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Cell lineage in vascularized bone transplantation

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Abstract

Background—The biology behind vascularized bone allotransplantation remains largely unknown. We aim to study cell traffic between donor and recipient following bone auto,-and allografting.

Methods—Vascularized bone allografts and isografts were analyzed in a rat model at 4 and 18 weeks. Bone remodeling areas were laser capture microdissected. Analysis by RT-PCR measured the relative Expression Ratio (rER) of donor to recipient cells.

Results—The rER was 0.456 (+/-0.266) at 4 weeks in allotransplants and 0.749 (+/-0.387) at 18 weeks, implying donor bone was gradually repopulated with recipient bone forming cells. In isografts, rER was 0.412 (+/-0.239) at 4 and 0.467 (+/-0.252) at 18 weeks. Cells in the inner and outer cortical bone remodeling areas were mainly donor derived (rER<0.5) at 18 weeks in isografts and mainly recipient derived in allotransplants (rER>0.5).

Conclusion—Applying novel methodology we describe detailed cell traffic in vascularized bone transplants, elaborating our comprehension on bone transplantation.

Introduction

Skeletal reconstruction of large segmental bone defects following trauma, infection, avascular bone necrosis or tumor challenges the reconstructive surgeon. Especially in difficult clinical circumstances, when soft tissue loss and ischemia is abundant, reconstruction with conventional (cryopreserved) graft is susceptible to complications^{1,2,3}. In such cases, vascularized bone autografts are preferably used to optimize revascularization and bone incorporation. However, there are limitations to this technique due to restricted availability from a few expendable sites, suboptimal size and shape match as well as potential for donor site morbidity.

An alternative source is vascularized bone allotransplantation (VBAT), defined as the transplantation of living allogenic bone with microsurgical reconstruction of its nutrient blood supply. A VBAT procured from a donor could combine the desirable healing characteristics of vascularized grafts with the structural stability of cryopreserved allografts. It would further eliminate morbidity, allow close matching of defect size and shape, and possibly maintain the desirable attributes of living autografts. Allotransplants require long-term immune modulation to prevent rejection and maintain transplant viability,⁴. This is problematic, as long term immunosuppressive therapy carries a considerable risk for neoplasm, infection as well as metabolic and toxic side-effects⁵. The search for more

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effective immune modulation protocols applicable for musculoskeletal tissues is promising and continues at present ⁶⁻⁹.

Prior to implementing bone allotransplantation clinically, it is essential to understand the complex underlying biology following the introduction of living donor bone into recipient tissue ⁴. While extensive research has been done on cell movement in parenchymal tissue transplantation, little is known of cell migration of recipient cells into segmental bone allotransplant, defined as intragraft chimerism.

In order to describe intragraft chimerism in detail we apply a new method with laser capture microdissection of accurately selected areas of new bone formation in bone allotransplants. We aim to describe the lineage of cells in allotransplants as compared to isografts and study its progress over time.

Materials and Methods

National Institute of Health guidelines were followed and approval was obtained from our Institutional Animal Care and Use Committee.

Transplantation model

A vascularized bone allotransplantation model previously designed in our laboratory was used (Figure 1A) ¹⁰. Female Dark Agouti rats (DA, RT1^a) served as donors in the allotransplant groups. Female Piebald Viral Glaxo (PVG; RT1^c) served as donors in the isograft groups. Male Piebald Viral Glaxo (PVG; RT1^c) served as recipient rats, providing a major histocompatibility mismatch for the DA donor rats. In the allotransplant group 22 rats were included with survival at two different time points: 4 weeks (group A, n=11) and 18 weeks (group B, n=11). Twenty rats were allocated to the isograft groups with two survival periods: 4 weeks (group C, n=10) and 18 weeks (group D, n=10). Rats were allocated randomly to each group.

The female donor rat was anesthetized with ketamine (90 mg/kg IM) and xylazine (10 mg/kg IM) and the right femur was dissected with its nutrient vascular pedicle including the proximal common iliac artery and vein for later anastomosis. Next, the proximal and distal parts of the femur were resected, leaving a 20 mm femoral diaphyseal segment with its pedicle. The intramedullary canal was reamed and the pedicle rinsed with heparinized saline.

Next a male PVG rat was anesthetized and the right femoral artery and vein were ligated. End to end anastomosis was performed. The contralateral saphenous arteriovenous bundle was dissected and implanted into the full length of the donor bone intramedullary canal. The allotransplant was wrapped in a silicone sheath and placed in an abdominal subcutaneous pocket.

Rats in all groups received daily intramuscular injections of FK-506 (1 mg/kg/day IM) (Tacrolimus, Fujisawa Pharmaceutical Co., Osaka, Japan) during the first two weeks postoperatively.

Animals were given calcein green and tetracycline orange fluorescent labels 14 and 2 days, respectively, prior to sacrifice. These labels are absorbed in active bone remodeling areas, which allow clear microscopic identification of these areas (Figure 1B). Rats were anesthetized with ketamine (90 mg/kg IM) and xylazine (10 mg/kg IM). To ensure that cortical bone was completely cleared from blood cells that could interfere with accurate cell lineage quantification, the vena cava and aorta were cannulated and the lower extremity

irrigated with heparinized saline. Next, the lower extremity was irrigated with a blue polymer silicone solution (Microfil, Flow Tech Inc., Carver, MA), in order to determine vascular patency. Animals were euthanized with an intraperitoneal injection of Sleepaway (pentobarbital sodium) at a dose of 200 mg/kg.

Laser capture microdissection

A 2 mm sample of the transplant was removed, decalcified and formalin-fixed. Three resin embedded 5 µm sections were cut and placed on a 1.35 µm thick PEN (polyethylene naphthalate) membrane metal framed slide (Arcturus Bioscience, Inc., Mountain View, CA) (Figure 1B). The membrane slide was then placed in the Veritas Laser Capture Microdissection System (Arcturus^{XT})¹¹. From one section, a half circumferential cortical sample was selected and laser cut (Figure 1C). From the two remaining sections, active bone forming areas, identified by fluorescent labels, were selected at 200 x magnification and laser cut. Separately, areas located from the inner (endosteal) border of the transplant and areas from the outer cortex (periosteal) were selected. This provided three different samples: overall cortical (C) bone, inner (I) active bone remodeling areas and outer (O) active bone remodeling areas.

The bone samples were captured on a specialized cap (CapSure Macro LCM caps, Arcturus Bioscience, Inc., Mountain View, CA). To prevent any soft tissue to be included after capturing, the bone samples were inspected at 40 x magnification for any adherent extraosseous tissue as well as capillary tissue, which were removed with the Ablation Laser.

Quantitative real time Polymerase Chain Reaction

DNA was extracted from the sample with stable Proteinase K (PicoPure DNA Extraction Kit, Arcturus Bioscience, Inc., Mountain View, CA) and 24 hours of incubation at 65°C (Figure 1D). Spin columns (Performa Spin columns - Catalog # 13266, Edge Bio Systems, Gaithersburg, MD) were used to further purify the extracted product, which averaged 21.1 ng/µl DNA. This procedure involved preparing the Performa Gel Filtration Cartridge by centrifuging at 750 x g for 2 minutes and then transferring the cartridge to a 1.5 ml microcentrifuge tube. Afterwards, the sample was added drop-wise to the center of the packed column and centrifuged again for 2 minutes at 750 x g. The eluate was retained and frozen in a -20° C freezer for further evaluation. Real time reverse transcription-polymerase chain reaction (RT-PCR) was performed using a Bio-Rad MyiQ Real-Time Instrument (description) and Bio-Rad Sybr Green Super mix (Bio-Rad Laboratories catalog # 170-8880, Hercules, CA.). RT-PCR was carried out using primer sets for the SRY gene (Sex Determining Region on the Y chromosome) as the gene of interest and Cyclophilin, a commonly used housekeeper gene. The SRY gene is used in sex-mismatched transplantation models to detect recipient or donor specific cells. Sequences used were *Rattus norvegicus* Sry (NM 012772.1) and Cyclophilin (M19533.1). Primer sets were designed using 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'.

Temperature, primer concentration and DNA concentration were optimized using a Bio-Rad I cycler with a gradient block. PCR amplicons were run on a 3% agarose gel to confirm proper size. They were then extracted and sequenced on an Applied Biosystems Incorporated 3730XL DNA analyzer (Foster City, CA) to confirm product. Quantitative real time PCR reactions were then 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 using linear regression analysis. Efficiencies were all within 95-103%, and correlation coefficients were all $R^2 > 0.980$

Data analysis

The raw data from the PCR runs as produced by the MyiQ Real-Time instrument and program was transferred to Linereg Software to calculate the efficiency for each individual well ¹²¹³. The Gene Expression Ct Difference (GED) formula according to Schefe was used to calculate the relative Expression Ratio (rER) ¹⁴. This method determines the individual efficiencies of amplification for each well while allowing for normalization to a reference sample (male control).

Three threshold cycle values (C_t1 , C_t2 , C_t3) were obtained from separate amplification products of each gene. This produces three rER values for each specimen, which represents 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 according to the formula:

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

This formula represents R_{norm} as 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) were assumed to be part of a normal distribution (as the C_t and E values are), which allows 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 approximating 1.0 signifies a majority of recipient (male) derived cell population, which reflects a high amount of intragraft chimerism. A low rER (< 0.5) represents minor intragraft chimerism with a majority of donor (female) bone cells present.

Statistics

The Kolmogorov-Smirnov test was used to analyze for normal distribution. One-way Anova was used as appropriate to analyze rER variances of areas (I, O and C) within each survival group. Differences between individual bone forming areas within samples were analyzed with paired *t*-tests. Differences between isografts and allografts and between survival periods were compared with unpaired *t*-tests. Data is presented as mean ratio with standard deviation ($\pm$ SD). Significance is set at $p < 0.05$.

Results

In all animals, the pedicle was patent at inspection of polymer filling of the vasculature.

The rER in allografts at 4 weeks (A) was 0.456 ($\pm$ 0.266) in the overall cortical area, while it was slightly higher at the outer cortex; 0.549 ($\pm$ 0.184) and lower at the inner

cortex; 0.362 (+/- 0.081). The rER at 18 weeks (group B) had increased in all areas, with an overall cortical rER of 0.749 (+/- 0.387), however this difference did not reach significance ($p=0.07$). The rER at the inner cortex at 18 weeks was 0.532 (+/- 0.188), at the outer cortex 0.586 (+/- 0.175).

In the isograft group at 4 weeks (group C), the overall cortical rER was 0.412 (+/- 0.239). The inner cortex had a rER of 0.398 (+/- 0.241), while at the outer cortex the rER was 0.247 (+/- 0.181). At 18 weeks in isografts (group D) the overall rER was slightly higher 0.467 (+/- 0.252) than group C (not significant), with an inner cortex rER of 0.356 (+/- 0.113) and an outer cortex rER of 0.392 (+/- 0.229).

The short term survival groups (A and C) had a comparatively equal overall cortical rER. At 18 weeks the rER was higher in allografts (group B) as compared to the isografts (group D), however no statistical significant difference was found ($p=0.09$). At the outer cortical areas the rER was significantly lower at 4 weeks in isografts as compared to allografts ($p<0.05$). This difference at the outer cortex was not found at 18 weeks. In the allograft group, a slight increase over time was found at the inner cortex, while in isografts the rER remained lower than 0.5 over time with a majority of cells of donor origin.

Discussion

For successful incorporation and optimal biological properties of bone grafts, remodeling is a prerequisite. To understand the biology behind this process, knowledge of cellular heritage and the movement of cells in the transplant over time is essential. We applied a sex mismatch rat model that has been used successfully in our previous bone transplantation research^{15,16,17}. This transplantation model allows the study of cell lineage with quantitative RT-PCR on the Sry and cyclophilin housekeeper genes to detect the relative amount of recipient cells to donor cells within the transplant. Laser capture microdissection facilitates highly selective harvest of tissue, without contamination of adjacent soft tissue including capillary tissue. In this study, we describe a new technique to select specific bone remodeling areas, identified by fluorescent labels so that the lineage of cells within cortical bone and cells in specific inner and outer cortical active bone forming areas can be determined. To our knowledge, such detailed description of bone intragraft chimerism has not been accomplished before. These methods can be applied in future research to study the effect of transplant enhancement techniques or various immunosuppressive regimens on intragraft chimerism.

Pelzer et al. determined the overall lineage of cells in transplants treated with short-term immunosuppression and donor-derived neoangiogenesis.¹⁵ Their study describes the effect of short-term immunosuppression (2 weeks), resulting in a lower percentage of cells of recipient lineage present in the donor transplant in short-term immunosuppressed rats as compared to nonimmunosuppressed rats, due to protection of donor cells from rejection. In this study, therefore, a higher rER would be expected in allografts if no immunosuppression was administered leading to increased rejection of donor cells. Conversely, a lower rER might be expected if even longer term immunosuppression was used. With intramedullary arteriovenous bundle implantation, the rER increases, likely due to a higher supply of recipient-derived bone forming cells and increased immunogenic exposure resulting in donor cell death and a relatively higher amount of recipient cells present.¹⁵

In this study we describe the progress of intragraft chimerism within specific areas and compare this with cell lineage as it would occur in autogenous transplantation. The fact that

the allotransplant is repopulated rapidly with almost half of the cells of recipient origin at 4 weeks, increasing to 3/4th of the recipient cells at 18 weeks, proves that intragraft chimerism is a rapid process in vascularized allotransplants. This extend of chimerism at 18 weeks was also found by Pelzer et al, who describes 81% of bone cells in immunosuppressed allotransplants to be recipient derived at 18 weeks¹⁵. Equally, Muramatsu et al determined allotransplant cell lineage in rats with semi-quantitative PCR techniques and found that by 24 weeks approximately 90% of fresh allotransplant bone had been repopulated by recipient cells¹⁷. Despite the dimensional differences between rat and human bone, the rate of bone remodeling has been found to be comparable between rodent and human bone¹⁸. Therefore, these high rates of transplant chimerism could be translated to human bone transplant biology.

In this study, a short-term (2 weeks) course of Tacrolimus was administered since the combined use of 2 weeks immunosuppression with donor-derived neoangiogenesis has proven to sustain bone blood flow and bone transplant viability long term.^{10,19}. This may be explained in part by the neoangiogenic circulation and resulting influx of donor-derived cells repopulating the bone. After the initial 2-week immunosuppression, immune competence also gradually improves. Thus, a number of factors may explain differences in rER seen between iso- and allotransplant groups, including a more rapid influx of recipient derived cells, and allotransplant cell death from transient ischemia, immune-mediated cell injury or both. The rER was significantly higher in allotransplant outer cortical bone than in the isotransplant group. Any such difference would be the result of immune differences, as the groups were otherwise identical. Both increased influx of recipient derived cells and lower surviving number of allotransplanted cells are possible explanations.

At 18 weeks, this process continued, with marked differences observed between allo- and isotransplanted bone. The rapid repopulation process in allotransplants is further illustrated by the higher amount of recipient cells at 18 weeks in allotransplants as compared to isotransplants, in which no immunogenic response is elicited and the rER had only slightly increased to 0.47 at 18 weeks.

Interestingly, the repopulation of isotransplant bone has progressed considerably at 4 weeks (0.41) but has increased only slightly long term (0.47). This implies that in autotransplants, there is rapid repopulation by recipient cells initially while at later time points this does not increase significantly. This could be explained by the fact that no immune response is elicited and the transplant's cells are not subject to rejection and can still contribute to bone remodeling at 18 weeks.

Cell heritage within active bone remodeling areas provides us with a valuable insight into the contribution of donor or recipient derived cells to bone formation within a vascularized allotransplant or autotransplant. Cells in the inner cortical and outer cortical bone remodeling areas were mainly donor derived (rER<0.50) at 18 weeks in isotransplants, while in allotransplants these were mainly recipient derived (rER>0.50). When considering different bone remodeling areas in isotransplants we found that the rER was lower at the outer cortex than at the inner cortex at 4 weeks, while at 18 weeks the rER had increased at the outer cortex up to values equal to that of the inner cortex. This implies that in vascularized isotransplants in this model, the bone remodeling process is initially mainly carried by cells that are transplant derived (rER< 0.50). However, at 4 weeks intragraft chimerism is already fairly active at the inner cortex (rER 0.398), where recipient derived cells have already infiltrated the cortical remodeling process. At the outer cortex (rER of 0.247 at 4 weeks), recipient derived cells are not yet predominant, likely due to less revascularization at the outer cortex and therefore limited provision of recipient derived cells at the outer cortical areas. At longer term analysis, as revascularization and invasion of

recipient derived cells increases, outer cortical transplant chimerism has reached values equal to the inner cortex.

When comparing bone-remodeling areas within allotransplants, no significant changes were found. However, at 4 weeks, the lower inner cortical rER is noted, while at 18 weeks the inner cortex had reached values equal to the outer cortex, suggesting slight progress of transplant chimerism at the inner cortex, and a more stable situation at the outer cortex.

This study has several limitations: Bone remodeling increases once it has been subjected to weight bearing and bone-to-bone contact.^{20,21} In this study, a heterotopic model was used in which bone remodeling was identified by fluorescent labeling, and bone remodeling was observed in all samples. In future research, we aim to apply orthotopic transplantation in larger animal models to determine iso- and allograft cell lineage, which will be one step further toward the study of physiologic cell lineage.

Second, bone remodeling areas contain osteoblasts, osteocytes, and osteoclasts. We did not determine specific cell amounts or biologic activity as it was the aim of this study to determine the overall lineage of cells in specific bone remodeling areas using a new technique to selectively acquire fluorescent labeled bone remodeling areas with the laser capture microdissection procedure. However, it will be interesting to correlate quantitative bone remodeling data in selected cortical remodeling areas with cell lineage data in future research. Furthermore, the effect of Tacrolimus immunosuppression on bone remodeling has been studied by Voggenreiter et al. in a fracture model.²² They found no significant effects of Tacrolimus on biomechanical or histological properties. However, they did observe increased bone remodeling with net bone loss in trabecular bone. While remodeling was observed throughout the cortex in both isotransplants and allotransplants in this study, the effect of Tacrolimus on bone remodeling was not quantified.

Third, while little significant differences were found in this study, likely due to a small number of animals per group, we do present interesting descriptive data of cell heritage within selected bone forming areas. In future research, we will therefore use larger groups with longer term analysis to acquire further insight into bone transplantation cell lineage.

Conclusion

We describe the cell lineage within vascularized isotransplants and allotransplants accurately with a new combination of methodology consisting of fluorescent labeling, selective laser capture microdissection and quantitative RT-PCR. The changes over time and differences between remodeling areas offer a distinct insight into cellular movement within the transplant and provide more knowledge of bone transplanting biology and transplant chimerism specifically.

Acknowledgments

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Figure 1.

A) Rat model with transplantation of vascularized femoral allograft and intramedullary revascularization. B) Overview and cross section of cortical segments that are loaded on a PEN membrane slide (Arcturus Bioscience, Inc., Mountain View, CA). C) Active bone forming areas (fluorescent green and orange labeled) are laser captured with the Veritas Laser Capture Microdissection System (Arcturus^{XT}). D) relative expression ratio (rER) was determined with RT-PCR of the SRY gene and Cyclophilin housekeeper gene.

Table 1

relative Expression Ratio, presented in ratio means with standard deviation

rER					
			Overall Cortex	Outer Cortex	Inner Cortex
group	transplant type	survival (wks)			
A	allotransplant	4	0.456 (+/- 0.266)	0.549 (+/- 0.184)	0.362 (+/- 0.081)
B	allotransplant	18	0.749 (+/- 0.387)	0.586 (+/- 0.175)	0.532 (+/- 0.188)
C	isotransplant	4	0.412 (+/- 0.239)	0.247 (+/- 0.181)	0.398 (+/- 0.241)
D	isotransplant	18	0.467 (+/- 0.252)	0.392 (+/- 0.229)	0.356 (+/- 0.113)