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Decellularized skin/adipose tissue flap matrix for engineering vascularized composite soft tissue flaps ☆

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ABSTRACT

Using a perfusion decellularization protocol, we developed a decellularized skin/adipose tissue flap (DSAF) comprising extracellular matrix (ECM) and intact vasculature. Our DSAF had a dominant vascular pedicle, microcirculatory vascularity, and a sensory nerve network and retained three-dimensional (3D) nanofibrous structures well. DSAF, which was composed of collagen and laminin with well-preserved growth factors (e.g., vascular endothelial growth factor, basic fibroblast growth factor), was successfully repopulated with human adipose-derived stem cells (hASCs) and human umbilical vein endothelial cells (HUVECs), which integrated with DSAF and formed 3D aggregates and vessel-like structures *in vitro*. We used microsurgery techniques to re-anastomose the recellularized DSAF into nude rats. *In vivo*, the engineered flap construct underwent neovascularization and constructive remodeling, which was characterized by the predominant infiltration of M2 macrophages and significant adipose tissue formation at 3 months postoperatively. Our results indicate that DSAF co-cultured with hASCs and HUVECs is a promising platform for vascularized soft tissue flap engineering. This platform is not limited by the flap size, as the entire construct can be immediately perfused by the recellularized vascular network following simple re-integration into the host using conventional microsurgical techniques.

Statement of Significance

Significant soft tissue loss resulting from traumatic injury or tumor resection often requires surgical reconstruction using autologous soft tissue flaps. However, the limited availability of qualitative autologous flaps as well as the donor site morbidity significantly limits this approach. Engineered soft tissue flap grafts may offer a clinically relevant alternative to the autologous flap tissue. In this study, we engineered vascularized soft tissue free flap by using skin/adipose flap extracellular matrix scaffold (DSAF) in combination with multiple types of human cells. Following vascular reanastomosis in the recipient site, the engineered products successful regenerated large-scale fat tissue *in vivo*. This approach may provide a translatable platform for composite soft tissue free flap engineering for microsurgical reconstruction.

1. Introduction

Traumatic injury or tumor resection can result in substantial soft tissue loss that requires surgical reconstruction with autologous soft tissue flaps. However, this approach is often limited by a lack of high-quality autologous flaps and by donor site morbidity.

One alternative strategy to using autologous tissue flaps is using allogenic tissue flaps. Vascularized composite tissue allotransplantation is a clinical reality in plastic and reconstructive surgery [1]; more than 100 composite tissue allotransplantations have been successfully performed in humans and have included such diverse sites as the hand, abdominal wall, femoral diaphysis and knee, peripheral nerve, larynx, and face [2,3]. Unfortunately, the survival of composite tissue allotransplantation without rejection depends on the use of chronic nonspecific and novel specific immunosuppressive medications, many of which carry a risk for neoplasms, opportunistic infections, and/or end-organ toxicity [4].

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Engineered composite tissue flaps, which do not require the use of immunosuppression, may offer an ideal, clinically viable alternative to both autologous flaps and allogenic tissue flaps. Recently, tissue engineers have reached several milestones in the regeneration of organs including the heart, lung, and liver using decellularized whole-organ matrix scaffolds [5–7]. The native platform, especially its vascular network within the organ extracellular matrix (ECM), has an important role in vascularized organ engineering. A similar concept has been applied in vascularized composite tissue engineering. In one study, researchers decellularized a segment of porcine small bowel with its artery and vein structure and repopulated it with multiple types of cells *in vitro* [8]. Another study showed that with blood perfusion, this kind of bioartificial vascularized scaffold, which had been recellularized with porcine bladder smooth muscle cells and urothelial cells and endothelial progenitor cells, remained intact for 1–3 h in a porcine model [9]. Furthermore, a similar bioartificial vascularized scaffold, which had been recellularized with a patient's autologous muscle cells and fibroblasts and endothelial cells and implanted into a patient's arm, had patent vessels with functional circulation and tissue viability 1 week postoperatively [10]. On the basis of these promising results, which indicated the feasibility of bioengineering a human tissue with innate vascularization, Aubin et al. decellularized the native cardiac muscle of a rat to establish a coronary artery tissue flap; its re-endothelialization *in vitro* indicated that it had great potential for further application [11]. More recently, Jank et al. created decellularized limb composite tissue matrix. The recellularized limb tissue matrix-multiple cells construct showed perfusion in a re-endothelialized vascular conduit in a short-term non-survival procedure in a rat model [12]. Overall, the use of decellularized composite tissue matrix to engineer vascularized composite tissue has progressed much in recent years. However, unlike the closed system of the whole-organ matrix, the open system of the composite tissue flap matrix poses many challenges, and most models are still in need of long-term observation *in vivo*.

Skin/adipose tissue flaps are the workhorses of routine reconstructive microsurgery. Thus, skin/adipose tissue flap engineering has tremendous impact in the field of reconstructive surgery. Studies have shown that a completely decellularized skin flap matrix retains its vascular structure intact [13,14]. However, details about the characterization of the acellular skin flap matrix are lacking, and the interaction between the cells and scaffold matrix during the remodeling process has not yet been investigated *in vitro* or *in vivo*.

As one of the biggest reconstructive microsurgery centers in the world, we have performed more than 3200 free flap transfers in the last 5 years (unpublished data). Our experience indicates a need for engineering composite tissue flaps to repair large tissue defects to ultimately avoid donor site injury and morbidity. Thus, the purpose of the present study was to establish a platform for using a decellularization approach to engineer soft tissue free flaps that have more flexible clinical applicability. We used a perfusion decellularization protocol to process skin/adipose tissue flaps from rats. The decellularized skin/adipose tissue flap (DSAF) was comprehensively characterized with respect to its three-dimensional (3D) architecture, biomolecular patterning, and bioactivity. The biocompatibility of DSAF was tested by integrating it with human adipose-derived stem cells (hASCs) and human umbilical vein endothelial cells (HUVECs). Prevascularized and recellularized DSAF with a dominant vascular pedicle and integrating multiple cell types was then implanted into a rodent model using conventional microsurgical techniques, and the *in vivo* response, biocompatibility, and remodeling properties of DSAF were evaluated. The findings of the present study may inform the development of a platform for designing and fabricating 3D vascularized dermal/adipose tissue constructs that can be used to repair extensive soft tissue defects.

2. Materials and methods

2.1. Creation of the DSAF bioscaffold

All animal handling and experimental procedures strictly followed the research protocol approved by MD Anderson's Institutional Animal Care and Use Committee and the National Institutes of Health guidelines for animal welfare. Groin skin/adipose tissue flaps ($2 \times 4\ cm^2$) were harvested from euthanized 8- to 10-week-old male Fischer 344 rats (Harlan Laboratories, Indianapolis, IN). The flap pedicle included the superficial epigastric artery and vein plus segments of the femoral artery and vein and the femoral nerve. Immediately after harvest, the femoral artery was catheterized with a 24G catheter (BD Biosciences, San Jose, CA), and the flap was irrigated with normal saline through the femoral artery until only clear normal saline flowed from the femoral vein. The flaps were frozen at −80 °C and thawed at room temperature for 3 cycles and were then processed with chemical detergents as described previously [15]. For each flap, the artery was catheterized, and the flap was connected via the catheter to a Masterflex pump perfusion system (Cole-Parmer, Vernon Hills, IL) and perfused with ultrapure water (2 ml/min) for 1 day at room temperature. The flap was then treated with 0.5 M NaCl for 4 h, 1 M NaCl for 4 h, and ultrapure water overnight; this saltwater perfusion procedure was repeated once. After treatment with 0.25% trypsin/ethylenediaminetetraacetic acid for 2 h at 37 °C and washing with deionized water for 1 h, the flaps were processed with isopropanol overnight with agitation. The flaps were then treated with 1% Triton X-100 for 2 days (1 change daily), washed in ultrapure water for 2 days (3 changes daily), and rinsed in phosphate-buffered saline (PBS) for 1 day within the perfusion system. DSAF was sterilized using 70% ethanol and rinsed in PBS. DSAF was stored at 4 °C in PBS containing 1% penicillin/streptomycin until use (Fig. 1A–D).

2.2. Characterization of DSAF

2.2.1. Histological and immunohistochemical analysis

Native skin/adipose tissue (NSAF) and DSAF ($n = 3$, respectively) were fixed in 10% formalin, embedded in paraffin, and sliced into 5-μm sections. Slides of the paraffin-embedded samples were processed for histological and immunohistochemical (IHC) staining. The slides were stained with hematoxylin and eosin (H&E), Masson's trichrome, and 4′,6-diamidino-2-phenylindole (DAPI). The slides were also stained using antibodies against vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF; both from Oncogene Science, Cambridge, MA) and antibodies against major histocompatibility complex antigen class I (MHC-I) and laminin (both from Abcam, Cambridge, MA). For IHC staining, the slides were placed in antigen retrieval citrate buffer (Biogenex, Fremont, CA) in a 95 °C steamer for 10 min. Endogenous peroxidases were blocked with a peroxide block (Innogenex, San Ramon, CA), and nonspecific binding was blocked with normal goat serum (Vector Laboratories, Burlingame, CA). Sections were incubated with the primary antibodies at 4 °C overnight. After the slides were washed, they were subjected to the application of a biotinylated secondary antibody for 30 min, treatment with a streptavidin-horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories) and diaminobenzidine solution (DAB kit, Vector Laboratories), and counterstaining with hematoxylin. After staining, the slides were dehydrated and mounted and then imaged using an Olympus IX71 microscope (Olympus, Center Valley, PA).

2.2.2. DNA assessment and quantification

Cell removal was quantified by measuring the nucleic acid concentration with the Quant-iT PicoGreen dsDNA assay kit