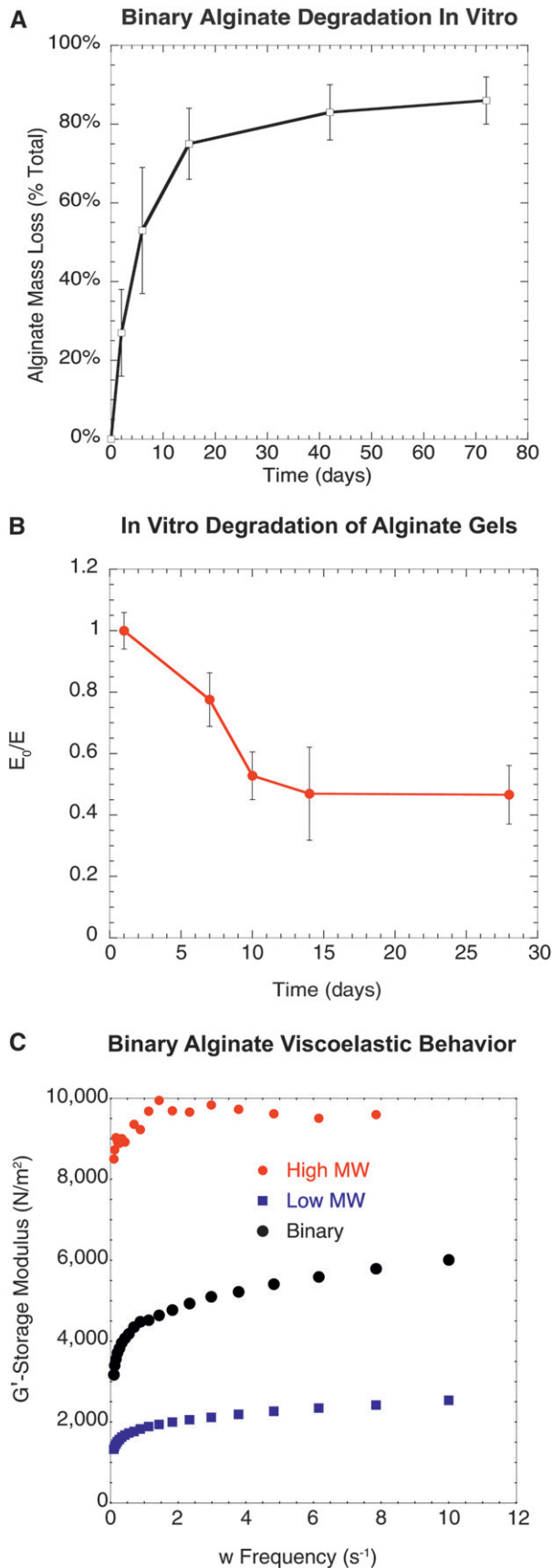
A Binary Alginate Degradation In Vitro

B In Vitro Degradation of Alginate Gels

C Binary Alginate Viscoelastic Behavior

significant ($P = 0.043$) relative to the control group (25.1 vessels/mm^2; 7.9) (Fig. 2B). There was no statistical difference between the control group and the VEGF group (33.1 vessels/mm^2; 9.7) or between the two groups treated with VEGF.

During examination of the treated sites at the 8-week time point, the statistically significant increase in neovascularization seen at the early time point was no longer evident among the groups, although the VEGF–Alg group had more vessels than the other two groups at this time point (Fig. 2C). Additionally, when looking at within-group changes in blood vessel density through time, there were no significant differences in blood vessel densities from 4 to 8 weeks within any of the three groups (Fig. 2D).

μCT Analyses of Bone Regeneration

Figure 3A shows representative three-dimensional μCT images from each of the three treatment groups at 4 and 8 weeks. In all three groups, there was early (4 weeks) evidence of mineralized tissue formation within the defect, yet qualitatively, there appeared to be no difference in mineralized tissue formation among groups. At 8 weeks, μCT images showed that more regenerated mineralized tissue was present within the VEGF–Alg-treated defects than in the other two groups (Fig. 3B). It was also observed that the best regenerative response at 8 weeks (as measured by highest BMD, BVF, and BA/TA) among all three conditions (n = 15) occurred in the VEGF–Alg-treated group, as shown in Figure 3B. Quantitative μCT analyses confirmed the qualitative evaluation and revealed that, although early bone formation was achieved in each of the three groups, among the three groups, there were no differences in regenerated mineralized tissue as measured by BMD and BVF. Although there were no differences in these parameters among groups at the early time point, with time in the VEGF–Alg group, there were significant increases ($P <0.01$) in BMD (mean difference = 184 mg/cc) and BVF (mean difference = 0.27). Additionally, at 8 weeks, quantitative μCT analyses of BMD showed that the VEGF–Alg group had significantly higher BMD (median = ±234.5 mg/cc; IQR = 48.4) than the VEGF group

Figure 1.

*Characterization of hydrogels. **A)** In vitro degradation of binary alginate gels as monitored by mass loss through time. The dry masses of gels formed from binary MW partially oxidized alginate were analyzed as a function of time after incubation in phosphate-buffered saline (pH 7.4) at 37°C. Values represent mean and standard deviation (n = 5). **B)** E of binary hydrogels as a function of time in vitro (1% oxidized). E_0 represents the initial E of the gels measured after incubation for 24 hours, and all values were normalized to this initial modulus. Values represent mean and standard deviation (n = 4). **C)** G′ of binary hydrogels (Binary), gels comprised of only high MW alginate (High MW), and gels comprised of only low MW polymer (Low MW), as a function of the strain frequency.*
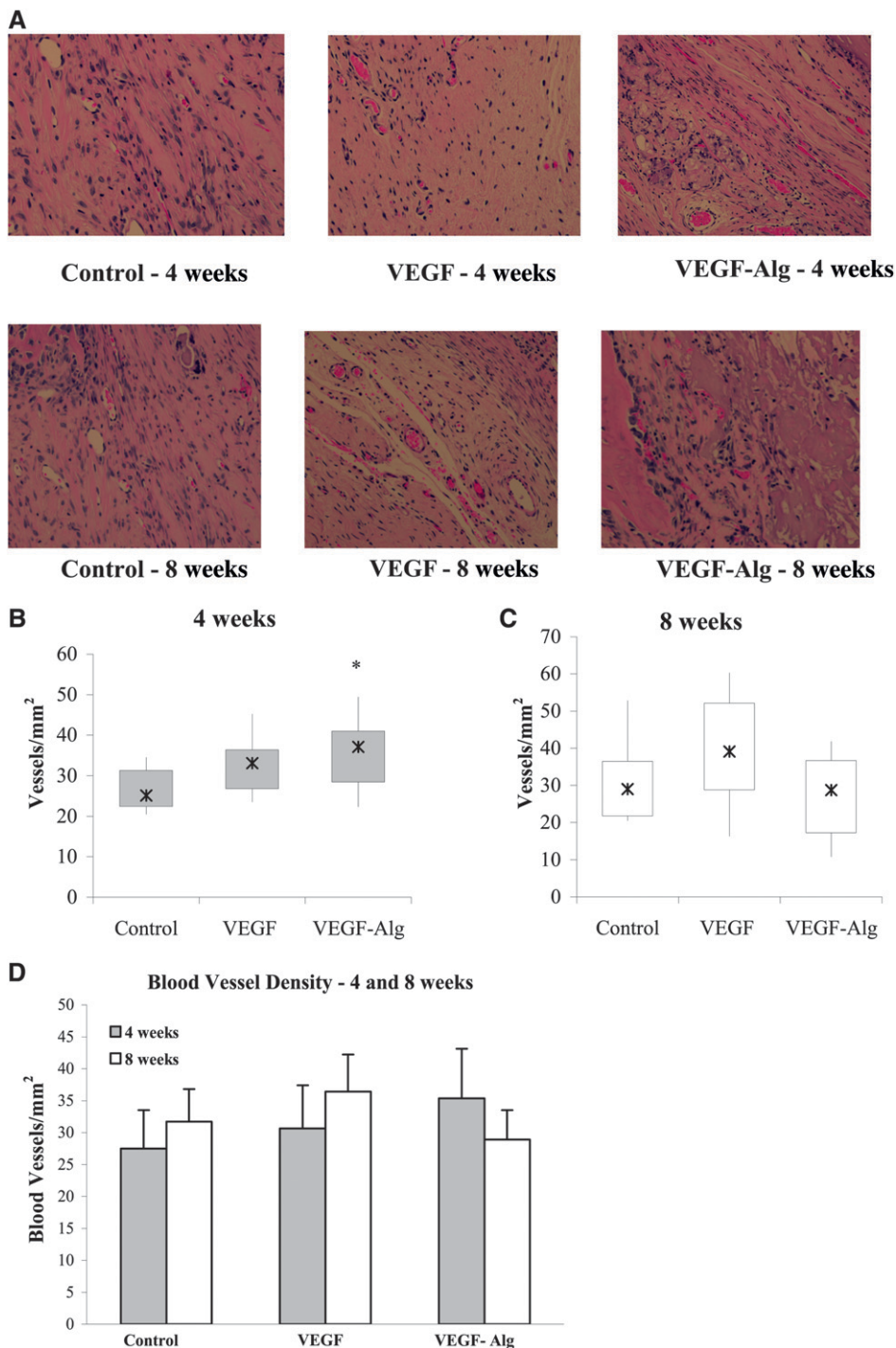
Figure 2.

*Angiogenesis within defect sites. A) Photomicrographs of representative histologic (hematoxylin and eosin-stained) sections taken from control, VEGF-treated, and VEGF–Alg-treated defect sites at 4 and 8 weeks. Vessels are characterized by the presence of red blood cells within defined lumens lined by endothelial cells. B) Box plots represent the median (x), IQR (limits of box), minimum (lower limit of bottom line), and maximum (upper limit of top line) numbers of blood vessels among control, VEGF, and VEGF–Alg conditions at 4 weeks (n = 5). C) Box plots representing blood vessel densities at 8 weeks. D) Graphs represent the mean ± SEM of blood vessel densities at 4 and 8 weeks among and within conditions. *P <0.05 relative to control condition*

(164.7 mg/cc; 37.2; $P = 0.041$) and the control group (138.8 mg/cc; 76.2; $P=0.036$) (Fig. 3C). Similarly, BVF in the VEGF–Alg group (median = 0.33; IQR = 0.07) was significantly higher than in the VEGF (0.25 mm/cc; 0.09; $P = 0.031$) and control (0.16 mg/cc; 0.12; $P = 0.023$) groups at 8 weeks (Fig. 3D).

Histologic Analyses of Bone Regeneration

Histologic evaluation of specimens at this time point confirmed that the mineralized tissue seen in the μCT analyses had morphology consistent with that of bone tissue (Fig. 4A), and at this later time point, there were also specimens among the three groups that exhibited complete bridging of the critical-sized defect with regenerated bone tissue (Fig. 4B). Three of the five specimens in the VEGF–Alg group had complete regeneration across the defect, whereas only one specimen in each of the other two groups exhibited complete bridging. Quantitative histomorphometric analysis of bone area was consistent with the μCT analysis in that, at 4 weeks, there was no difference in bone area (BA/TA) among the three groups, but with time in the VEGF–Alg group, BA/TA significantly increased (mean difference = 25%) (Fig. 4C). Finally, at the 8-week time point, the VEGF–Alg-treated group (median = 35.5%; IQR = 19.2) had the highest BA/TA relative to the VEGF (18.5; 13.1; $P = 0.039$) and control (23.9; 22.7; $P = 0.047$) treatment groups (Fig. 4C).

DISCUSSION

Central to the success of GBR procedures is the ability of the barrier membrane to control ("guide") the cells that populate the regenerative site through
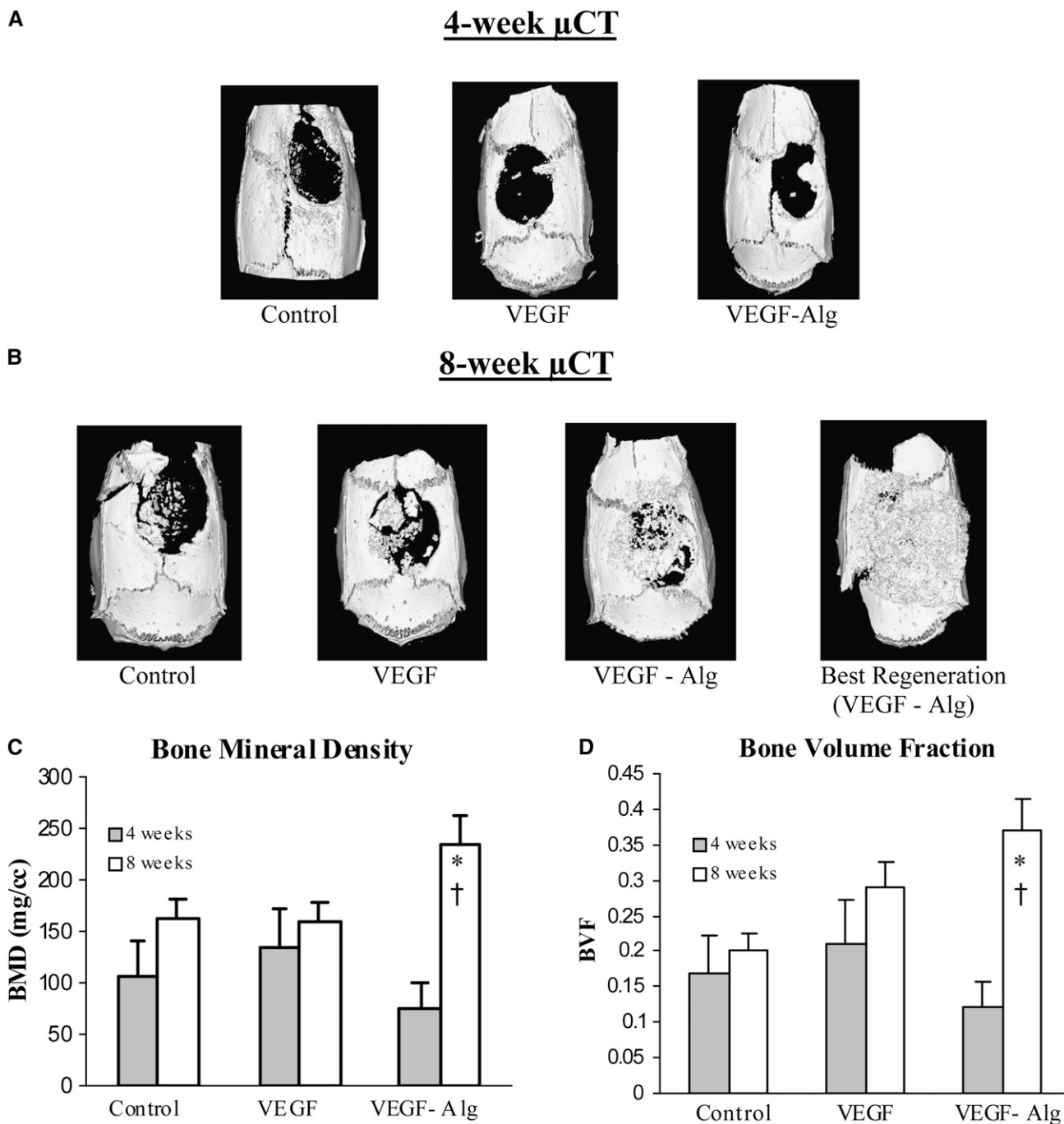
A

4-week µCT

Control   VEGF   VEGF-Alg

B

8-week µCT

Control   VEGF   VEGF - Alg   Best Regeneration
(VEGF - Alg)

C

Bone Mineral Density

□ 4 weeks
□ 8 weeks

BMD (mg/cc)

Control   VEGF   VEGF- Alg

D

Bone Volume Fraction

□ 4 weeks
□ 8 weeks

BVF

Control   VEGF   VEGF- Alg

Figure 3.
*µCT analyses of bone regeneration. Representative three-dimensional reconstructed µCT images across calvarial defects from control, VEGF, and VEGF–Alg conditions at 4 **(A)** and 8 **(B)** weeks (also shown is best regeneration of defect among all conditions, as measured by BMD and BVF). µCT analyses of BMD **(C)** and BVF **(D)** at 4 and 8 weeks. *P <0.05 relative to the same condition at 4-week time point; †P <0.05 relative to control and VEGF conditions at the 8-week time point.*

cell exclusion of undesirable (epithelial) cells and inclusion of desired (osteogenic and vascular) cells. In this regard, the membrane functions in a passive manner, yet the key factor underlying success of the regenerative response is vascularization of the site.[11] In this report, we aim to make GBR more "active" through using a controlled growth-factor delivery system to enhance the vascular response to the regenerating site. Combining oxidized low and high MW alginates enabled control of the degradation rate to maintain favorable viscous and mechanical properties for use in this GBR indication.
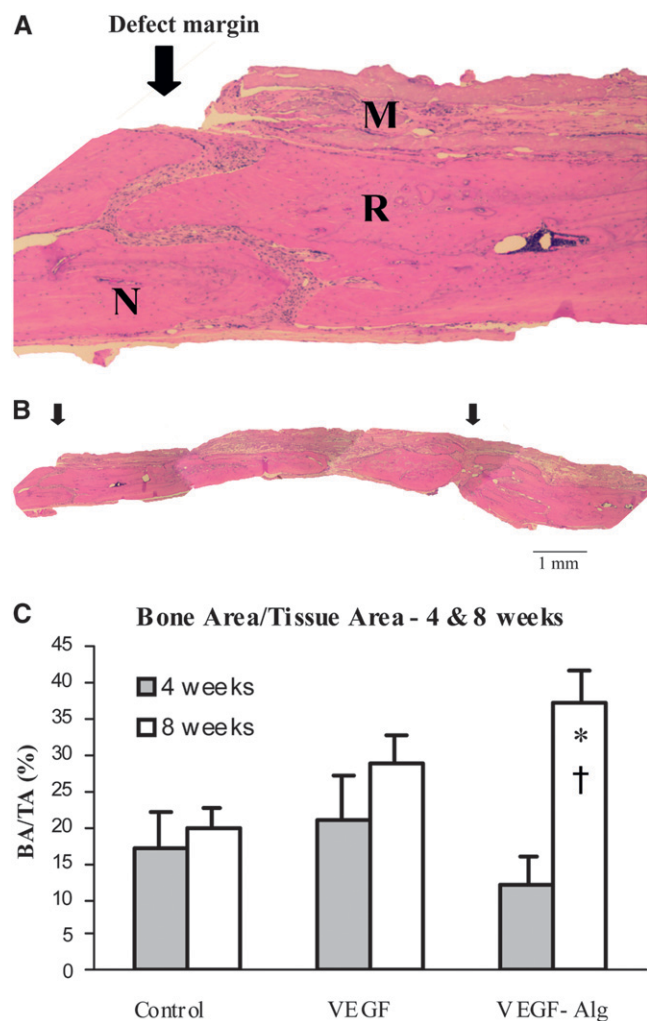
Figure 4.
Histologic analysis of bone regeneration. **A)** *Histologic section (×10) of the interface (at the defect margin) among native bone (N), regenerated bone (R), and the membrane (M) from the VEGF–Alg condition.* **B)** *Histologic section (×4) demonstrating complete bridging across the calvarial defect, seen at 8 weeks in three of the five VEGF–Alg conditions and in one of the five of each of the control and VEGF conditions at this time point (black arrows represent margins of calvarial defect).* **C)** *Graph representing histomorphometric bone area analyses (n = 5).* *P <0.05 relative to the same condition at 4-week time point; †P <0.05 relative to control and VEGF conditions.*

GBR procedures were performed in critical-sized osseous defects, and the barrier membranes used were coated with VEGF-releasing hydrogels. The results demonstrated that, relative to the comparison groups, the VEGF–Alg group increased blood vessel density early in the regenerative process (4 weeks) and ultimately enhanced bone regeneration by 8 weeks.

It has been shown recently that delivery of VEGF-releasing polylactide coglycolide (PLGA) microspheres into critical-sized defects enhances bone regeneration when used in combination with a PLGA membrane.[36] However, in this report, there is

no evaluation of blood vessel formation, and the primary outcome measure of bone regeneration was performed with soft x-ray imaging. We have also previously evaluated the regenerative potential of VEGF-releasing scaffolds in non-critical-sized irradiated bone defects, showing that it enhances bone regeneration and angiogenesis.[32] In the present study, we specifically evaluate the angiogenic response at an early time point (4 weeks) to determine whether the hydrogel releasing system affected the vasculature in the defect site. Although there were no differences in bone formation at this early time point, blood vessel formation was significantly enhanced relative to the control GBR condition.

At the later 8-week time point, we found that there were no differences in blood vessel densities among groups or within groups (with time). It is not surprising that early differences in blood vessel densities among groups "wash out" with time because the early events of wound healing rely most heavily on the angiogenic response. Nonetheless, at the later time point, we could see significant histologic and radiographic (μCT) differences in bone regeneration among the VEGF–Alg group and the other two groups. Also, interesting to note was that the VEGF–Alg condition was the only one in which bone formation significantly increased with time. The combinatorial effect of sustained VEGF delivery with GBR would appear to be the cause for this enhanced regenerative response. It also supports previous work that suggests that delivery of VEGF in combination with other materials affects bone healing and maturation, through indirect processes that enhance angiogenesis.[37,38] In fact, when only VEGF is delivered to critical-sized defects, there is an increase in angiogenesis but no effect on overall bone regeneration relative to control conditions.[38] However, if another variable (e.g., osteoinductive factor, additional material) is incorporated in these systems, it appears as though it can have additive effects to enhance the overall bone regeneration.[37] One of the limitations of our study is that we do not include a GBR plus alginate alone group (without VEGF); thus, one could argue that, although unlikely, the mere presence of alginate itself could be responsible for the results seen. However, there is no sound basis for this conclusion because in our previous studies, we have evaluated the angiogenic effects of alginate, and, regardless of the regenerative model system evaluated (i.e., bone, muscle), have never found significant upregulation of angiogenesis in conditions with only alginate.[20,35,39,40]

There have been a number of different studies examining the delivery of different growth factors to defect sites, yet VEGF, because of its highly potent angiogenic activities, makes clear sense for GBR applications. Additionally, VEGF has been

implicated in having direct chemotactic and mitogenic effects on osteoblasts and osteogenic cells.[41,42] Thus, it could have direct and indirect effects on bone regeneration as part of GBR procedures. Failures of these procedures are partially attributed to inadequate blood supply to support the regenerating site. This challenge is the rationale for incorporating other angiogenesis-promoting modalities (i.e., platelet-rich plasma, recombinant human PDGF) in the clinical treatment of large oral reconstructions using GBR. However, because these angiogenic agents are delivered as a bolus, their "availability" to the local microenvironment is not well controlled or sustained with time. Thus, GBR using a controlled delivery system, which could be either easily injected into defects or incorporated as part of the material (i.e., VEGF-coated membrane), could provide great benefit.

CONCLUSIONS

Overall, this study sought to evaluate the regenerative effects of an injectable VEGF-releasing hydrogel used as a coating on a bioabsorbable membrane as part of GBR. Extensions of these findings could have clinical implications in the development of GBR adjuncts and even GBR membranes that not only function passively to direct cell migration but also function to actively direct the regenerative process through enhancing angiogenesis and, ultimately, bone regeneration.

ACKNOWLEDGMENTS

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Correspondence: Dr. Darnell Kaigler, Department of Periodontics and Oral Medicine, University of Michigan, 1011 N. University, Ann Arbor, MI 48109. E-mail: dkaigler@umich.edu.