Bone graft revascularization strategies

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Chapter 6

Induction of Angiogenesis and Osteogenesis in Surgically Revascularized Frozen Bone Allografts by Sustained Delivery of FGF-2 and VEGF

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ABSTRACT

Purpose
Large conventional bone allografts are susceptible to fracture and nonunion due to incomplete revascularization and insufficient bone remodeling. We aim to improve bone blood flow and bone remodeling using surgical angiogenesis combined with delivery of fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF).

Methods
Frozen femoral allografts were heterotopically transplanted in a rat model. The saphenous arteriovenous bundle was implanted within the graft medullary canal. Simultaneously, biodegradable microspheres containing phosphate buffered saline (control), FGF-2, VEGF, or FGF-2 + VEGF were placed within the graft. Rats were sacrificed at 4 and 18 weeks. Angiogenesis was determined by quantifying bone capillary density and measuring cortical bone blood flow. Bone remodeling was assessed by histology, histomorphometry, and alkaline phosphatase activity.

Results
VEGF significantly increased angiogenesis and bone remodeling at 4 and 18 weeks. FGF-2 did not elicit a strong angiogenic or osteogenic response. No synergistic effect of FGF-2 + VEGF was observed. VEGF delivered in microspheres had superior long-term effect on angiogenesis and osteogenesis in surgically revascularized frozen bone structural allografts as compared to FGF-2 or FGF-2 + VEGF.

Conclusion
Continuous and localized delivery of VEGF by microencapsulation has promising clinical potential by inducing a durable angiogenic and osteogenic response in frozen allografts.

INTRODUCTION

Cryopreserved bone is commonly used to replace large osseous defects resulting from oncologic resection, traumatic loss, or osteomyelitis. Allograft bone may be closely matched in size and shape to the resected bone, and is readily available from bone banks. Furthermore, storing at -80°C reduces antigenicity but maintains the osteoconductive properties of fresh bone. Osteoinduction, which is the cytokinetic potential within the graft to elicit bone remodeling, is impaired by freezing. These large structural grafts remain substantially necrotic long-term, as revascularization and graft viability are limited by a slow and incomplete bone remodeling process (1). Consequently, segmental frozen allografts have a high incidence of complications (25–35%), including infection, nonunion, and stress fracture (2), (3).

We have previously demonstrated the ability to improve or maintain blood flow in bone allotransplantation revascularization by use of implanted vascularized tissue (4), (5). This technique is termed surgical angiogenesis. In this study, we evaluate the role local delivery of growth factors may offer when combined with surgical angiogenesis in improving both bone vascularization and new bone formation. Basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor-A (VEGF) are strong angiogenic cytokines, promoting proliferation of endothelial cells. Both stimulate osteoblastic differentiation as well (6), (7). We encapsulated FGF-2 and VEGF solely and combined in biodegradable poly lactic-co-glycolic acid (PLGA) microspheres and delivered these to the grafts intramedullary. We previously investigated the effect of growth factors on cryopreserved bone allografts at 4 weeks (7). In this short-term study VEGF proved to have angiogenic and osteogenic potential, while FGF-2 was not beneficial, either alone or synergistically with VEGF. In this study we have applied the same model to investigate the long-term effects of local and sustained delivery of microencapsulated FGF-2 and VEGF on angiogenesis and osteogenesis in frozen allografts.

METHODS

Experimental Groups

Eighty transplantations were performed. Four groups were created with 20 rats each receiving either PBS (Group I), FGF-2 (Group II), VEGF (Group III), or FGF-2 + VEGF (Group IV). The survival time was either 4 or 18 weeks. The group size of ten recipient rats was determined prior to the experiment, based upon a power calculation for the primary outcome of interest, capillary density. Using a two sample t-test with α=0.05, we would have 80% power to detect an effect size of at least 1.33. Effect size is defined as the difference in group means divided by the common standard deviation. In a previous study,
the mean capillary density was 28.6 ± 16.8%. Anticipating similar results, this study was designed to have 80% power of detecting a difference in means of 22.3% or greater between any two groups.

**Bone Transplantation Procedure**

Female Dark Agouti rats (RT1a) were anesthetized with pentobarbital sodium at a dose of 35 mg/kg IP. Femoral diaphyseal bone grafts (20 mm length) were removed bilaterally. After harvest, donor rats were euthanized with an intraperitoneal injection of Sleepaway (pentobarbital sodium) at a dose of 200 mg/kg. The grafts were reamed with a 2 mm hand drill, rinsed with saline to remove all bone marrow and stored at -80 °C.

In order to create a major histocompatibility mismatch, male Piebald Virol Glaxo rats (RT1c) were used as recipients. A dose of 10 IU Fragmin (SC) was given preoperatively and once daily for 5 days postoperatively. Rats were anesthetized with ketamine (90 mg/kg IM) and xylazine (10 mg/kg IM). Additional ketamine (20 mg/kg IM) was injected if necessary during the procedure. Buprenorphine (SC) of 0.1 mg/kg was given postoperatively and once daily for 2 days. The graft was thawed in sterile saline at room temperature immediately prior to implantation. Using our previously described model, the graft was placed in an abdominal subcutaneous pocket, wrapping it with silicone sheeting to block spontaneous angiogenesis from surrounding soft tissues. The left saphenous arteriovenous (AV) bundle was dissected from the leg, including adjacent fascia and inserted as a pedicled flap into the intramedullary canal of the graft. Animals were randomly assigned to one of four groups, receiving biodegradable microspheres encapsulating either phosphate buffered saline, FGF-2, VEGF, or VEGF + FGF-2. Postoperatively Tacrolimus FK-506 (Fujisawa Pharmaceutical Co., Osaka, Japan) immunosuppression was administered daily (1 mg/kg/day IM) for 2 weeks. Previous published and unpublished research in our laboratory demonstrates that a short course of immunosuppression decreases inflammatory response and improves arteriovenous bundle patency rate in surgically revascularized allografts (5), (8). As it was our aim to apply a practical model to investigate the effect of growth factors on angiogenesis and osteogenesis we therefore applied a short course of immunosuppression. All animals were treated according to directions of the Institutional Animal Care and Use Committee (IACUC).

**Growth Factor Delivery**

We prepared poly D,L-lactide-co-glycolide microspheres (PLGA) containing phosphate buffered saline (PBS) as control, FGF-2, VEGF, or FGF-2 + VEGF, as has been described in previous research from our laboratory (7). We delivered 15 mg of microspheres into each bone graft. Microspheres were formulated to contain 0.7 mg of each growth factor per mg microsphere, thus providing a total dose of 10.5 mg of growth factor. Gradual degradation of the microspheres by hydrolysis results in an initial burst release followed...
by a sustained zero order kinetic release for 28 days (9), (10), (11). PLGA microsphere preparation, the optimization of microsphere entrapment efficiency, and drug release kinetics have been described in detail by Kempen et al (10), (12), (13) and were applied accordingly.

**Bone Blood Flow**
At sacrifice, rats were anesthetized and cortical bone blood flow was measured with the hydrogen washout technique. This technique has been validated in our laboratory and is described in detail elsewhere (7), (14), (15). In short, a superficial cortical hole was drilled in the graft. The rat was given a breathing mixture of 30% oxygen and 70% hydrogen and after tissue saturation the hydrogen inhalation was stopped and the decline of hydrogen was measured. The rate of hydrogen washout represents bone blood flow and was calculated using customized LabVIEW™ software (Austin, TX).

**Microangiography: Capillary Density**
After bone blood flow measurement, the aorta and vena cava were both cannulated. The rat was then euthanized with Sleepaway at a dose of 200 mg/kg IV. The lower extremity vasculature was thoroughly irrigated with heparinized saline under physiologic pressure followed by a colored polymer silicone solution (Microfil, Flow Tech Inc., Carver, MA). The patency of the AV bundle was determined by investigating the filling of the AV bundle with the polymer solution. Samples with a thrombosed AV bundle were excluded from analysis. The grafts were fixed in 10% formalin for 24 h and decalcified in 14% ethylenediaminetetraacetic acid (EDTA) for 7 h in a calibrated laboratory microwave at 750 W (Pelco Biowave 3450 Laboratory Microwave, Ted Pella, Inc., Redding, CA). To obtain translucency of the bone and visualize the bone vasculature (filled with Microfil), the bone was then exposed to increasing concentrations of ethanol using the modified Spalteholz methylsalicylate optical bone clearing process (16), (17). Microangiography was performed and images were analyzed with scientific image analysis software (Scion Image 4.03). Capillary density is calculated as the number of vessel pixels/total pixels (= vasculature area/total bone area).

**Quantitative Histomorphometry**
Two-weeks and 2-days prior to sacrifice, calcein and tetracycline fluorescent labels (20 mg/kg) were administered, respectively. Unstained transverse sections were analyzed at 200X with imaging analysis software (Osteomeasure; Osteometrics, Atlanta, GA). The ratio of bone surface with a single fluorescent label to total bone surface, defined as Single Labeled Surface to Bone Surface (SLS/BS) was determined, as well as the Double Labeled Surface (Calcein and Tertacycline labels) to total Bone Surface (DLS/BS). We additionally measured mineralizing bone surface to total bone surface (MS/BS), which is
the ratio of total labeled (single and double) bone surface to total bone surface. Mineral apposition rate (MAR) was measured which represents the rate at which new bone is deposited. Bone formation rate to bone surface (BFR/BS) was measured which is the product of MAR and MS/BS, representing the annual fractional volume of bone formed per unit bone surface area. Bone surface to bone volume (BS/BV), and bone volume to total volume (BV/TV) were determined as well.

**Osteocyte Count**

The osteocyte count is a measure of bone viability. Ten random fields of transverse bone sections stained with hematoxylin and eosin examined at 400X magnification. The number of lacunae was recorded, as well as the number of lacunae occupied by viable osteocytes. We defined a viable osteocyte as a cell containing a nucleus and having normal cytoplasmic staining. The average amount of filled lacunae from the 10 fields was calculated and presented as a percentage from the total amount of counted lacunae.

**Alkaline Phosphatase Activity**

Osteoblastic activity was determined by alkaline phosphatase activity at 18 weeks survival. A 2 mm bone segment was removed after sacrifice, rinsed with PBS and stored at -80 °C. Each segment was then ground and mixed with alkaline lysis buffer (1.0 ml, 0.75 M 2-amino-2-methyl-1-propanol, pH 10.3) containing p-nitrophenyl phosphate substrate (2 mg/ml). The mixture was incubated 37 °C for 30 min. Next, an equal volume of 50 mM NaOH was added to stop the reaction and the absorbance was measured after dilution of standards and samples with 20 mM NaOH. A Bradford protein assay (Bio-Rad, Hercules, CA) was performed to determine total protein for each sample to allow normalization of the phosphatase activity for total cellular protein (18).

**Statistics**

Results were analyzed using 2-way anova. The Wilcoxon rank sum test was used as appropriate to analyze differences between groups. Correlation between variables was assessed with Spearman’s rank correlation coefficient. Significance was set at \( p < 0.05 \). Statistical analysis was performed with SAS, version 9.1 (SAS Institute Inc, Cary, NC)

**RESULTS**

**AV Bundle Thrombosis**

Thrombosed arteriovenous bundles were found in 15% of the samples and were therefore excluded from analysis. This left, 9, 10, 8, and 7 rats in groups I, II, III, and IV, respectively, at 4 weeks, and 9, 8, 8, and 9 rats at 18 weeks, respectively.
**Bone Blood Flow**

Bone blood flow was 0.39 (± 0.4) ml/min/100 g at 4 weeks and 1.7 (±1.6) ml/min/100 g at 18 weeks in the control group (I). In the VEGF group (III), blood flow was significantly higher than control at both time points with a bone blood flow of 3.84 (±2.0) at 4 weeks and 5.7 (±2.9) at 18 weeks (Fig. 1). Group IV also had significantly higher bone blood flow compared to control at both time points, 3.85 (±2.2) at 4 weeks and 3.8 (±1.8) at 18 weeks. Bone blood flow remained comparatively stable over time in group IV, which was also observed in the FGF-2 group; 2.39 (±1.8) at 4 weeks to 2.5 (±1.7) at 18 weeks. Group II did not show differences compared to control at both time points. Bone blood flow in groups I and III were higher at 18 weeks as compared to 4 weeks, however no significant differences over time were found.

![Bone Blood Flow](image)

**Figure 1.** Bone blood flow at 4 and 18 weeks (ml/min/100 g).

**Capillary Density**

There were no significant differences in capillary density between groups at either time point (Fig. 2). At 4 weeks capillary density in groups I, II, III, and IV were respectively 7.4 (±6.3), 10.8 (±3.6), 9.8 (±6.9), and 8.5 (±6.4). At 18 weeks these values remained statistically unchanged, with mean capillary density measurements of 7.4 (±5.3), 11.3 (±4.2), 7.0 (±5.1), and 10.2 (±7.4), respectively.

**Osteocyte Counts**

At 4 weeks, Osteocyte counts ranged from 0% to 11% (mean: 3.6%), without significant difference between growth factor groups. There was, however, an overall significant increase in osteocyte counts over time with a mean value of 7.7% (range 0–22%) at 18 weeks (p = 0.0002). Group III (VEGF) osteocyte counts increased from 4 to 18 weeks, with mean osteocyte counts improving from 4.5% (±2.5) to 12.5% (±5.9), respectively. In groups II and IV these changes were not significant.
Quantitative Histomorphometry

Histomorphometric results are displayed in Table 1. The percentage of single labeled surface (SLS/BS) overall (all groups combined) decreased significantly over time ($p = 0.004$), while the overall double-labeled surface (DLS/BS) increased significantly ($p = 0.002$). Equally, the mineral apposition rate (MAR), increased significantly over time ($p < 0.0001$). When comparing individual groups, the control group (I) and the VEGF treated group (III) had significantly higher MAR at 18 weeks than at 4 weeks. At 4 weeks, VEGF

![Capillary Density](image)

**Figure 2.** Capillary density at 4 and 18 weeks (%).

**Table 1.** Histomorphometric Results in Mean (±SD). MS/BS, mineralizing surface/bone surface; SLS/BS, single labeled surface/bone surface; DLS/BS, double labeled surface/bone surface; MAR, mineral apposition rate; BFR/BS, bone formation rate/bone surface; BV/TV, bone volume/total volume.

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<th>Group nr</th>
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<th>III</th>
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<td></td>
<td></td>
<td>MS/BS (%)</td>
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Long term analysis of FGF-2 and VEGF in conventional allograft bone

and FGF + VEGF groups had significantly higher MAR compared to control. At 18 weeks, only the VEGF group had a significantly higher MAR. At 4 weeks, mineralized surface/total bone surface (MS/BS) ratio was higher in group III as compared to control, but did not change significantly thereafter. MAR correlated positively with histologic osteocyte counts (Spearman r: 0.4506, p = 0.0003). The bone formation rate/total bone surface (BFR/BS) ratio was higher at 4 weeks in groups III and IV as compared to control, but not at 18 weeks. The control group showed a significant decrease in the bone volume/total volume ratio between 4 and 18 weeks (p = 0.0002), as did the VEGF group (p = 0.02). These groups also had a significant increase in the bone surface/total bone volume ratio over time (p = 0.0002 and p = 0.003, respectively). A comparison between groups at 18 weeks showed the FGF-2, VEGF, and FGF-2 combined with VEGF groups, all had a higher BV/TV ratio than the control group.

Alkaline Phosphatase

Alkaline phosphatase activity was significantly lower in group IV; 302 (±268) than in the control group; 681 (±372). Group III trended towards higher osteoblastic activity; 1,248 (±937), however this did not reach statistical significance. Alkaline phosphatase activity correlated with both osteocyte repopulation (Spearman r: 0.5630, p = 0.0018) and bone blood flow (Spearman r: 0.4088 and p = 0.0308, respectively).

DISCUSSION

Having previously described a benefit of micro-encapsulated VEGF and FGF-2 delivery to revascularize conventional bone allografts at 4 weeks (7), we theorized that either or both cytokines would also increase new bone formation over time. In this study we found that bone blood flow and angiogenesis to have occurred by 4 weeks post-implantation, with no major changes thereafter. Flows greater than control were seen in the VEGF and VEGF + FGF-2 groups, but not FGF-2 alone at 4 weeks. This reflects the period of exposure to the growth factor. Not surprisingly, this effect is sustained but not increased further over time.

Osteocyte counts, a measure of bone viability, increased between 4 and 18 weeks. Osteocyte repopulation in surgically revascularized conventional grafts has been described previously (19). We found that repopulation not only significantly increases over time, but is augmented by exposure to VEGF at the time of surgery. These data suggest a possible role for combined allograft revascularization combined with VEGF administration, and will be the focus of future research.

Osteogenic parameters proved to change over time. Overall, the proportion of DLS/BS to MS/BS increased over time while SLS/BS decreased over time. This portrays an
interesting qualitative and quantitative change in bone formation from 4 to 18 weeks. A relative larger initial contribution of single label represents a broad and slow increasing bone remodeling process at 4 weeks, while at 18 weeks bone formation occurs at more narrowly determined areas, with relatively more double labels present. Additionally, the MAR increased over time as well, illustrating that the rate of bone formation at these more defined sites is significantly enhanced at longer-term periods.

In this heterotopic and non-weight bearing model, a slight decrease in bone volume can be expected, as observed in the control group. However, the growth factor groups did not undergo the net bone loss seen in group I as measured by BV/TV. This can be explained by either a reduction in bone resorption or an increase in bone formation, or both, following growth factor treatment. The overall significant increase of BS/BV (the result of fragmentation of bone by resorption, which increases the bone surface and decreases bone volume) indicates that bone resorption is indeed involved in this process, as was confirmed by observation of the H&E specimens. In group III a significant decrease was observed in BV/TV and a significant decrease in BS/BV from 4 to 18 weeks. VEGF induces bone remodeling by stimulation of chemotactic migration of osteoblasts as well as osteoclasts (20), (21). The changes in BV/TV and BS/BV are likely the result of higher osteoclastic activity, preceding osteoblastic activity. Biomechanical consequences of the effects of growth factors treatment therefore need to be clarified in future research.

Interestingly, osteoblastic activity was positively correlated with histologic graft repopulation as well as cortical bone blood flow. These results confirm that an increase of graft revascularization results in higher osteoblastic activity and resultant increased viability of the grafts.

**VEGF**

This study reveals a strong angiogenic and osteogenic potential of VEGF in revascularized frozen allografts. VEGF treated animals showed an initial higher cortical bone blood flow which persisted long term. Osteocyte repopulation proved to be more extensive in VEGF treated animals at 18 weeks and histomorphometry showed higher bone remodeling. The encapsulation of VEGF in biodegradable microspheres as applied in our transplantation model allows for a controlled and localized delivery of growth factors to frozen allograft and as such increases revascularization, bone remodeling, and viability throughout long term survival periods. This method of delivery offers strong clinical potential. Moreover, the localized and continuous exposure is efficient and allows for smaller amounts of growth factor needed to be effective.

**FGF-2**

FGF-2 demonstrated a minor or insignificant increase of angiogenic and osteogenic parameters when compared to control. FGF-2 is known to be involved in all stages of
Long term analysis of FGF-2 and VEGF in conventional allograft bone osteogenesis, including osteoblast differentiation, maturation, and apoptosis. Nevertheless the exact interactions are not completely understood. FGF-2 overexpression has been described to induce apoptosis of more differentiated osteoblasts in vivo and continuous FGF-2 signaling can initiate apoptosis in mature osteoblasts (22), (23). Furthermore, FGF-2 has a more narrow therapeutical concentration range than other growth factors such as VEGF (21). Additionally, expression of FGF-2 and VEGF during osteoblastic differentiation peak in a sequential pattern, with maximum VEGF expression preceding expression of FGF-2 (24). These findings suggest that prolonged exposure of FGF-2 might interfere and even prevent osteoblastic differentiation (25). Osteoblastic development appears to be more susceptible for dose and time changes of FGF-2 than VEGF. The delivery of FGF-2 with our methodology might have been too extended or out of range, ultimately resulting in apoptosis of mature osteoblasts. This is consistent with group II alkaline phosphatase activity, suggesting osteoblastic activity equivalent to control values. In group IV (FGF-2 + VEGF), the presence of continuous FGF-2 exposure may have significantly lowered osteoblastic activity despite the stimulus of VEGF. Moreover, angiogenic and bone remodeling parameters in group IV were the same or lower than in groups II and III. In the future, microspheres with more ideal release kinetics could possibly evoke a stronger osteogenic response than what we observed.

**FGF-2 + VEGF**

To our knowledge, this research is the first to study potential osteogenic and angiogenic synergism of FGF-2 with VEGF in revascularized necrotic bone allografts. Kano et al described a synergistic effect on angiogenesis by FGF-2 and VEGF in vitro through signaling of endogenous platelet-derived growth factor-B–platelet-derived growth factor receptor β (PDGF-B–PDGFRβ) (26). However, only few dose- and time-response studies for FGF-2 and VEGF have been performed with varying results. Consequently, exact optimal in vivo concentrations for the single or combined delivery of growth factors are difficult to resolve and can only be approximated. Otherwise, the osteogenic response to a certain dose or length of exposure might be different than the angiogenic response in growth factors with multiple functions (27). Synergism of various growth factors has been studied before using diverse delivery methods, with varying and somewhat confusing results. Allograft bone continuously exposed to FGF-2, solely or in combination with BMP-7 resulted in decreased bone formation, perhaps caused by interruption of osteoblastic differentiation due to prolonged FGF-2 exposure (25). Vonau et al (28) demonstrated a similar negative synergistic effect on bone formation when with continuous delivery of FGF-2 with BMP-2, while others conversely found BMP-2 and FGF-2 to improve osteogenesis, with only 6 days exposure (29). Long-term local exposure of bone to combined VEGF and PDGF also evoked a higher angiogenic response than their single delivery (11) as did long term delivery of VEGF and BMP-4 (30).
Angiogenic synergism of FGF-2 and VEGF has been seen in non-osseous tissues both in vivo and in vitro (26), (31), (32). In these studies growth factors were delivered either as a single bolus or continuously during a short-term period (<4 days), as opposed to the prolonged exposure used in this study. This suggests that a narrower dose and time dependent delivery of FGF-2 could be beneficial to induce synergism. Encapsulated in microspheres, the combination of FGF-2 and VEGF did not result in a synergistic response in frozen allograft bone in this study and inhibited osteoblastic activity. To consider clinical applicability of concurrent delivery of multiple growth factors to allograft bone, their interaction and effect on angiogenesis and osteogenesis need to be further clarified, as well as their time and dose dependent properties in in-vivo studies.

**CONCLUSION**

Angiogenic and osteogenic growth factors carry great potential to enhance revascularization and bone remodeling of surgically revascularized frozen bone allografts. We found that VEGF delivered in biodegradable microspheres increases bone vascularization and osteogenesis after long term survival period. Continuous combined delivery of VEGF and FGF-2 did not elicit a synergetic osteogenic or angiogenic response. Future experiments must explore the complex kinetics of cytokine delivery, as well as investigate the potential benefits of revitalized structural allograft bone for reconstruction of segmental bone defects in a large animal model. Such methods may eventually modify current clinical limb salvage practice.
REFERENCES