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Fibroblast Growth Factor-2 and Vascular Endothelial Growth Factor Mediated Augmentation of Angiogenesis and Bone Formation in Vascularized Bone Allografts

Mikko Larsen, MD PhD, Wouter F. Willems, MD, Pelzer Michael, MD PhD, Patricia F. Friedrich, AAS, Mahrokh Dadsetan, PhD[†], and Allen T. Bishop, MD

Department of Orthopedic Surgery, Microvascular Research Laboratory, Mayo Clinic, Rochester, Minnesota

[†]Tissue Engineering and Biomaterials Laboratory, Mayo Clinic, Rochester, Minnesota

Abstract

We previously demonstrated recipient-derived neoangiogenesis to maintain viability of living bone allogeneic transplants without long-term immunosuppression. The effect of cytokine delivery to enhance this process is studied.

Vascularized femur transplantation was performed from DA to PVG rats. Poly(D,L-lactide-co-glycolide) microspheres loaded with buffer (N=11), basic fibroblast growth factor (FGF2) (N=10), vascular endothelial growth factor (VEGF) (N=11), or both (N=11) were inserted intramedullarily alongside a recipient-derived a/v bundle. FK-506 was administered for 2 weeks. At 18 weeks, bone blood flow, microangiography, histologic, histomorphometric and alkaline phosphatase measurements were performed.

Bone blood flow was greater in the combined group than control and VEGF groups (p=0.04). Capillary density was greater in the FGF2 group than in the VEGF and combined groups (p<0.05). Bone viability, growth and alkaline phosphatase activity did not vary significantly between groups.

Neoangiogenesis in vascularized bone allografts is enhanced by angiogenic cytokine delivery, with results using FGF2 that are comparable to isografts from previous studies. Further studies are needed to achieve bone formation similar to isografts.

Introduction

A new alternative in the reconstruction of segmental loss of bone or joint is the use of vascularized (living) bone or composite tissue allografts (CTAs). Maintaining tissue viability in the face of acute and chronic rejection remains a challenge however, requiring long-term immunosuppression or induction of tolerance with attendant significant risks. We have investigated a novel method to enable transplantation of living bone without long-term immune modulation¹⁻³. This is accomplished by placing vascularized recipient tissue in the form of an arteriovenous (a/v) bundle or fascial flap within the bone at the time of

Address Correspondence to: Allen T. Bishop, M.D., Department of Orthopedic Surgery, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905, Phone: (507) 284-4149, Fax: 507-284-5539, bishop.allen@mayo.edu.

Mikko Larsen, M.D., PhD., Department of Plastic and Reconstructive Surgery, Bronovo Hospital and Medisch Centrum Haaglanden, Bronovolaan 5, 2597 AX The Hague, The Netherlands

Patricia F. Friedrich A.A.S., Wouter F. Willems, M.D., Mahrokh Dadsetan, PhD., Department of Orthopedic Surgery, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905

Michael Pelzer, M.D., PhD., Ethionum Klinik Heidelberg, Vosstrasse 6, 69115 Heidelberg, Germany

transplantation, together with microvascular repair of nutrient vessels. Only short-term immune modulation is necessary while neoangiogenesis from host tissue occurs. We have demonstrated maintenance of femoral bone viability using this method^{2,3}, and have evaluated the underlying mechanisms by quantifying angiogenesis and bone blood flow as well as measuring transplant chimerism^{4,5}. We have shown that this method is independent of donor-specific tolerance induction, and is dependent on transplantation of a vascularized transplant, with results comparable to isotransplants¹.

In the current study, we aimed to show long-term enhanced angiogenesis and new bone formation through the administration of biodegradable microspheres encapsulating basic fibroblast growth factor (FGF2), vascular endothelial growth factor (VEGF), or both within the transplanted femur adjacent to the a/v bundle. Previously, short-term augmentation of angiogenesis and bone formation was shown using this method⁶. We hypothesized that growth factor administration onto the a/v bundle would increase neoangiogenesis compared to control, that this would occur in unison with increased bone formation and tissue viability, and that these effects would be maintained on long-term follow-up.

Materials and Methods

Inbred female Dark Agouti (DA) rats (genetic expression: RT1^a, 150-175g) were used as donors in a nonsurvival procedure. Male Piebald Virol Glaxo (PVG) rats (genetic expression: RT1^c, 200-250g) were used as the recipient animals. All animals were obtained from Harlan Sprague Dawley, Madison, WI. Immunosuppression was administered in the form of FK-506 (1mg/kg/day IM) (Tacrolimus, Fujisawa Pharmaceutical Co., Osaka, Japan).

Sterile technique was maintained throughout the procedures. All experiments were performed according to established National Institutes of Health guidelines and under the direction of our Institutional Animal Care and Use Committee. Animals were allowed to move freely in their cages and fed standard rodent feed and water *ad libitum*.

Microsphere manufacture

Poly(D,L-lactide-co-glycolide) (PLGA) microspheres (50:50 lactic to glycolic acid ratio and average molecular weight of 23kD) were manufactured as previously described⁶. The manufacturing parameters were determined by our collaborating laboratory^{7,8} to provide a zero-order drug release for 28 days after an initial burst, on the condition of embedding in a substrate such as a collagen matrix. Microspheres loaded with buffered saline were used for the control group.

Animal Model

The vascular distribution to the bone was provided by proximal and distal nutrient branches arising from the lateral femoral circumflex artery and femoral artery, respectively (Figure 1A). The operative model is shown in Figure 1B.

Operative Procedure

In the donor procedure, the right femur was raised on its nutrient vascular pedicle. The proximal common iliac artery and vein were ligated and cut at their origin, to be used for the microvascular repair. The pedicle was irrigated with heparinized saline, the femoral head and neck and distal femoral condyles were resected and the medullar canal reamed by hand with a 2mm diameter drill bit. Allotransplant sizes varied from 20-22mm in length.

In a PVG recipient, the right femoral artery and vein were ligated at the saphenous origin. Microvascular anastomosis to the transplant vessels was performed in an end-to-end fashion. The transplant was wrapped in a reinforced silicone membrane to prevent angiogenesis from surrounding tissues and placed in an abdominal skin pocket. The left saphenous artery and vein were isolated along with a small distal fascial flap and implanted as an a/v bundle into the medullary canal to run the entire length of the allotransplant. Growth factors were delivered as encapsulated microspheres placed on the a/v bundle. The medullary canal was then filled with 20 μ l of collagen I solution at pH 7.4. Closure of the subcutaneous and skin layers was done using interrupted 4-0 absorbable and nylon sutures and staples.

Experimental Design

Groups were divided based on the growth factor(s) delivered as follows: Group 1 (control, N=11); Group 2 (10 μ g FGF2, N=10), Group 3 (10 μ g VEGF, N=11), and Group 4 (10 μ g FGF2 and 10 μ g VEGF, N=11). All animals received immunosuppression for 14 days, and the time of sacrifice was at 18 weeks.

Bone blood flow measurement: Hydrogen washout

Measurements were performed on all transplants, immediately prior to euthanasia^{9,10}.

Microangiography

Following euthanasia, the abdominal aorta and vena cava were filled with blue microangiographic solution (Microfil, Flow Tech Inc., Carver, MA) under physiologic pressure. The compound was allowed to polymerize for 24 hours at 4°C. Transplants were then fixed in 10% formalin for 24 hours and a 2mm trasverse section for histomorphometric analysis was removed by a diamond-coated circular drill. The transplants were then decalcified in 14% ethylenediaminetetraacetic acid for 7 hours in a calibrated laboratory microwave at 750W (Pelco Biowave 3450 Laboratory Microwave, Ted Pella, Inc., Redding, CA). The intraosseous vasculature was visualized by optical bone clearing^{11,12} and the capillary density (D) of each specimen was measured using image analysis software (Scion Image for Windows 4.0.2): $D = \text{Vessel pixels} / \text{Total pixels}$. The two-dimentional image of the optically cleared specimens was converted into a grey-scale image, from which the aforementioned ratio was calculated (Figure 2). The same observer performed all capillary density measurements, and intra-observer bias was determined by 10 measurements of 10 random samples to be non-significant ($p < 0.05$). Patency of the a/v bundle was documented from the intraluminal presence of the angiographic polymer after sacrifice.

Histologic grading of inflammation and bone necrosis

A 2mm transverse section was cut for histological analysis from the end of each decalcified bone prior to optical clearing. Inflammation was graded (none, mild, moderate, severe) on hematoxylin-eosin (H&E) stained specimens at 400 $\times$ optical magnification (Nikon Eclipse 50i). The grading system was based on previously determined criteria¹³. To measure bone viability, the number of osteocyte-occupied lacunae versus empty lacunae was counted in three randomly selected fields at 400 $\times$. The results were graded: *Grade 0*, normal histology; *Grade 1*, less than half empty lacunae and decreased peritrabecular osteoblast lining; *Grade 2*, more than half empty lacunae and no peritrabecular osteoblast lining; *Grade 3*, severe necrosis with empty lacunae and fragmentation.

Quantitative histomorphometry for bone remodeling

Calcein and tetracycline hydroxychloride were given by perivascular injection near the tail vein (both 20mg/kg) at 2 weeks and 2 days prior to sacrifice, respectively. A semiautomatic image analysis system (Osteomeasure®; Osteometrics, Atlanta, GA) was used for

quantitative histomorphometric assessment of new bone formation and resorption¹⁴. Perimeters of trabecular bone surface, osteoblast-covered surface, eroded surface, and osteoclast-covered surface were measured at the endosteal level and periosteal level at 200× optical magnification. Bone formation rates were calculated by the software package in units of bone volume per surface area measured, per unit of time ($\mu\text{m}^3/\mu\text{m}^2/\text{year}$) and was calculated for each level separately.

Alkaline phosphatase activity

The alkaline phosphatase activity was measured using a kit (Sigma Chemical Co.). Bone samples were rinsed with PBS, harvested, and processed for determining the alkaline phosphatase activity. The activity was normalized to total cellular protein, which was determined by the Bradford protein assay.

Statistical analysis

Continuous data, including bone blood flow, capillary density, histomorphometry and alkaline phosphatase measures were reported as means and standard deviations or medians and ranges, as appropriate. Categorical data, including histology, was described as medians and ranges. Bone blood flow, capillary density, histomorphometry and alkaline phosphatase activity were compared across all four growth factor treatment groups, using analysis of variance or the Kruskal-Wallis test as appropriate. If a significant difference between groups was detected, the Bonferroni multiple comparison test or Wilcoxon rank sum test was used to examine where the groups differed. Histology grades of inflammation and bone viability within the 4 groups were compared using the exact test for ordered contingency tables¹⁵ and cumulative logistic regression (Cochran-Mantel-Haenszel test)¹⁶. All statistical tests were two-sided and the threshold of statistical significance was set at $p < 0.05$. Coefficient of determination was noted as R^2 . Analyses were performed with SAS, version 9.1 (SAS Institute Inc, Cary, NC).

Results

All of the saphenous a/v bundles remained patent until sacrifice. There were no wound healing problems and all animals survived until the sacrifice date.

Results are shown in Table 1. Bone blood flow varied significantly between groups ($p = 0.026$). Significantly more blood flow was found in Group 4 (FGF2 and VEGF) than Groups 1 (control) ($p = 0.004$), and 3 (VEGF) ($p = 0.04$). Group 4 also had more blood flow than Group 2 (FGF2), although this was not significant ($p = 0.131$). No further significant differences between groups were found.

Capillary density varied significantly between groups ($p = 0.017$). Group 2 showed significantly greater capillary density than Groups 3 and 4 ($p < 0.05$). Group 2 also had greater capillary density than Group 1, although this was not significant ($p > 0.05$). Different morphologies are shown in Figure 2.

Bone viability did not vary significantly between groups ($p < 0.001$). Inflammation grade was significantly lower in Group 1 than Groups 3 and 4 ($p < 0.05$).

Neither bone formation in the endosteal plane ($p = 0.138$) or periosteal plane ($p = 0.257$), nor alkaline phosphatase activity ($p = 0.411$) varied significantly between groups.

The only positive and significant correlation between measures of circulation and bone formation was between the alkaline phosphatase activity and bone blood flow values in Group 3 ($R^2 = 0.83$, $p < 0.05$).

Discussion

Microvascular transfer of the rat femur was first described in 1984 based on anatomical dissection and vessel arborization¹⁷. We first described the introduction of a saphenous a/v bundle into the femur allotransplant through an open heterotopic approach^{2,3} and then through a subcutaneous pocket approach¹, the latter resulting in the virtual elimination of wound complications. Orthotopic models of allotransplantation incorporating a superficial inferior epigastric fascial (SIEF) flap with or without a saphenous a/v bundle is possible in larger models such as the rabbit^{18,19}. We have applied this approach in rat knee joint allotransplantation²⁰, using an inferior epigastric and saphenous a/v bundle for the femur and tibia, respectively, and have translated this to a rabbit surgical model of autotransplantation of the knee joint incorporating a SIEF flap for the femur and a saphenous a/v bundle for the tibia²¹.

The combination of DA with PVG strains provided a strong histocompatibility mismatch. The haplotypes of the rat groups used were DA (RT1.AaBa) and PVG (RT1.AcBc), comprising a fully allogeneic MHC mismatch. We previously demonstrated the absence of any donor-specific tolerance in this model, which therefore does not function as an effective vascularized bone marrow transplant (no tolerance is induced by any remaining bone marrow after reaming the medullary canal)¹.

VEGF works synergistically with FGF2 to stimulate angiogenesis in vitro²² and in vivo²³. Vascular remodeling in response to these growth factors is further dependent on arterial sufficiency and nitric oxide production²⁴. Apparent from our four-week follow-up study was a disconnect between greater capillary density and lower blood flow when FGF2 and VEGF were combined⁶. We speculated that the large number of capillaries formed in the combined group may have resulted in relatively lower regional flow rates, when a constant total bone blood flow is apportioned between more vessels⁶. After 18 weeks, however, the opposite is seen: FGF2 combined with VEGF resulted in significantly lower capillary density than FGF2 alone, and similar to VEGF alone, while blood flow values were higher. Thus the discord between capillary density and blood flow remains, but is reversed at some point between 4 and 18 weeks. Presumably the large immature capillary network at 4 weeks is replaced by a more stable system capable of transporting greater volumes of blood per weight of tissue at 18 weeks, comparable to the evolution from an exponential phase of capillary network development to the sigmoidal phase²⁵. However, on closer inspection of the actual values measured, blood flow in the FGF2 with VEGF combined group was the only one to be comparable at 18 weeks to that at 4 weeks. In all other groups, the values were lower at 18 weeks than at 4 weeks. In addition, at 21 weeks, much higher blood flow values were obtained in a group similar to the control groups in the growth factor studies, but without the use of microspheres (4.58 versus 1.8 and 0.22 ml/min/100g for 4- and 18 week follow-up, respectively)¹. That might imply a detrimental effect of the presence of microspheres on the long-term maintenance of bone blood flow. On the other hand, the isograft control group from the same 21-week study had blood flow values comparable to the FGF2 and VEGF groups at 18 weeks (0.44, 0.41, 0.48 ml/min/100g, respectively). Furthermore, the FGF2 group at 18 weeks was the only one to have levels of inflammation comparable to control, levels that were identical to isograft and control groups without microspheres at 21 weeks. The inverse relationship between both inflammation grade and bone viability with capillary density was also apparent from the 21-week study, as has been confirmed again in the current study. This implies that there is a level of chronic rejection in groups 3 and 4, absent in the FGF2 group (2), whereby the latter has maintained blood flow comparable to isograft, significantly greater capillary density, as well increased bone formation and alkaline phosphatase activity (although the latter two measures were not significant). The significantly higher levels of inflammation in Groups 3 and 4 could not be

caused by the byproducts of microsphere degradation as these were the same in amount and type in all groups (lactic and glycolic acid). In addition to this, VEGF administration, has in itself has been shown to enhance inflammatory processes, resulting in more severe inflammation²⁶. FGF2 was associated with higher inflammation levels at 4 weeks than at 18 or 21 weeks, although those values were the same across all four groups at four weeks⁶, and did not vary significantly compared to other studies with four week survival periods where no microspheres had been used². In summary, analysis of the results across the current and previous studies implies a significant role for FGF2 in achieving long-term results in bone allotransplantation that are comparable to isotransplants. Only the bone formation rate with FGF2 at 18 weeks remains 50% of that of the isotransplant at 21 weeks (22.2 versus 50.3 $\mu\text{m}^3/\mu\text{m}^2/\text{year}$). The achievement of the remaining level of bone growth in allotransplants is our goal for future studies.

The combined effect of FGF2 with VEGF could be summarized as showing the increased inflammatory response caused by VEGF, with the positive effect of FGF2 on bone growth, along with the possibly more robust microcirculation mediated by the combination of these two growth factors. As we did not perform essays to qualify the immune response, the increased inflammation seen in the combined group on histologic examination cannot be attributed solely to an immune reaction. As donor-specific tolerance has been previously ruled out as an underlying mechanism in this model¹, there may however be an underlying rejection process that is further enhanced by the combining of these growth factors, with the attenuation of bone viability and the lower levels of bone growth in the FGF2 with VEGF group as a result. The role of vasculogenic cytokines in chronic rejection is still unclear, and studies are focusing on VEGF inhibition as a stratagem in the prevention of chronic renal allograft rejection in rats, for example²⁷.

Previously we had demonstrated that FGF2 provided more bone formation over control, and VEGF more than both control and FGF2 groups at four-week follow-up⁶. New bone formation and alkaline phosphatase activity increased in the wake of stimulation with FGF2 over VEGF with or without FGF2 at 18 weeks, although these results were not significant. Further studies with larger group sizes would be necessary to distill significant differences between groups. A combination of local sustained bone morphogenic protein-2 BMP2 release from microspheres and VEGF from a hydrogel has been shown previously to significantly enhance ectopic bone formation compared to BMP2 alone^{28,29}. Similar studies using microspheres loaded with a combination of vasculogenic and bone morphogenic proteins may provide combined vessel and bone formation rates over control in the long term.

Future studies are also needed to compare different immunosuppression protocols with our model, such as the low dose cyclosporine A (CsA) monotherapy protocol, as has been used successfully in models of bone composite tissue allotransplantation in rats³⁰. This would allow for better control of VEGF release by osteoblasts on exposure to FGF2, which is enhanced by FK506 but not by CsA³¹.

The current study has allowed us to compare this model across different modalities, varying follow-up intervals, allograft versus allotransplant and isotransplant and the use of microspheres carrying combinations of cytokines to mimic the physiological conditions of an isotransplant. On final analysis of current and all previous results, we found FGF2 administration to give long-term results most similar to isotransplant. Further studies are needed that aim to augment the bone formation rate along with other variables, possibly by combining FGF2 with BMP2.

Conclusions

Administration of angiogenic cytokines in vascularized allotransplants positively influences cortical bone blood flow in the long term. Capillary density values diminish as cortical blood flow increases, possibly reflecting maturation of the vascular bed. Further studies are needed in order to determine the relationship between cortical blood flow and capillary density as a function of time.

The complex regulation of bone and vascular tissue genesis and the inflammatory by-effects of cytokine administration must be taken into account when they are to be used to modulate clinical vascularized bone allotransplantation.

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Figure 1.

Figure 1A. Drawing of the rat femoral transplant with (1) common iliac a. & v., (2) ligated superficial iliac circumflex a. & v., (3) lateral femoral circumflex a. & v. with proximal nutrient branch to femur, (4) femoral a. & v. with distal nutrient branch, (5) ligation at the saphenous a. & v. origin.

Figure 1B. Diagram showing the surgical model. The femur diaphysis based on the bone nutrient vessels with microvascular anastomosis in the recipient and a/v bundle implantation using saphenous vessels carefully pulled through the medullar canal.

Figure 2.

Figure 2A. Grey scale conversion of optically cleared bone sample for counting of vessel pixel versus total pixel ratio by image analysis software.

Figure 2B-E. Morphology photographs showing blue Microfil intravascular polymer in representative bone allotransplants after optical clearing using a modified Spalteholz method. Figure 2B: Group 1 sample with 19.0% capillary density, blood flow 0.44ml/min/100g. Figure 2C: Group 2, 32.5% capillary density, blood flow 1.09ml/min/100g. 2D: Group 3, 9.1% capillary density, blood flow 0.12ml/min/100g. 2E: Group 4, 14.9% capillary density, blood flow 3.92ml/min/100g.

Table 1

Results of blood flow, capillary density, inflammation grade, level of bone necrosis, bone formation rate and Alkaline Phosphatase activity.

Group	Number	Blood Flow [ml/ min/100g mean ±SD]	Capillary Density [% mean ±SD]	Inflammation Grade [median(range)]	Level of Bone Necrosis [median(range)]	Bone Formation Rate [$\mu\text{m}^3/\mu\text{m}^2/\text{year}$ mean(range)]	Alkaline Phosphatase activity [units mean(range)]
1 – Control	11	0.22±0.32 [^]	19.5±5.9	0(0-2) [^]	2(1-3)	0(0-0)	1135(238-2121)
2 – FGF2	10	0.41±0.38	30.2±18.0 [^]	1(0-3)	1(1-3)	22.2(0-158.9)	1553(30-6583)
3 – VEGF	11	0.48±0.45 [^]	10.6±11.4 [^]	2(0-3) [^]	1.5(1-3)	1.8(0-7.5)	893(101-2826)
4 – FGF2 and VEGF	11	1.16±1.15 [^]	12.2±8.1 [^]	2(1-3) [^]	2(1-3)	4.9(0-49.2)	791(148-3097)

[^] denotes statistical significance.