In Vivo Activity of Genetically Modified Cells Preseeded in Rat Vascularized Composite Allografts

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ABSTRACT

Objective. Transplantation of the hand or face, known as vascularized composite allotransplantation (VCA), has revolutionized reconstructive surgery. Notwithstanding, there are still several areas of improvement to mitigate immune rejection while sparing systemic adverse effects. The goal of this study was to evaluate the engraftment and viability of a genetically modified cell population pre-engrafted into a VCA transplant, to potentially act as a local biosensor to report and modify the graft in vivo. A rat fibroblast cell line genetically modified to secrete Gaussia-Luciferase (gLuc), which served as a constitutive biomarker of cells, was incorporated into a VCA to study the viability of biosensor cells in a syngeneic rat heterotopic partial hindlimb transplantation model.

Results. Five perfusions were first performed as engineering runs to have a stable limb perfusion protocol, followed by 3 perfusions to analyze the cell engraftment during machine perfusion, and finally 4 perfusions to study in vivo persistence of the cell biosensors. Blood samples were collected to monitor gLuc secretion during perfusion and postoperatively. A time-dependent increase in gLuc secretion in the limb perfusion outflow during machine perfusion indirectly verified the presence of biosensors within the graft. After the ex vivo perfusion, VCA hindlimbs were analyzed for near infrared fluorescence emission that showed a presence of dyed engineered cells in all areas of the limbs. Postoperatively, gLuc was detectable 4 to 5 days after transplantation ($W = 16$, $P = .02857$). This study demonstrated that engineered cells could be successfully preimplanted into VCAs—an important step toward development of an in vivo biosensor platform to use in modulating acute VCA outcomes.

VASCULARIZED composite allotransplantation (VCA) is an established option for reconstruction of complex injuries that are inadequately addressed by autologous surgery [1,2]. However, VCA is often not performed [3] due to a number of factors: 1) a risk of immunologic rejection (a risk considered higher than in solid organ transplantation due to the immunogenicity of the skin [4]), 2) the need for aggressive immunosuppressive drug regimens with concomitant side effects, and 3) the potential for

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long term graft loss [5,6] secondary to chronic rejection. Furthermore, the monitoring [7] and management of early VCA rejection lacks specific clinical and biological markers [8,9]. Currently, only gross observations and histological features can confirm a rejection diagnosis [10] and grade using the BANFF classification [11].

This pilot study evaluated a new approach to potentially monitor and modulate VCA rejection by using a genetically-modified cell biosensor to release a protein biomarker in VCA. The primary objective was to verify if biosensors were viable in VCA grafts after transplantation. Machine perfusion, a promising approach for organ preservation [12−14], was used as an ex vivo platform to engraft these genetically modified cells during VCA preparation. As recently described by our group, bioengineered cells can be successfully infused into a liver ex vivo using machine perfusion along with methods to evaluate their engraftment properties [15] but no in vivo attempts were evaluated thus far. Here, we report the use of cell sensors in the preparation of a VCA along with in vivo detection of cell sensors after heterotopic transplantation in a syngeneic rat model.

MATERIAL AND METHODS

Animals

All animals were kept in accordance with the National Research Council guidelines. The experimental protocol was approved by the Institutional Animal Care and Use Committee, Massachusetts General Hospital. We used 350g to 400g syngeneic male Lewis rats (Charles River Laboratories, Boston, Mass, United States).

Genetically Modified Cell Preparation

Allogeneic rat fibroblasts were sourced from the American Type Culture Collection (Rat2 ATCC CRL-1764; ATCC, Manassas, Va, United States). These cells were engineered via lentiviral transduction to secrete Gaussia-Luciferase (gLuc), as previously described [15], and purified by fluorescence activated cell sorting (FACS) sorting based on the expression of green fluorescent protein. Cells were frozen and used immediately post thaw to simulate an organ transplant setting. Ten million cells were preseeded into the limb through machine perfusion before transplantation.

Procurement

Donor right partial hindlimbs were procured during terminal procedures in a sterile manner as described previously [16]. Five minutes after intravenous infusion of 300 U of heparin, the hindlimb was isolated. The femoral pedicle was cannulated with 24-gauge angiocatheters and flushed with 10 mL of 100 U/mL heparin saline using a pressure monitor to avoid vessel injuries.

Machine Perfusion

The hindlimb was connected to a subnormothermic machine perfusion through the femoral artery and perfused with our preservation media [17]. We realized 12 limb perfusions: 5 perfusions to optimize the protocol, 3 to analyze the cell engraftment during machine perfusion, and eventually 4 limbs perfusions that were transplanted. Ten million bioengineered fibroblasts were added to the media and the perfusion was carried on for a duration of 3 hours. The perfusion flow rate was adjusted for a target pressure of 25 to 35 mm Hg. Venous outflow was collected at 0, 30, 60, 120, and 180 minutes, and analyzed for gLuc.

In Vivo Transplantation

Three animals were then transplanted with VCA in a heterotopic manner. The femoral vessels of the syngeneic recipient rat were dissected out [16] and were ready for transplantation after 3 hours of perfusion. The hindlimb was flushed with heparin saline, and vascular anastomosis was performed under microscope with 10/0 Ethilon (Ethicon, Somerville, NJ). The partial hindlimb was then sutured in heterotopic position in the left flank. Animals were monitored for pain and distress and treated accordingly.

gLuc Assay

Blood samples were collected from the lateral tail vein daily during the first week. Plasma was isolated and stored frozen. A gLuc luminescence assay was performed using its substrate, coelenterazine (NanoLight Technology, Arizona, USA) [15].

Fibroblast Ex Vivo Imaging

Fibroblasts were marked with a near infrared fluorescent membrane dye as previously described [15]. Ex vivo near infrared fluorescence imaging was used to visualize cell engraftment and distribution in the VCA immediately after machine perfusion.

End of Study

Animals were kept alive for more than 1 month to ensure good viability of the transplants, and at the end of study vascular anastomosis patency was confirmed, skin and muscle biopsy specimens were taken, and animals were euthanized.

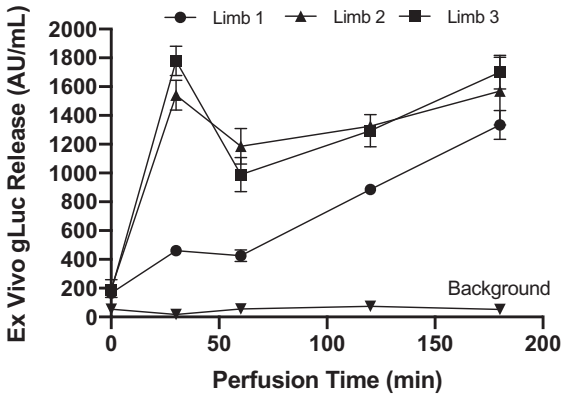

Fig 1. Bioengineered cell engraftment during vascularized composite allotransplantation machine perfusion: Gaussia-Luciferase (gLuc) secretion increase with time of perfusion. Samples were collected in the venous outflow of rat hindlimbs during 3-hour time machine perfusion after addition of 10 million genetically modified fibroblasts at time 0. Three different hindlimb perfusions were performed with cell engraftment and one without cell engraftment that appears as the control on the graph.

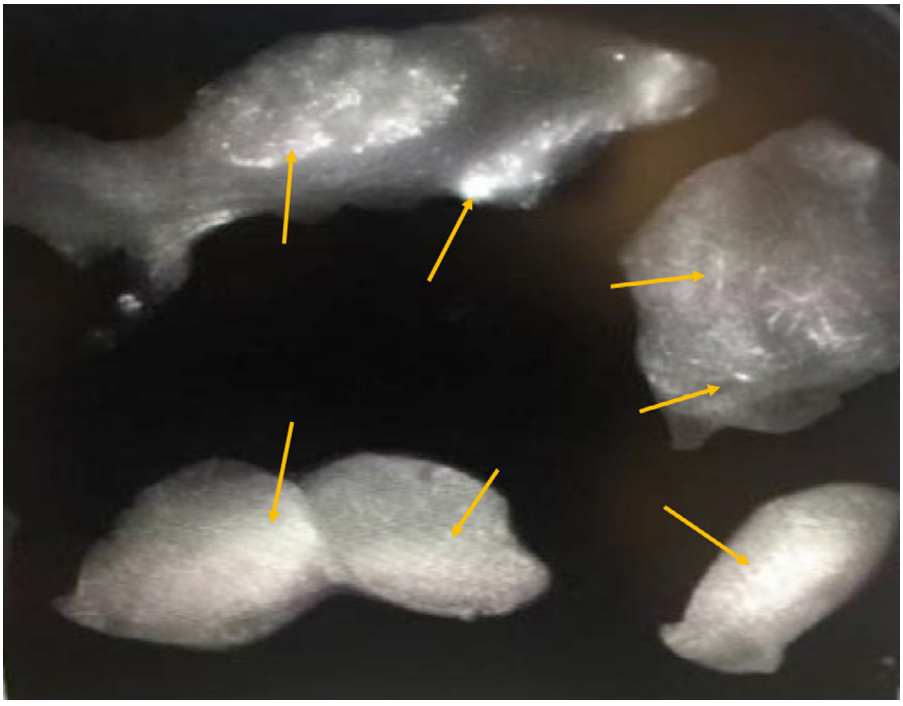

Fig 2. Bioengineered cells successfully engrafted in whole vascularized composite allotransplantation after machine perfusion. Near infrared fluorescence imaging of sliced rat hindlimb immediately after 3-hour machine perfusion showing a global distribution of dyed fibroblasts. Arrows are pointing to different highly fluorescent spots present in every slice representing various clusters of dyed cells.

Statistical Analysis

Statistical analysis was performed using R software (3.3.1 version; The R Foundation, Vienna, Austria, available online). To calculate if the detection of gLuc in the transplanted rats serum was statistically different compared with naive rats from the herd, we used a Wilcoxon rank sum because of the small number of subjects.

RESULTS

Allogeneic rat fibroblasts were genetically engineered to express a secreted luciferase (gLuc) as a simple biomarker to indirectly assess viability of cell sensors within an organ by measurement of circulating fluids. Allogeneic cells were used to simulate the clinical scenario where an "off-the-shelf" cell therapy could be available in a frozen format for rapid use in transplant settings. Five VCA perfusions were performed to develop a process for engineered fibroblast engraftment that began to show detectable gLuc in the perfusate before conducting a protocol-driven study (data not shown).

Upon developing a stable engraftment process for biosensors, a protocol-driven perfusion study was performed with 3 VCA grafts with cell sensors. gLuc was detected in the venous outflow with an initial spike at 30 minutes after cell perfusion and then a linear increase of expression during the duration of the perfusion (Fig 1). The biosensor cells also were labeled with an intracellular, near infrared dye for noninvasive tracking after ex vivo perfusion. All VCA hindlimbs were analyzed for near infrared fluorescence emission and showed presence of dyed engineered cells distributed across the limbs (Fig 2).

Finally, 4 VCA transplantations performed were successful as evidenced by viable transplanted limbs and absence of

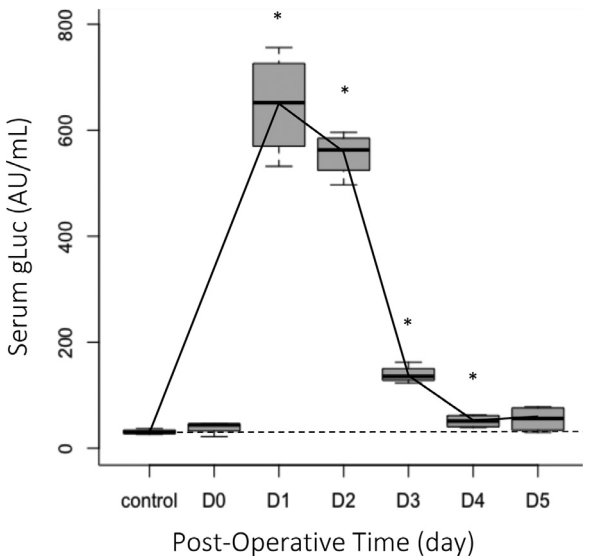

Fig 3. In vivo activity of genetically modified preseeded fibroblasts detectable up to 4 days after transplantation. gLuc luminescence assay performed on postoperative plasma showing the presence of plasmatic expression of gLuc 96 to 120 hours after transplantation. The control is the gLuc expression found in naive rats from the same herd that were not transplanted, nor had genetically modified fibroblasts infusion. The asterisks represent statistically significant differences ($P < .05$) between the gLuc expression on day 1 compared with the control values using a Wilcoxon rank sum test. D0, day of transplantation immediately after surgery; Dx, x day after surgery; gLuc, Gaussia-Luciferase.

wound dehiscence up to euthanasia. All animals were healthy after surgery and underwent a similar postoperative history as previously experienced on this surgical model. Postoperatively, gLuc was detectable during the first several days after transplantation and then became undetectable by approximately day 5 (Fig 3). During the first 4 days after transplantation gLuc expression was significantly different from control rat serum ($W = 16$, $P = .02857$), and at day 5 after transplantation there is no more difference from the control animals ($W = 14$, $P = .1143$). The viability of the VCA was good at the end of the study as noted by gross and microscopic histology (4 weeks postoperative; data not shown).

DISCUSSION

This study evaluated the engraftment of genetically engineered cells in an organ before transplantation while tracking transient cell engraftment and function in vivo. Our results demonstrate that genetically modified cells are viable and detectable after perfusion into organs and transplantation. However, the release of gLuc was transient, which suggests that genetically modified fibroblasts may not have survived or the expression of gLuc was lost in vivo. Potential causes of transient expression include the use of allogeneic cells without immunosuppressive therapy leading to rejection of engineered cells, or apoptosis of cells due to ischemic or reperfusion injury on transplantation, and antibody neutralization of gLuc in rodents. Further studies to explore the mechanism of transient expression and improve the signal for longer-term persistence are warranted. This research establishes that an engineered cell population can remain viable in vivo to potentially help the acute detection and management of VCA rejection.

This pilot study shows early verification data that engineered cells can be preimplanted into VCA, with the aid of machine perfusion, and be tracked after transplantation. The power of the study was limited to minimize animal usage while trying to detect dramatic outcomes in cell viability, though further validation with a greater number of animals and control groups is warranted. The duration of the genetically modified cells engraftment in the limb has to be increased to gain clinical relevance because the objective would be to create a cell line that would ideally survive for at least the first few months, when the rejection episodes are more often and severe, or even better for years.

CONCLUSIONS

This study demonstrates that engineered cells can be preimplanted into VCA, with the aid of machine perfusion, and be tracked after transplantation as a first step toward developing biosensor cells for the early detection and treatment of rejection episodes in VCA.

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