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Vascularized Bone Graft Chimerism Mediated by Vascular Endothelial Growth Factor

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Abstract

Background—Vascular Endothelial Growth Factor (VEGF) induces angiogenesis and osteogenesis in bone allotransplants. We aim to determine whether bone remodeling in VEGF-treated bone allotransplants results from repopulation with circulation-derived autogenous cells, or survival of allogenic transplant-derived cells.

Methods—Vascularized femoral bone grafts were transplanted from female Dark Agouti rats (DA;RT1^a) to male Piebald Viral Glaxo (PVG;RT1^c). Arteriovenous bundle implantation and short term immunosuppression were used to maintain cellular viability. VEGF was encapsulated in biodegradable microspheres and delivered intramedullary in the experimental group (n=22). In the control group (n=22), no VEGF was delivered. Rats were sacrificed at 4 or 18 weeks. Laser capture microdissection of bone remodeling areas was performed at the inner and outer cortex. Sex-mismatched genes were quantified with reverse transcription-polymerase chain reaction to determine the amount of male cells to total cells, defined as the relative Expression Ratio (rER).

Results—At 4 weeks rER was significantly higher at the inner cortex in VEGF treated transplants as compared to untreated transplants (0.622 ± 0.225 vs 0.362 ± 0.081 , $p=0.043$). At 4 weeks, the outer cortex in the control group had a significantly higher rER ($p=0.038$), while in the VEGF group, the inner cortex had a higher rER ($p=0.015$). Over time, in the outer cortex the rER significantly increased to 0.634 ± 0.106 at 18 weeks in VEGF treated rats ($p=0.049$). At 18 weeks, the rER was > 0.5 at all cortical areas in both groups.

Conclusion—These in vivo findings suggest a chemotactic effect of intramedullary applied VEGF on recipient derived bone and could imply that more rapid angiogenesis of vascularized allotransplants can be established with microencapsulated VEGF.

Introduction

Restoration of function after segmental bone loss may be accomplished with vascularized bone *autografts* (such as fibula or iliac crest). Cryopreserved structural *allografts* provide close matching of the missing bone size and shape and diminished immunogenicity compared to fresh allografts¹. Devoid of circulation, the remodeling potential of

cryopreserved allograft is less than that of living tissue, resulting in significant risks of stress fracture, nonunion and infection²³. Vascularized bone allotransplants (fresh allogenic bone transplanted with microsurgical vascular repair) potentially offer the superior healing and remodeling properties of vascularized bone autografts, provided circulation is maintained. This is typically enabled by drug immunosuppression, but may also be accomplished experimentally with short-term drug therapy combined with angiogenesis from implanted autogenous vessels⁴⁵. Using these methods, new bone formation, bone healing and remodeling have been observed⁶. Previous studies, using sex-mismatched bone transplantation models and semi-quantitative and quantitative reverse transcription-polymerase chain reaction (rtPCR) analysis on pooled bone tissue for a Y-chromosome marker, have shown that many of the viable cells in the allotransplant are derived from the recipient animal⁷⁸.

Another method to induce angiogenesis and bone remodeling in bone allotransplant is to deliver cytokines locally or systemically⁹¹⁰. Vascular endothelial growth factor (VEGF-A) is a potent growth factor inducing both angiogenesis and osteogenesis¹¹¹²¹³. In this study we aim to describe cell traffic in bone allotransplants following exposure to VEGF-A.

Materials and Methods

Approval from our Institutional Animal Care and Use Committee was obtained and the National Institutes of Health guidelines were followed. We used dual-fluorescent labeling, laser capture microdissection and rtPCR in a sex-mismatched rat allotransplant model (44 female donor rats and 44 male acceptor rats) to acquire quantitative data on intra-graft chimerism (defined as the presence of recipient-derived cells in the graft) at two different time points following local VEGF-A delivery.

Biodegradable Microspheres Preparation

Biodegradable PLGA (Poly D,L-lactide-co-glycolide) microspheres were prepared to encapsulate VEGF-A and allow a sustained exposure of growth factor in the intramedullary canal of the allotransplant¹⁴¹⁵. Using the solid-encapsulation/single-emulsion/solvent extraction method, microspheres were prepared and loaded with VEGF-A¹⁶. In 2.5 µl dichloromethane per mg PLGA (50:50 lactic to glycolic acid ratio and average molecular weight of 23 kilodalton) 1 µl of a 1.0 mg/ml VEGF solution was emulsified with a vortex at 3050 rpm. A double emulsion was created by re-emulsifying the solution in 1% w/v aqueous polyvinyl alcohol (PVA). 0.3% w/v PVA and 2% w/v isopropanol were added and stirred during one hour. Finally, the microspheres were filtered and desiccated overnight and stored at -7 °C.

Transplant Surgery

A previously published heterotopic vascularized bone allotransplantation model was used¹⁷. Femoral diaphyseal segments from female Dark Agouti rats (DA; RT1^a) were transplanted over a major histocompatibility mismatch to male Piebald Viral Glaxo (PVG; RT1^c) rats. Harvest of the femoral segment was performed under ketamine (90 mg/kg IM) and xylazine (10 mg/kg IM) anesthesia. The right femur was dissected including its vascular pedicle.

Prior to transplantation and microsurgical repair, the proximal common iliac artery and vein were ligated. A 20 mm femoral mid-diaphyseal vascularized graft was created by resecting the head and neck and distal femoral condyles. The pedicle was rinsed with heparinized saline and the intramedullary canal was reamed with a 2-mm-diameter drill bit. The recipient male rats were anesthetized with ketamine (90 mg/kg IM) and xylazine (10 mg/kg IM). The right femoral artery and vein were ligated at the saphenous origin while sufficient limb vascularization was maintained due to collateral flow. Microsurgical arterial and venous end-to-end anastomosis was performed. Next, a silicone membrane was used to wrap the graft and the graft was inserted into a subcutaneous abdominal pocket. The silicone membrane was used to prevent vascular ingrowth from adjacent tissue, allowing specific analysis of the effect of growth factor application intramedullary in vascularized bone allograft. The left saphenous artery and vein were dissected and inserted as an arteriovenous bundle into the intramedullary canal of the graft. In group I (control), no growth factor was delivered. In group II, 15 mg of microspheres containing 10 µg of VEGF-A were delivered intramedullary (Figure 1). The microspheres degrade slowly, resulting in a short burst release, followed by a zero order kinetic release of at least 4 weeks¹⁶¹⁸¹⁹²⁰²¹. Subcutaneous tissue was closed with 4.0 absorbable sutures and skin was closed with interrupted 4.0 nylon sutures and staples. The control group contained 22 rats and the VEGF group 22 rats, creating 4 groups in total. Analysis was set at two time points: 4 and 18 weeks to determine short term and long term results. Within each group 11 rats were analyzed at 4 weeks and 11 rats were analyzed at 18 weeks. Selection for VEGF application was performed in a random fashion intra-operatively. Selection for survival period was randomized as well.

Laser Capture Microdissection

Fourteen and two days prior to sacrifice (at 4 weeks or 18 weeks post-transplantation surgery) Calcein and Tetracycline fluorescent labels were administered, respectively. These fluorescent labels are absorbed in active bone remodeling areas, which can be accurately identified microscopically (Figure 2).

At sacrifice surgery, rats were anesthetized with ketamine (90 mg/kg IM) and xylazine (10 mg/kg IM). The vena cava and aorta were cannulated and irrigated with heparinized saline at physiologic pressure. After irrigation, self-polymerizing silicone (Microfil®, Flow Tech Inc., Carver, MA) injection was performed to check for patency of the vasculature. Rats were sacrificed with intraperitoneal injection of pentobarbital sodium (Sleepaway, 200 mg/kg).

Grafts were removed, decalcified and formalin-fixed. Three sections of 5 µm were cut, resin embedded and placed on a 1.35 µm thick PEN (polyethylene naphthalate) membrane metal framed slide (Arcturus Bioscience, Inc., Mountain View, CA). From the PEN membrane, selected areas of bone were imaged with fluorescence microscopy at 200x magnification. Active bone remodeling areas were located between the two fluorescent labels. Three specific areas were collected from the sections using the Veritas Laser Capture Microdissection System (Arcturus^{XT}, Arcturus Bioscience, Inc., Mountain View, CA) (22) and specialized caps (CapSure Macro LCM caps, Arcturus Bioscience, Inc., Mountain View, CA): all active bone remodeling areas (containing fluorescent labels) at the inner

cortex, all active bone remodeling areas (containing fluorescent labels) at the outer cortex and a half circumferential cortical sample to determine overall cell lineage. These samples were retrieved from all animals and analyzed in a blinded fashion. We inspected the captured bone, selecting and removing any adjacent extraosseous tissue or soft tissue at 40 X magnification using the ablation laser.

Quantitative real time Polymerase Chain Reaction

The relative Expression Ratio (rER) ratio of recipient (male) cells to the total number of cells (male + female) was determined with quantitative reverse transcription-polymerase chain reaction (RT-PCR). Stable Proteinase K (PicoPure DNA Extraction Kit, Arcturus Bioscience, Inc., Mountain View, CA) was added to each sample followed by 24 hours of incubation at 65°C to extract DNA. To further purify the extracted product, spin columns (Performa Spin columns - Catalog # 13266, Edge Bio Systems, Gaithersburg, MD) were used. The Performa Gel Filtration Cartridge was prepared by centrifuging at 750×g for 2 minutes and transferring the cartridge to a 1.5 ml microcentrifuge tube. Next the sample was added drop-wise to the center of the packed column and again centrifuged at 750×g for 2 minutes. The eluate was then frozen at -20°.

RT-PCR was performed with Bio-Rad MyiQ Real-Time Instrument and Bio-Rad Sybr Green Super mix (Bio-Rad Laboratories catalog # 170-8880, Hercules, CA. Primer sets for the SRY gene (Sex Determining Region on the Y chromosome) as the gene of interest and cyclophilin, the housekeeper gene were used for RT-PCR in this sex-mismatch transplantation model. *Rattus norvegicus* SRY (NM 012772.1) and cyclophilin (M19533.1) sequences were used. Primer sets were created with Beacon Designer software (Premier Biosoft International, Palo Alto CA.). All sequences were confirmed using the Basic Local Alignment and Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI, Bethesda, MD). SRY primers used were: 5'- GGG ACA ACA ACC TAC ACA CTA TC -3' and 5'-CTG GTG CTG CTG TTT CTG C - 3'. Cyclophilin primers used were 5'- ATC AAA CCA TTC CTT CTG TAG CTC - 3' and 5' - GGA ACC CAA AGA ACT TCA GTG AG - 3'. A Bio-Rad I cycler with a gradient block was used to optimize temperature, primer concentration and DNA concentration. PCR amplicons were run on a 3% agarose gel to confirm proper size. Next, they were extracted and sequenced on an Applied Biosystems Incorporated 3730XL DNA analyzer (Foster City, CA) to confirm product. Quantitative real time PCR reactions were run using the Bio-Rad MyiQ system with sybr green and melt curve analysis. PCR was carried out using the following conditions, (i) 3 minutes denaturation at 95 degrees for 1 cycle, (ii) 15 seconds of denaturation at 95 degrees, 1 minute of annealing and extension at 66 degrees for 51 cycles followed by (iii) generation of a melting curve. Melt curves were performed as follows: (i) 1 minute at 95°C, (ii) 1 minute at 55°C, (iii) 81 repeats at 55°C with reading of fluorescence every 10 seconds. Serial dilutions were run in triplicate for both SRY and Cyclophilin synthetic amplicon, from which a standard curve was calculated with linear regression analysis. Efficiencies were all within 95–103%, and correlation coefficients were all $R^2 > 0.980$.

The data produced by the PCR runs was analyzed with Linereg Software to calculate the efficiency for each individual well ^{23,24}. To calculate the relative Expression Ratio (rER), the Gene Expression Ct Difference (GED) formula as described by Schefe et al was applied ²⁵. The individual efficiencies of amplification for each well with normalization to a reference sample (male control) can be determined.

Three threshold cycle values (C_t1 , C_t2 , C_t3) are acquired from separate amplification products of each gene. This generates three rER values for each specimen, representing a normal distribution. On each real time PCR run female and male control samples were also included in triplicate. In each calculation, the male-only control sample served as the reference sample. Including the individual PCR efficiencies (E), the three relative expression ratios were averaged following the formula:

$$rER = \frac{R_{norm}(SOI)}{R_{norm}(ref)} = \frac{(1+E(HKG))^{CT(HKG;SOI)-CT(HKG;ref)}}{(1+E(GOI))^{CT(GOI;SOI)-CT(GOI;ref)}}$$

R_{norm} is the relative quantity of the Gene of interest (GOI: SRY) to the Housekeeper gene (HKG: Cyclophilin). The calculated rERs for one sample-of-interest (SOI) and the C_t and E values were assumed to be normally distributed, allowing calculation of the mean value and the standard deviation of these rERs. This produces a relative quantification of the amount of male cells to the total amount of cells. A rER of > 0.5 indicates that recipient (male) derived cells are predominant in the cell population, reflecting a high amount of transplant chimerism. A low rER (< 0.5) indicates that the majority of cells are donor derived.

Statistical Analysis

Distribution was tested using the Kolmogorov-Smirnov test. rER variances of selected areas (overall cortex, inner cortex and outer cortex) within each group were analyzed with one-way Anova. Paired t-tests were used for analysis of differences between individual bone forming areas within samples. Differences between the VEGF group and control group and between survival periods were compared with unpaired t- tests. Significance was set at $p < 0.05$. Data is presented as mean ratio with standard deviation.

Results

In all rats the vasculature was patent as confirmed by filling with polymer silicone. Overall Cortical samples had a rER of 0.456 ± 0.266 at 4 weeks in the control group and 0.455 ± 0.260 in VEGF treated grafts. At 18 weeks the overall rER had increased to 0.749 ± 0.387 ($p = 0.07$) in the control group and to 0.637 ± 0.259 ($p = 0.16$) in VEGF treated grafts. No significant changes were observed in overall cortical rER when comparing VEGF treated rats with the control group at both time points.

Results for rER at inner and outer cortical active bone remodeling areas are displayed in table 1. In VEGF treated transplants, rER was significantly higher at the inner cortex at 4 weeks as compared to untreated transplants (0.622 ± 0.225 vs 0.362 ± 0.081 , $p = 0.043$). When comparing separate remodeling areas in each group, at 4 weeks, the outer cortex in the

control group had a significantly higher rER ($p=0.038$), while in the VEGF group, the inner cortex had a higher rER ($p=0.015$). At 18 weeks the rER was higher than 0.5 in all inner and outer cortical areas and no significant differences were found between areas at that time point. When comparing differences over time, VEGF treated grafts had a significantly increased rER at the outer cortex from 4 weeks (0.369 ± 0.182) to 18 weeks: (0.634 ± 0.106 , $p=0.049$).

Discussion

Vascular endothelial growth factor is an essential growth factor in the continuous bone remodeling process. In vivo and in vitro studies have shown that VEGF directly enhances vascular endothelial cell proliferation including vasculogenesis (formation of new blood vessels) and angiogenesis (neovascularization)²⁶. Furthermore VEGF induces differentiation of osteoblasts¹⁴²⁶, chemotactic migration of osteoblasts¹⁵²⁷ and osteogenesis with bone remodeling and callus formation¹⁰¹²²⁸. Extensive research has been performed on the effect of VEGF in parenchymal tissue, while its effect has been studied only to a limited extent in preclinical osseous revascularization studies. Applying VEGF to osseous tissue poses a challenge since VEGF has a short half-life and continuous exposure is necessary to acquire angiogenesis²⁹. Furthermore, VEGF needs to be delivered locally in an appropriate dose to result in a biologic response³⁰.

Several techniques of continuous VEGF delivery with systemic or local exposure have been used to induce bone angiogenesis and osteogenesis, including continuous VEGF infusion and gene transfection³¹³², with variable results. The methodology as used in this paper to deliver VEGF continuously has been applied in the form of slow release microspheres in previous pre-clinical research with success. Karagoz et al applied microsphere encapsulated VEGF to induce axonal sprouting in nerve grafts. Equally, in various tissues subject to ischemia, successful revascularization has been acquired using VEGF loaded microspheres¹⁰³³³⁴. The longstanding exposure to growth factor following the release from slow degradation of the microspheres has therefore proven to result in sufficient neoangiogenesis to overcome stagnation of tissue growth due to avascular conditions.

Prior to clinical implementation of growth factor induced bone revascularization and remodeling, it is necessary to understand the underlying physiology and determine cell lineage within the graft, defined as transplant chimerism⁸. We determined the lineage of cells that were harvested from inner and outer cortical areas where active bone remodeling was verified by fluorescent labeling. We hypothesized that VEGF, delivered intramedullary would increase the relative amount of recipient cells at active bone remodeling areas. To test this hypothesis, we analyzed areas of active cortical bone remodeling at 4 and 18 weeks following allotransplantation and determined the amount of recipient derived cells (male: SRY) to the amount of total cells (male plus female: cyclophilin) present, defined as the relative Expression Ratio. This ratio represents the relative donor to total cells ratio and it is important to note that an increased rER can result from either increased numbers of recipient-derived cells, or a decline in the amount of donor-derived cells, or both.

A limitation of this study is the rat model. Rodents are recognized as feasible models for long bone research³⁵. Rat bone has similarities to human bone, however some structural properties such as composition and mineral density are different from human bone and therefore direct correlation to human bone physiology can not be made³⁶.

Furthermore, the methodology does not differentiate between osteoblasts, osteocytes and osteoclasts. Quantification of these cells separately would provide even more insight into the complicated process of bone remodeling in vascularized allografts. This is the subject of future research in our laboratory. Moreover, it is necessary to determine outcome of bone grafting procedures at even longer term analysis than as applied in this study. Marumatsu studied intragraft chimerism in composite tissue allotransplants in rats up to 24 weeks using semi quantitative techniques⁸. In this sex-mismatch model using adult Piebald Viral Glaxo rats we decided to use 4 weeks survival for short term analysis and 18 weeks survival for long term analysis as has been applied in previous studies using this rat strain¹³. Long term analysis is relevant since possible complications can occur only after many months to years (non-union, graft fracture, graft rejection) and successful long term survival of bone grafts is dependent of many factors. Therefore, larger animal models that allow longer term analysis would contribute to our knowledge of VEGF induced bone remodeling and cell lineage physiology.

In *overall* cortical samples, we found the rER to reflect predominantly donor-derived bone cells at 4 weeks (rER<0.5), becoming mainly recipient-derived at 18 weeks (rER>0.5) in both groups. No difference between control and VEGF application was seen at either 4 weeks or 18 weeks. At the *inner cortical* remodeling areas, however, VEGF administration increased the relative proportion of recipient-derived bone cells as compared to no growth factor controls. These differences are logically explained by the intramedullary placement of VEGF-encapsulated microspheres. We have previously demonstrated VEGF to increase the angiogenic and osteogenic effect of implanted AV bundles³⁷. Thus, the increased angiogenesis and local blood flow provided by VEGF results in repopulation of inner cortical bone with recipient-derived bone cells. At 18 weeks VEGF had no additional effect, and both inner and outer cortical bone contained mainly recipient derived cells (rER>0.5).

Interestingly, we found VEGF treated rats to have a lower rER than controls in the *outer cortex*. This might be explained by a combination of Tacrolimus-mediated survival of donor bone cells at the outer cortex at 4 weeks and osteogenic stimulation of these cells by VEGF. At the same time, recipient-derived revascularization (which is initiated from the intramedullary implanted vascular bundle) has likely not reached the outer cortex at 4 weeks³⁸. Recipient-derived bone cells will therefore not be able to infiltrate the outer cortical area, yet, while at 18 weeks the rER had increased significantly, indicating a higher proportion of recipient derived cells present. At 18 weeks in both inner and outer cortical areas bone cells were largely recipient derived in the control group and in the VEGF group. This finding likely reflects both apoptosis of donor cells no longer protected by Tacrolimus and influx of recipient-derived bone cells.

Only a few prior studies have evaluated cell traffic from the recipient to bone allotransplants (transplant chimerism). Marumatsu et al studied transplant chimerism in bone allotransplants

and composite tissue allotransplants using semi-quantitative techniques⁸. They found that by 24 weeks bone cells in rat bone transplants were in approximately 99 % from recipient origin, implying extensive replacement by recipient derived bone cells. Pelzer et al found that transplant chimerism in bone allotransplants was influenced by both immunosuppressive therapy and arteriovenous bundle implantation (surgical revascularization)⁷. Abstinence of immunosuppression and surgical revascularization with a patent arteriovenous bundle instituted a higher proportion of recipient derived cells, likely due to higher donor cell apoptosis and increased supply of recipient derived bone cells.

Previously, we applied fluorescent labeling, laser capture microdissection and quantitative RT-PCR to study cell lineage in bone autografts and allotransplants and described detailed spatial changes in cell lineage over time³⁹. Vascularized allogenic bone transplantation can offer the unique benefits of vascularized autograft bone graft with strong osteoinductive and osteoconductive properties, while providing good size and shape matching and no donor site morbidity⁴⁰.

Currently, clinical allotransplantation of bone remains experimental, due in part to the significant potential morbidity and considerable expense of the life-long immunosuppression required to maintain tissue viability. Donor-specific tolerance is another method with strong potential to facilitate clinical allotransplantation. Siemionow et al studied induction of tolerance extensively in vascularized composite allografts using various animal models with promising results with improved allograft survival⁴¹.

In bone, an alternative may be to 'switch the circulation' from allogeneic nutrient blood supply, microsurgically-reconstructed and maintained with short-term drug therapy, to a neoangiogenic autogenous blood supply. We have demonstrated the method to maintain blood flow and remodel bone in animal models and have studied the use of growth factors to enhance both blood supply and new bone formation⁵¹³.

In this study, we have examined the lineage of bone cells in areas of new bone formation following allotransplantation. We determined that VEGF accelerates transplant chimerism significantly on bone surfaces close to the areas of autogenous neoangiogenesis in the first weeks following bone allotransplantation. These findings implicate that once microsurgical transplantation of vascularized bone allograft is clinically feasible, combining autogenic derived vasculature with VEGF allows modification of bone cell traffic following transplantation. Further study is needed to quantify the resultant bone remodeling processes in larger animal models, prior to clinical application.

Conclusion

This report presents detailed descriptions of transplant chimerism in vascularized allotransplants following delivery of VEGF. Since the clinical application of growth factors in bone allotransplant surgery will likely increase in future regimens that intend to accelerate graft remodeling, it is necessary to comprehend the physiology behind the complex process of transplant chimerism in detail.

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Figure 1.

(A) A rat model was applied with transplantation of vascularized femoral allograft, intramedullary revascularization and microsphere-encapsulated vascular endothelial growth factor. (B) Cross-section of three cortical segments which were loaded on a PEN membrane slide (Arcturus Bioscience, Inc., Mountain View, CA) and specific areas selected for analysis: I half circumferential, II outer cortical active bone remodeling areas and III inner cortical bone remodeling areas. (C) Active bone remodeling areas were detected by fluorescent labeling and laser captured using the Veritas Laser Capture Microdissection

System (ArcturusXT). (D) Extraction of DNA was performed to determine relative expression ratio (rER) with RT-PCR of the SRY gene and Cyclophilin housekeeper gene.

Figure 2.
Detailed image of outer cortical graft area with active bone remodeling visualized by fluorescent labeling (Calcein Green and Tetracycline) (400X).

Table 1

Results of rER at inner cortical active bone remodeling areas and outer cortical active bone remodeling areas at 4 and 18 weeks, with 44 rats total, each group containing 11 rats. (GF= Growth Factor, VEGF= Vascular endothelial growth factor, rER= relative Expression Ratio)

rER	survival	Bone area	No GF	VEGF	p
	4 weeks	Inner cortex	0.362±0.081	0.622±0.225	0.043
		Outer cortex	0.549±0.184	0.369±0.182	0.023
		p	0.038	0.015	
	18 weeks	Inner cortex	0.532±0.188	0.556±0.281	0.930
		Outer cortex	0.586±0.175	0.634±0.106	0.707
		p	0.750	0.906	