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Engineering vascularized soft tissue flaps in an animal model using human adipose–derived stem cells and VEGF+PLGA/PEG microspheres on a collagen-chitosan scaffold with a flow-through vascular pedicle☆

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ABSTRACT

Insufficient neovascularization is associated with high levels of resorption and necrosis in autologous and engineered fat grafts. We tested the hypothesis that incorporating angiogenic growth factor into a scaffold–stem cell construct and implanting this construct around a vascular pedicle improves neovascularization and adipogenesis for engineering soft tissue flaps. Poly(lactic-co-glycolic-acid/polyethylene glycol (PLGA/PEG) microspheres containing vascular endothelial growth factor (VEGF) were impregnated into collagen-chitosan scaffolds seeded with human adipose-derived stem cells (hASCs). This setup was analyzed *in vitro* and then implanted into isolated chambers around a discrete vascular pedicle in nude rats. Engineered tissue samples within the chambers were harvested and analyzed for differences in vascularization and adipose tissue growth. *In vitro* testing showed that the collagen-chitosan scaffold provided a supportive environment for hASC integration and proliferation. PLGA/PEG microspheres with slow-release VEGF had no negative effect on cell survival in collagen-chitosan scaffolds. *In vivo*, the system resulted in a statistically significant increase in neovascularization that in turn led to a significant increase in adipose tissue persistence after 8 weeks versus control constructs. These data indicate that our model—hASCs integrated with a collagen-chitosan scaffold incorporated with VEGF-containing PLGA/PEG microspheres supported by a predominant vascular vessel inside a chamber—provides a promising, clinically translatable platform for engineering vascularized soft tissue flap. The engineered adipose tissue with a vascular pedicle could conceivably be transferred as a vascularized soft tissue pedicle flap or free flap to a recipient site for the repair of soft-tissue defects.

1. Introduction

Soft tissue defects resulting from tumor resection, trauma, and congenital deformities represent an ongoing challenge in reconstructive surgery. Autologous flaps serve as the workhorses in reconstructive microsurgery. However, the availability of autologous flaps and donor site morbidities significantly limit their application. Adipose tissue engineering strategies offer a promising alternative solution [1]. However, adipose tissue engineering, especially adipose flap engineering, is still far from providing a clinically translatable product on a large scale.

Human adipose-derived stem cells (hASCs), one cellular component of adipose tissue, play an important role in fat grafting and adipose tissue regeneration and are considered an ideal autologous cell source for adipose tissue engineering [2–4]. hASCs can be easily derived from excised or liposuctioned adipose tissues, which are conventionally discarded. Large quantities of hASCs can be recovered from adipose tissues because adipose tissues, which contain approximately 100 times as many stem cells as bone marrow does, are abundant reservoirs of multipotent mesenchymal

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stem cells [5]. These stem cells have been characterized at length to differentiate along multiple lineage pathways useful in soft tissue repair [6]. The implantation of hASCs with various other materials has begun to expand the usefulness of soft tissue engineering as reconstructive options [7–9]; however, problems with engineered adipose tissue retention, graft persistence, and neovascularization remain.

Effective vascularization is the predominant factor that supports the persistence of engineered adipose tissue. Adipose tissue is metabolically active and requires a rich vascular supply to nourish it. Therefore, the retention of the graft and the growth of the transplanted tissue *in vivo* should be improved by enhancing the graft's vascular support. Problems with fat resorption after implantation have been proposed to stem from a lack of sufficient vascularization [10–12]. This concept has indeed been shown time and again to be true: promoting vascularization increases graft survivability [13]. To capitalize on the close relationship between angiogenesis and adipogenesis, researchers have used angiogenesis-related factors to support adipogenesis. Yuksel et al. demonstrated that graft maintenance can be improved with insulin and insulin-like growth factor [14], and Rophael et al. reported that adding vascular endothelial growth factor (VEGF) and fibroblast growth factor enhances angiogenesis and graft volume retention [15]. Lu et al. demonstrated that genetically manipulating cells in the graft to express VEGF improves graft volume retention [13].

Models that introduce a vascular pedicle to support the engineered soft tissue graft inside a chamber have been shown to facilitate the large-scale engineering of soft tissue flaps for clinically translatable applications [16–19]. We have used Matrigel scaffold combined with basic fibroblast growth factor within a silicone chamber to engineer adipose soft tissue [16]. However, because Matrigel is derived from tumor, using Matrigel raises concerns about maintaining adipose tissue normality. Therefore, in the current study, we took advantage of the use of a vascular pedicle inside a chamber but changed the scaffold material and other essential elements to improve the engineered vascularized soft tissue. In previous studies, we have demonstrated that a scaffold composed of a blend of the natural polymers collagen type I and chitosan is both biocompatible and effective for *in vivo* delivery of a preadipocyte cell population. In addition, this blended scaffold supports *de novo* adipose tissue growth within a subcutaneous pocket model in Lewis rats [20]. Collagen type I is a biological fibrous protein polymer derived from the body's natural extracellular matrix (ECM). Chitosan, which mimics the glycosaminoglycans of the ECM, provides additional structural support, sequesters water, and promotes the even distribution of growth factors. The result of combining these two proteins with a small amount of the homobifunctional crosslinker glutaraldehyde is a scaffold that closely mimics natural ECM and delivers cells effectively *in vivo* [12,20]. Poly(lactic-co-glycolic-acid/polyethylene glycol (PLGA/PEG) microspheres have been proven to be biocompatible with this scaffold and are an effective way to deliver peptide growth factors like VEGF *in vivo* [21–23]. Such carrier systems, whose degradation and release mechanics are tunable according to the composition of the polymers, have been used as stand-alone scaffolding material [24–27].

This study aimed to develop fully vascularized adipose tissue flaps suitable for autologous transplantation for the repair of soft tissue defects. We hypothesized that incorporating VEGF-containing PLGA/PEG microspheres into engineered constructs consisting of hASCs seeded to a cross-linked collagen-chitosan scaffold and then encasing the scaffold and vascular pedicle in a chamber increases neovascularization in the remodeled tissue, thereby increasing the growth and retention of the formed soft tissue. To test this hypothesis, we analyzed the interaction between hASCs and VEGF-containing and non-VEGF–containing microsphere scaffolds *in vitro* and analyzed the growth, longevity, and vascularization of the implanted constructs *in vivo*.

2. Materials and methods

2.1. PLGA/PEG microsphere preparation

PLGA/PEG microspheres were prepared using the solid-encapsulation/single-emulsion/solvent extraction technique [28] with modification. Briefly, 200 mg of the PLGA powder was combined with 5 mg of PEG powder (Sigma, St. Louis, MO) and 1 mL dichloromethane (Fisher Scientific, Pittsburgh, PA) in a 30 mL glass test tube and shaken for 60 min 10 μg of recombinant human VEGF (PeproTech, Rocky Hill, NJ) was introduced by co-encapsulation with 50 mg of BSA at a 1:5000 ratio of recombinant human VEGF:BSA. This 50 mg of Bovine serum albumin (BSA; Fisher Scientific) was then added to the test tube, and the mixture was vortexed for 1 min. Nine milliliters of 0.3% polyvinyl alcohol (Sigma) was added to the test tube, and the mixture was vortexed vigorously for 30 s to induce microsphere formation. The contents of the test tube were then transferred into a beaker containing 90 mL of 0.3% polyvinyl alcohol and allowed to incubate for 5 min. One hundred milliliters of 2% isopropyl alcohol was then added, and the solution was stirred uncovered for 60 min at room temperature to allow for organic solvent evaporation. Microspheres were collected by centrifugation and rinsed by resuspension in distilled water multiple times, frozen at −80 °C, and finally dried by lyophilization overnight. Particle size distribution was measured with bright-field imaging. Random samples of microspheres were resuspended in water, mounted to a microscope slide, and visualized using 10X, 20X, and 40X objectives. Multiple random fields were imaged, and particle diameters were automatically calculated using ImageJ software (National Institutes of Health [NIH], Bethesda, MD). Following incorporation into the collagen-chitosan scaffolds, particle size and morphology were confirmed with scanning electron microscopy (SEM).

2.2. Collagen-chitosan scaffold preparation

Chitosan (Sigma) was dissolved at 5 mg/mL in 2% acetic acid with mechanical stirring and then blended with 5 mg/mL rat tail collagen type I solution (BD Biosciences, Bedford, MA) at a 9:1 collagen:chitosan ratio [20,29]. A 0.1% final concentration of glutaraldehyde, which served as a homobifunctional crosslinker, was added, along with a 10 mg/mL concentration of PLGA/PEG microspheres. The aqueous scaffold solution was covered and stirred for 3 days at 4 °C. Scaffold solution aliquots were pipetted into polystyrene plates, frozen at −80 °C overnight, and then subjected to lyophilization for 3 days. Scaffolds used for *in vitro* tests were prepared directly on 96-well cell culture plates with 100 μL of solution; *in vivo* scaffolds were prepared using 6-well cell culture plates with 5 mL of solution. All scaffolds were sterilized by immersion in 70% ethanol for a minimum of 12 h and then rinsed with sterile phosphate-buffered saline (PBS) multiple times under a laminar flow hood to remove excess ethanol and neutralize pH. Scaffolds were immediately seeded following sterilization for all experiments.

2.3. hASC isolation and culture

All procedures were conducted with Institutional Review Board approval and in accordance with research guidelines at The University of Texas MD Anderson Cancer Center. Patients had provided informed consent for their tissues to be used in basic research.