Published in final edited form as:

J Periodontol. 2013 February ; 84(2): 230–238. doi:10.1902/jop.2012.110684.

Guided bone regeneration (GBR) utilizing injectable Vascular Endothelial Growth Factor (VEGF) delivery gel

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Abstract

Background—Vascularization underlies the success of guided bone regeneration (GBR) procedures. This study evaluated 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 either alone (control), plus bolus delivery of VEGF, or plus application of VEGF releasing hydrogels (VEGF - Alg). Four and eight weeks following treatment, defect sites were evaluated with microcomputed tomographic and histomorphometric analyses for blood vessel and bone formation.

Results—At four 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 the VEGF+ Alg significantly ($p < 0.05$) increased blood vessel density. Though there was no difference in bone regeneration at four weeks, at eight weeks, there was a significant ($p < 0.05$) increase in bone regeneration in the VEGF + Alg treated defects.

Conclusions—These data demonstrated that the application of VEGF + Alg enhanced early angiogenesis while at a later timepoint, 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.

Keywords

regenerative medicine; bioengineering; growth factors; hydrogel; bone; angiogenesis

INTRODUCTION

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 utilize autogenous, allogeneic, or alloplastic bone grafts for bone regeneration.^{5, 6} Regardless of the

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The authors declare that there is no conflict of interest associated with this report.

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 employed in order 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 for 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 which have aimed to address this issue by adjunctively using a commercially available growth factor, PDGF (platelet-derived growth factor), in combination with bone grafting procedures for enhancement of bone regeneration.^{13–17} Though PDGF acts primarily through its chemotactic and proliferative properties, it has demonstrated the ability to enhance angiogenesis as well. Though regenerative success has been shown with this approach, there have been no controlled studies to determine whether or not 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 previously demonstrated the ability to develop hydrogels which incorporate growth factors that can be predictably released over a sustained period of time in a localized fashion.^{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 polymers components: low and high molecular weight alginate polymer solutions, as previously described.²⁹ Briefly, ultrapure alginates obtained from Novamatrix (Pronova UP MGv) were utilized as the high molecular weight (~250 KDa) component. Low molecular weight alginate (~50 KDa) was obtained by gamma (γ)-irradiating high molecular weight alginate at a γ -dose of 3.0 Mrad (Environment, Health and Safety Office, Massachusetts Institute of Technology - MIT). The injectable delivery alginate system was formulated by combining the two different molecular weight polymers at a ratio of 7.5:2.5. Both alginate polymers were diluted to 1% w/v in ddH₂O, and 1% of

the sugar residues in the polymer chains were oxidized with sodium periodate (Sigma-Aldrich). To gel the alginates solutions, the sterile oxidized alginate stock was reconstituted to a 2% w/v solution in basal EBM media phenol red free (Lonza) overnight and subsequently was mixed with recombinant human VEGF₁₆₅ (0.06 ug/ul of alginate gel) (R and D Systems), and a 0.21g/mL calcium sulfate slurry (25:1 alginate to 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 prior to animal applications. For the in vitro mechanical analysis, the gels were stored at 37 °C in EBM2 basal media for 24hrs before testing.

Characterization of Hydrogels

The capability of alginate hydrogels to be used as an injectable delivery system was evaluated by characterizing the rheological properties of pre-gelled solutions, as previously described.²⁹ Briefly, The viscosities of alginate solutions (pre-gelled) were monitored by using a controlled-stress rheometer (CS-50, Bohlin Instrument) at a constant temperature of 25 °C. Prior to the quantification, all samples were pre-sheared 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.s. 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 s⁻¹ at a strain of 0.005. During testing, storage modulus (G') were recorded as a function of frequency.

The degradation of hydrogels formed from a combination of high and low molecular weight 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 over time in vitro. The elastic moduli (E) of the gels were measured by compressing at a constant deformation rate of 1 mm/s with a mechanical tester (MTS Bionix 100, MTS systems) at 25 °C, as described in detail by Kong.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 NIH animal handling procedures were observed. Critical-sized defects in 30 Fischer rats (175–200 g, Charles River Laboratories) were created, with one defect being created in each rat, as previously described.³² Briefly, a mid-longitudinal 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 (1.5–2 mm) of the calvarial bone. Following creation of the defect, resorbable collagen membranes[§] were immediately placed over the defects either alone as a guided bone regeneration 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 (μ CT) analysis

Four and eight weeks following GBR, regenerated tissue within the defect site was evaluated with μ CT and histomorphometric analyses. 3-dimensional reconstructions of μ CT images of the non-decalcified calvariae were used to determine bone mineral density (BMD) and bone volume fraction (BVF) as previously described.³² Briefly, MicroView® software was used to generate 3-D reconstructions of the calvaria using images scanned in 2-D μ CT (μ CT MS8X-130; EVS Corp.). For analysis of mineralized bone, a threshold value of 1100 was used. Using 3-D images of the specimens, the total volume (TV, mm³), bone volume (BV; mm³), and bone mineral density (BMD) of tissue within the defect site were measured

directly, and the fractional bone volume (BV/TV; %) was calculated from the BV and TV measures.

Blood Vessel Formation/ Bone Histomorphometry

Four and eight weeks after GBR, the regenerated tissue was harvested and neovascularization was assessed through histological analysis. Samples from H & E-stained tissues in each condition were analyzed for blood vessel and bone formation manually and digitally as previously described.³³ Briefly, total blood vessel were counted in each histological section based upon 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/mm². 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, %).

Statistical Analysis

The statistical analyses were performed with the Instat Software Program. For between group comparisons, the median and interquartile ranges (IQR) for the blood vessel counts and bone measures were reported, unless otherwise noted. For within group comparisons (over 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 nonparametric (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 timepoints, a Student's 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 (~50%), and almost complete mass loss by 40 days (Figure 1A). Changes in the elastic moduli over time were quantified *in vitro* and showed that the binary gels displayed a gradual reduction in E over the first two weeks, supporting their degradation in physiologic medium (Figure 1B). In examination of the rheological properties of pre-gelled solutions formed from a combination of low and high molecular weight alginates (binary gels), they exhibited an intermediate storage modulus, as compared to gels formed from the high or low molecular weight polymers alone (Figure 1C). All three gel types demonstrated a modest dependence of G' on the shear frequency, as expected for viscoelastic gels. The *in vivo* localization of VEGF released from these hydrogels has been previously established.^{29, 34, 35}

Effects of VEGF on Neovascularization in GBR sites

At four weeks, defect sites from all three conditions exhibited an abundant vascular response indicative of granulation tissue formation within a wound healing site. Though numerous capillaries were present in all conditions, qualitatively, blood vessel densities in the sites treated with alginate-VEGF (VEGF-Alg) (Figure 2A) appeared higher than densities in regenerated tissues of the sites treated with bolus delivery of VEGF (VEGF) or only GBR (Control). Quantification of vessel densities at this earlier timepoint revealed 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 significant ($p = 0.043$) relative to the Control group (25.1 vessels/mm²; 7.9) (Figure 2B). There was no statistical difference between the

control group and the VEGF group (33.1 vessels/mm²; 9.7) or between the two groups treated with VEGF.

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

μCT Analysis of Bone Regeneration

Figure 3A shows representative 3-D 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 between 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 (Figure 3B). It was also observed that the best regenerative response at eight 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 though early bone formation was achieved in each of the three groups, between the three groups, there were no differences in regenerated mineralized tissue as measured by bone mineral density (BMD) and bone volume fraction (BVF). Though there were no differences in these parameters between groups at the early timepoint, over time, in the VEGF-Alg group, there were significant increases ($p < 0.01$) in BMD (mean difference = 184 mg/cc) and BVF (mean diff. = 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 (164.7 mg/cc; 37.2; $p = 0.041$) and the control group (138.8 mg/cc; 76.2; $p = 0.036$) (Figure 3C). Similarly, BVF in the VEGF-Alg group (median = 0.33; IQR = 0.07) was significantly higher than in the VEGF (0.25; 0.09; $p = 0.031$) and control (0.16; 0.12; $p = 0.023$) groups at 8 weeks (Figure 3D).

Histological Analyses of Bone Regeneration

Histological evaluation of specimens at this timepoint confirmed that the mineralized tissue seen in the μCT analyses had morphology consistent with that of bone tissue (Figure 4A), and at this later timepoint, there were also specimens among the three groups which exhibited complete bridging of the critical-sized defect with regenerated bone tissue (Figure 4B). Three of the five specimens in the VEGF-Alg group had complete regeneration across the defect while 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) between the three groups but over time, in the VEGF-Alg group, BA/TA significantly increased (mean diff. = 25%) (Figure 4C). Finally, at the 8 week timepoint, 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 (Figure 4C).

DISCUSSION

Central to the success of GBR procedures is the ability of the barrier membrane to control ("guide") the cells which populate the regenerative site through 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 aimed to make GBR more “active” through utilizing 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. 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 (four weeks) and ultimately enhanced bone regeneration by eight weeks.

It has recently been shown that delivery of VEGF-releasing poly-lactide co-glycolide (PLGA) microspheres into critical sized defects enhances bone regeneration when used in combination w/ a PLGA membrane.³⁶ However, in this report, there was 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 size irradiated bone defects, showing that it enhances bone regeneration and angiogenesis.³² In the present study, we specifically evaluated the angiogenic response at an early timepoint (4 weeks) to determine if the hydrogel releasing system affected the vasculature in the defect site. Though there were no differences in bone formation at this early timepoint, blood vessel formation was significantly enhanced relative to the control GBR condition.

At the later eight week timepoint, we found that though there were no differences in blood vessel densities between groups or within groups (over time). It is not surprising that early differences in blood vessel densities between groups “wash out” over time in that it is the early events of wound healing which rely most heavily upon the angiogenic response. Nonetheless, at the later timepoint we could see significant histologic and radiographic (μ CT) differences in bone regeneration between 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 over 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 which suggests that delivery of VEGF in combination with other materials affects bone healing and maturation, through indirect processes which enhance angiogenesis.^{37, 38} In fact, when only VEGF is delivered to critical sized defects, there is an increase in angiogenesis, though no effect on overall bone regeneration relative to control conditions.³⁸ However, if another variable (ie. osteoinductive factor, additional material) is incorporated in these systems, it appears as though these can have additive effects to enhance the overall bone regeneration.³⁷ One of the limitations of our study is that we did not include a GBR + alginate alone group (without VEGF), thus, one could argue that though unlikely, the mere presence of alginate itself could be responsible for the results seen. However, there is no sound basis for this conclusion in that in our previous studies we have evaluated the angiogenic effects of alginate and regardless of the regenerative model system evaluated (ie 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, due to 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 partly attributed to inadequate blood supply to support the regenerating site. This challenge is the rationale for incorporating other angiogenesis promoting

modalities (ie. platelet rich plasma, rhPDGF) in the clinical treatment of large oral reconstructions utilizing GBR. Yet, because these angiogenic agents are delivered as a bolus, their ‘availability’ to the local microenvironment is not well controlled or sustained over time. Thus, GBR using a controlled delivery system, which could either be easily injected into defects or incorporated as part of the material (ie. VEGF coated membrane), could provide great benefit.

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 which 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

Financial support: Burroughs Wellcome Fund (CAMS) and the National Institutes of Health (R01 HL069957)

The authors would like to thank Jaclynn Kreider (University of Michigan, Ann Arbor MI, USA) for technical support of this study. The authors would like to also acknowledge the Burroughs Wellcome Fund (CAMS award to DK) (Research Triangle Park, NC, USA) and the National Institutes of Health (DM) for financial support of this research (R01 HL069957).

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Figure 1. Characterization of hydrogels

A) In vitro degradation of binary alginate gels as monitored by mass loss over time. The dry masses of gels formed from binary molecular weight partially oxidized alginate were analyzed as a function of time following incubation in PBS (pH 7.4) at 37 °C. Values represent mean and standard deviation (n=5). B) The elastic moduli (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 h, and all values were normalized to this initial modulus. Values represent mean and standard deviation (n=4). C) The storage modulus (G') of binary hydrogels (Binary), gels comprised only of high molecular weight alginate (High MW), and gels comprised only of low molecular weight polymer (Low MW), as a function of the strain frequency.

Figure 2. Angiogenesis within defect sites

A) Photomicrographs of representative histological (H&E stained) sections taken from Control, VEGF, 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), interquartile range (IQR, limits of box), minimum (lower limit of lower line), and maximum (upper limit of upper line) numbers of blood vessels between 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 ($\pm$ standard error of the mean) of blood vessel densities at 4 and 8 weeks between and within conditions.

* denotes $p < 0.05$ relative to Control condition

Figure 3. μ CT analyses of bone regeneration

Representative 3D reconstructed μ CT images across calvarial defects from Control, VEGF, and VEGF-Alg conditions at A) 4 and B) 8 weeks (also shown is best regeneration of defect among all conditions, as measured by BMD and BVF). μ CT analyses of C) Bone Mineral Density and D) Bone Volume Fraction at 4 and 8 weeks. $p < 0.05$ relative to the same condition at 4 week timepoint; ** denotes $p < 0.05$ relative to relative to Control, and VEGF conditions at 8 week timepoint

Figure 4. Histological analysis of bone regeneration

A) Histological section (10X) of the interface (at the defect margin) between native bone (N), regenerated bone (R), and the membrane (M) from the VEGF-Alg condition. B) Histological section (4X) demonstrating complete bridging across the calvarial defect, seen at 8 weeks in 3 of the 5 VEGF-Alg conditions and in one of the five of each of the control and VEGF conditions at this timepoint. C) Graph representing histomorphometric Bone Area analyses (n=5). * denotes $p < 0.05$ relative to the same condition at 4 week timepoint; ** denotes $p < 0.05$ relative to Control, and VEGF conditions