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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.

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The authors have nothing to disclose in relation to this study.

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Keywords

Soft tissue flap engineering; Adipose-derived stem cell; Vascular endothelial growth factor; PLGA/PEG microsphere; Collagen-chitosan scaffold; Vascularization

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 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 pre-adipocyte 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 minutes. 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 minute. Nine milliliters of 0.3% polyvinyl alcohol (Sigma) was added to the test tube, and the mixture was vortexed vigorously for 30 seconds 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 minutes. One hundred milliliters of 2% isopropyl alcohol was then added, and the solution was stirred uncovered for 60 minutes 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 10×, 20×, and 40× 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 hours 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. Adipose tissue samples (subcutaneous adipose tissue in the abdominal wall area) were collected from patients undergoing reconstructive surgery. The fat tissue was minced by hand and digested in 0.075% collagenase type-I (Sigma) with 5% antibiotics for 2 hours. The processed fat tissue was then passed through 100 µm cell strainers and centrifuged at 200 g. The cell pellet was resuspended in red blood cell lysis buffer, centrifuged again, and then plated for expansion. Stem cell culture was performed with α minimum essential media supplemented with 15% fetal bovine serum and 1% antibiotics in a 37°C incubator with 90% humidity and 5% CO₂ (NuAire, Plymouth, MN). hASCs within 3 passages were harvested and plated onto scaffolds for *in vitro* experiments. hASC identification was confirmed by differentiation assay and flow cytometry analysis with an LSRFortessa cell analyzer (BD Biosciences) using antibodies against CD29 and CD90 (ASCs/stromal cells); CD11b (immune cells); and CD45 (hematopoietic cells) (Supplementary Figure 1). All mouse anti-human primary antibodies were from BD Biosciences.

2.4. Green fluorescent protein gene transfection for in vivo tracking of hASCs

To trace the seeded cells *in vivo*, hASCs were transfected with green fluorescent protein (GFP)-lentiviral vector following the manufacturer's instructions. Briefly, cells were seeded in multi-well plates and transfected with GFP-lentivirus (GenTarget, Inc, San Diego, CA) with cells at 70% confluence. After the initial GFP gene transfection, a media change, and subsequent culture, the cells were sorted using a FACS ARIA II cell sorter (BD Biosciences). GFP-positive hASCs were confirmed by fluorescence imaging (Supplementary Figure 1).

2.5. Scanning electron microscopy

Collagen/chitosan scaffolds with or without VEGF-containing microspheres were cut (diameter, 6 mm) to fit into the wells of 96-well cell culture plate and placed at the bottom of the wells. The scaffolds were seeded with hASCs at 4×10^4 cells/cm² and incubated at 37°C for different times. Following incubation, the scaffolds were washed 2 times with PBS to remove unattached cells and then fixed in 2% paraformaldehyde and 3% glutaraldehyde solution. Cell-seeded scaffolds were imaged using SEM before and during cell studies to illustrate physical degradation and assess the surface morphology of both the microspheres and scaffolds over time. Microsphere morphology was visualized with bright-field microscopy at 200× magnification. All imaging was performed using a JSM-590 scanning electron microscope (JEOL USA, Inc., Peabody, MA).

2.6. Cell viability and proliferation on scaffolds

Cell viability on scaffolds was assessed with live cell staining using calcein AM (Biotium, Hayward, CA) as described previously [30]. Samples were examined with an Axiovert 200 fluorescence microscope (Zeiss, Thornwood, NY) on days 2 and 4 after cell seeding. The cells' morphologic features (perimeter, area, and roundness) were measured using Adobe Photoshop CS5.1 image-processing software (Adobe, San Jose, CA). Roundness was defined as $4\pi \cdot \text{area}/(\text{perimeter})^2$.

The proliferation of hASCs on VEGF-containing and non-VEGF-containing microsphere scaffolds was assessed using CellQuanti-MTT Cell Viability Assay Kits (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. hASCs were seeded onto scaffolds prepared in 96-well tissue culture plates or onto blank wells at varying densities (1×10^4 , 2×10^4 , and 4×10^4 cells/cm²). For test conditions, all scaffolds contained PLGA/PEG microspheres, and half of the scaffolds contained VEGF. Cells seeded onto blank wells were the negative controls. Cells were washed and treated with a 1:5 ratio of 3(4,5-dimethylthiazol-2Yl)-2,5-diphenyltetrazolium bromide (MTT):culture media at time points of 1, 2, 5, 7, 10, 14, and 20 days after seeding. Following incubation at 37°C for 2 hours, MTT was taken up by active cells and reduced inside the mitochondria to insoluble purple formazan granules. Cells were subsequently lysed, and formazan granules were solubilized in dimethyl sulfoxide on a shaker. The supernatant was transferred to fresh 96-well plates to reduce background contamination from the scaffold, and the optical density was read at 540 nm using a photometric plate reader (BioTek, Oklahoma city, OK).

2.7. VEGF release in vitro

While the hASCs were being cultured on the scaffolds, the culture medium was changed, and VEGF levels were measured in the medium that was removed. Seven groups were compared: group 1, scaffold+VEGF microspheres+hASCs (40k seeding); group 2, scaffold +VEGF microspheres+hASCs (10k seeding); group 3, scaffold+hASCs (40k seeding); group 4, scaffold+hASCs (10k seeding); group 5, scaffold+VEGF microspheres; group 6: scaffold only; and group 7, blank well. Enzyme-linked immunosorbent assay (Invitrogen, Camarillo, CA) was utilized to measure the concentration of VEGF in these media at different times. Briefly, 50 μ L of the samples were added to wells, and the plate was incubated for 2 hours in the dark. Wells were washed, incubated with 100 μ L of biotinylated anti-VEGF for 1 hour, washed again, and incubated with 100 μ L of streptavidin–horseradish peroxidase solution for 30 minutes. The wells were washed a final time, and 100 μ L of stabilized chromogen was added and incubated for 20 minutes before the reaction was stopped and absorbance measured at 450 nm using a photometric plate reader (BioTek).

2.8. Aortic ring assay

An aortic ring assay was used to test the effects of VEGF-containing microspheres and stem cell culture systems on angiogenesis *in vitro*. Rat thoracic aortas were obtained from three female nude rats (8 to 10 weeks old, NIH). The aortas were sectioned into 1 mm-thick rings, which were then immersed in 25 μ L of rat-tail collagen type I (BD Biosciences) and incubated at 37°C for 30 minutes to allow the collagen solution to transform into a gel. Aortic rings embedded in the collagen gels were treated with one of four media: 1) regular endothelial growth medium (EGM; Lonza, Walkersville, MD); 2) medium collected from group 5 on day 5 and combined with the same volume of EGM (“VEGF media”); 3) medium collected from group 3 on day 5 and combined with the same volume of EGM (“hASCs-conditioned media”); or 4) medium collected from group 1 on day 5 and combined with the same volume of EGM (“VEGF+hASCs– conditioned media”). Phase contrast images of these samples were taken on day 7 using an Olympus IX70 microscope before and after hematoxylin staining. Cell outgrowth and migration from the rings were analyzed using ImageJ software. Aortic rings with outgrowth of cells and branches were stained with a mouse monoclonal primary antibody against CD31 (1:200, AbD SeroTec, Raleigh, NC) and a rabbit monoclonal primary antibody against α -smooth muscle actin (1:200, Abcam, Cambridge, MA) followed by staining with goat anti-mouse DyLight 488 and goat anti-rabbit DyLight 594 (each 1:500; both from JacksonImmuno Research Laboratories, West Grove, PA). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (1:5000; Molecular Probes, Eugene, OR). The stained rings were viewed with an Olympus IX81 confocal fluorescence microscope.

2.9. Silicone chamber preparation

Silicone chambers (length, 1.5 cm; internal diameter, 1.0 cm; volume, 1.1–1.2 mL) were prepared and cut open lengthwise as described previously [16]. Small circles of thin silicone membrane, whose radii were slightly larger than that of the chambers, were placed over the open ends of the chambers and attached with a 5/0 polypropylene suture (Ethicon, Somerville, NJ). The silicone membranes were then trimmed and cut in such a way that the

construct could be placed around major vessels without obstructing their flow while remaining sealed off from surrounding local tissues. In this way, we were able to evaluate scaffolds *in vivo* without having to account for major influence from neighboring tissues. Four separate configurations were packed inside the silicone chambers: 1) scaffold plus VEGF plus hASCs, 2) scaffold plus VEGF, 3) scaffold plus hASCs, and 4) scaffold alone. For the experimental groups containing hASCs, GFP⁺ hASCs (4×10^4 cells/cm²) were seeded in each scaffold type in 6-well plates the night before surgery. Immediately before surgery, the medium was aspirated and the scaffolds gently rinsed with PBS. Each chamber construct was packed with one scaffold's worth of material of all 6 wells.

2.10. Animal model

All animal procedures were approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center and met all requirements of the U.S. Animal Welfare Act. Sixty 8- to 10-week-old syngeneic athymic female nude rats from the NIH were used to minimize cellular and humoral immuno-responses to human cells. Anesthesia in rats was induced and maintained with isoflurane (0.5–2%, 3–5 L/min) in oxygen. Animals underwent bilateral groin dissection to isolate the deep femoral artery-vein pedicles (AVPs); nerves were preserved to avoid interfering with the animal's movement. A single silicone chamber was carefully placed around the AVPs on the left and right sides of the animal, and each chamber was filled with one of the four aforementioned combinations of composite scaffold materials. Cell-seeded constructs with and without VEGF-containing microspheres were implanted in each side of the same rat, and non-cell-seeded constructs with and without VEGF microspheres were implanted in each side of another rat. The silicone chambers were closed lengthwise with a polypropylene suture, and a small amount of fibrin gel sealant (Baxter Healthcare, Westlake Village, CA) was applied at either end to help ensure the system's isolation from local tissues. The constructs were left in place for 2, 4, 8, or 12 weeks, at which time the animals were sacrificed and the constructs explanted (n = 6 rats). The constructs were opened, and the engineered tissue was removed from the chambers. The samples were then dissected or cross-sectioned for frozen sectioning, or fixed in 10% formalin and embedded in paraffin for histological analysis.

2.11. Histological and immunohistochemical analysis

Tissue sample blocks were cut into 5 µm sections. Sections were deparaffinized, rehydrated, washed in distilled water, and mounted on slides. Slides were subjected to histological hematoxylin and eosin staining and Masson's trichrome staining. Frozen samples embedded in optimal cutting temperature compound (O.C.T) were cut into 5 µm sections for Oil Red O staining. For immunohistochemical staining, slides were placed in antigen retrieval citrate buffer (Biogenex, Fremont, CA) in a 95°C steamer for 10 minutes. Endogenous peroxidases were blocked by incubation with peroxide block (Innogenex, San Ramon, CA), and nonspecific binding was blocked with normal goat serum (Vector Laboratories, Burlingame, CA). Sections were incubated at 4°C overnight with primary antibodies (anti-collagen I, anti-collagen III, anti-CD31, anti-CD68, and anti-GFP, from Abcam; and anti-CD163 and anti-CD80 from AbD Serotec). After the sections were washed, biotinylated secondary antibody was applied for 30 minutes, followed by treatment with streptavidin-horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories) and diaminobenzidine

solution (DAB kit, Vector Laboratories) and counterstaining with hematoxylin. Slides were dehydrated, mounted, and imaged using an Olympus IX70 microscope (Olympus, Center Valley, PA) using Q image Retiga 4000R digital capture camera using QCapture Pro 6.0 digital capture software. The data analysis of the captured images was performed using ImageJ software (NIH). The analysis measured the following quantities in each slide: total area of the sample, area of the major vascular structures of the pedicle (main artery and vein), the perimeter of the major vascular structures of the pedicle, the total area of the adipose tissue and the total area of remodeled tissue. These data were used to calculate the percentage of the total area that was adipose tissue and the percentage of the total area that was remodeled in individual groups. Positive cell staining was quantified visually using random fields over the entire construct, from the capsular area to the pedicle area.

2.12. Statistical analysis

Data were presented as means $\pm$ standard deviations. Unpaired *t*-tests were used to compare independent groups and conditions. Paired *t*-tests were used to compare individual and subsequent tests between times. One-way analysis of variance with pair-wise multiple comparison was performed to compare multiple groups where applicable. All analyses were performed using the SigmaStat software program (version 3.5, SyStat, USA) and Microsoft Excel 2010 data analysis add-on. Differences were considered to be significant at a level of $p < 0.05$.

3. Results

3.1. Microsphere/scaffold morphology and characterization

Individual microspheres were spherical and similar in shape to one another (Figure 1A). A representative particle size distribution revealed that virtually all microspheres (95%) were between 10 μm and 50 μm in diameter. The microspheres had a mean diameter of $21.1 \pm 7.8 \mu\text{m}$; 2.2% of the microspheres were less than 10 μm in diameter, and less than 1% was greater than 50 μm in diameter (Figure 1B). The vast majority of the microspheres' diameters were within one standard deviation of the mean. Loading microspheres with VEGF made no discernible difference in microsphere size distributions (data not shown).

Overall, the collagen-chitosan scaffold was largely fibrous and had heterogeneous fiber sizes and small areas with flat sheet-like appearances consistent with cross-linked type I collagen. Microspheres and hASCs were both present within the scaffold. Microspheres in aqueous scaffold material were evenly distributed within the collagen/chitosan scaffolds and incorporated into the surface structure. Initially, microspheres cross-linked with scaffold material exhibited a smooth spherical morphology similar to that of unbound microspheres (Figure 1C). After 14 days, these microspheres begin to swell and degrade, appearing to “deflate” and lose their spherical morphology (Figure 1D). hASCs integrated and migrated into the porous scaffold and formed firm adhesions (Figure 1E&F).

3.2. hASC incorporation and proliferation on scaffolds

Live cell staining with calcein AM showed that the collagen-chitosan scaffold supported hASC survival and proliferation (Figure 2A). hASCs attached well to the scaffold 2 days

after plating. When cultured *in vitro*, hASCs proliferated on the scaffold. Quantitative analysis of cell morphologic features confirmed significant differences between cells cultured on the scaffold and cells cultured on two-dimensional glass slides. The cells had significantly higher circularity in a three-dimensional environment; in a two-dimensional environment, the cells tended to elongate, increasing their perimeter and decreasing their area (Table 1).

MTT assay was used to assess the effects of microspheres alone and the effects of microspheres loaded with VEGF on cellular proliferation on the collagen-chitosan scaffolds. Compared with that on polystyrene petri dishes alone, the cellular proliferation on the collagen-chitosan scaffolds was much higher and lasted much longer. Cells on scaffolds exhibited exponential growth almost immediately after being plated. Cells on petri dishes exhibited similar initial growth curves; however, cell growth slowed to senescence within 2 days in wells seeded with $4 \times 10^4/\text{cm}^2$ cells and within 5 days in wells seeded with $1 \times 10^4/\text{cm}^2$ cells. This was expected, however, as the wells had limited surface area and hASCs double rapidly in culture and are inhibited by cell contact. Cells seeded to plates alone reached a maximum average confluence of 5×10^5 cells and exhibited the lowest proliferation, whereas cells seeded to scaffolds reached a maximum confluence of more than 1.5×10^6 cells before becoming senescent. Microspheres, with or without VEGF loading, did not significantly affect cell proliferation or death. Compared with cells plated on petri dishes, cells plated on scaffolds had significantly greater total numbers and proliferation as early as 5–10 days after plating ($P < 0.05$; Figure 2B). These data indicated that the collagen-chitosan scaffold, with or without VEGF-containing microspheres, provided a compatible and supportive environment for hASC integration and proliferation. VEGF did not interfere with the biological behavior of hASCs on the scaffold.

3.3. VEGF release kinetics in vitro

VEGF-containing microspheres cross-linked to collagen-chitosan scaffolds (group 5) exhibited linear VEGF release over time (Figure 2C); VEGF release averaged 6.7 ng/mL/day over 10 days. After 14 days, the microspheres in group 5 had bulk degradation but continued to release VEGF. For hASCs seeded at a high density (4×10^4 cells/ cm^2), the group with VEGF-containing microspheres (group 1) initially had a lower average VEGF concentration in the growth media than did the group without VEGF-containing microspheres (group 3); however, the VEGF concentration in group 1 followed a positive upward slope until reaching a maximum concentration of around 50 ng/mL on days 7 and 10, which was significantly higher than that of group 3 (Figure 2C). For cells seeded at a low density (1×10^4 cells/ cm^2), the group with VEGF-containing microspheres (group 2) had significantly higher VEGF levels than the group without VEGF microspheres (group 4) from days 5 to 10 in culture medium. There was no significant difference in the VEGF release patterns of groups 1 and 2 when the initial seeding density was changed from $1 \times 10^4/\text{cm}^2$ to $4 \times 10^4/\text{cm}^2$, although higher seeding did result in higher average VEGF levels (Figure 2C). These data indicate that both VEGF microspheres and hASCs contribute to VEGF levels, but after day 5, VEGF microspheres, not hASCs, are the major contributor to VEGF levels in the system.

An aortic ring assay was utilized to test the effect of the VEGF and hASCs culture system on cell outgrowth and new vessel formation (Figure 3). Cell outgrowth was observed in all groups. At day 7, there were no significant differences in cell migration among the VEGF media, hASCs-conditioned media, and VEGF plus hASCs-conditioned media groups, but cell migration in all three of these groups was significantly greater than that in the control group ($P < 0.05$, Figure 3A). In addition to cell migration, cell number and new vessel formation were significantly increased in the VEGF plus hASCs-conditioned media group (Figure 3B-D). Vessel branches extending from the aortic ring in 3-dimensional collagen gels were positive for both a smooth muscle marker (α -SMA) and an endothelial cell marker (CD31) (Figure 3B and Supplementary Video 1). These data suggest that the VEGF plus hASCs culture system induces angiogenesis *in vitro*.

3.4. Evaluation of engineered vascularized soft tissue in vivo

We next sought to determine whether the VEGF plus hASCs culture system promotes the angiogenesis and vascularization of engineered soft tissue after implantation *in vivo*. Rats had no noticeable physiological complications related to the silicone implants. The constructs were harvested at 2, 4, 8, and 12 weeks. All samples retained three-dimensional volume through 12 weeks post-implantation, and there were no significant differences in volume among the groups. However, the stiffness of the engineered soft tissue in the scaffold+VEGF microspheres+hASCs group was stronger than that of the soft tissue in the other three groups after 4 weeks; the tissue in the other three groups was fragile and easily broken during the explantation process, even at week 12 (Figure 4). The hematoxylin and eosin staining analysis showed that a fibrous layer encapsulated all tissue samples. There was a vascular pedicle (artery and accompanying vein) in the center of the connective tissue (Figure 5A). New adipose tissue was seen as early as 2 weeks after implantation; Oil Red O staining revealed that adipose tissue formed along the scaffold (Figure 5B). For all groups, adipose tissue growth reached a maximum at 4 weeks and then decreased until 12 weeks on average. Compared with control groups without hASCs or VEGF microspheres, groups with both VEGF and hASCs had greater adipose tissue growth and persistence from 4 to 12 weeks (Figure 5C). The presence of hASCs was evaluated by immunohistochemical staining for an anti-GFP antibody. In the engineered soft tissue, the “donor” cells (i.e., the original GFP⁺ hASCs) were distinguishable and persistent at 4 weeks (Figure 5D); by 8 weeks, however, the signal was fading, and by 12 weeks, the donor cells could not be distinguished from the “host” cells (i.e., the GFP⁻ rat cells) (data not shown). Cells in the vascular and adipose structures stained positively for GFP, indicating that seeded hASCs contributed to angiogenesis and adipose tissue development.

Engineered soft tissue remodeling was determined by assessing cell infiltration, scaffold degradation, and new ECM deposition. As shown by Masson Trichrome staining (Figure 6A), dense soft tissue formed in the scaffold+VEGF microspheres+hASCs group and was characterized by an abundance of collagen tissue distribution and higher cellular infiltration. This connective tissue was observed throughout the constructs from the capsular area to the pedicle area. However, in the other groups, cellular infiltration and tissue remodeling was only seen around the pedicle area, and scaffold alone comprised the majority of the graft in those groups. Collagen staining confirmed the Masson Trichrome staining results. Large

amounts of collagen III and collagen I were seen in the scaffold+VEGF microspheres +hASCs group, indicating a substantial amount of new ECM deposition in the engineered soft tissue (Figure 6B). Compared with assessing adipose tissue growth, assessing tissue remodeling was more consistently tracked construct progression over time. Initially at 2 weeks, the scaffold+VEGF microspheres+hASCs group had vastly greater host cellular infiltration than the non-hASC-seeded scaffold groups did. By week 4, scaffold remodeling, as well as fat growth, increased. Although adipose tissue growth slowed after 4 weeks in all groups, soft tissue remodeling increased in the scaffold+VEGF microspheres+hASCs group (Figure 6C). In addition, significantly less collagen, especially collagen III, and lower levels of cellular infiltration were seen in the three control groups, suggesting that tissue remodeling without hASC and VEGF support is slow or nonexistent.

Because macrophages play an important role in the tissue remodeling process, we examined the macrophage phenotypes in this study. Cells in the engineered tissues stained positively for CD68, CD163, and CD80 (Figure 7A). CD163+ (M2) macrophages were located predominantly in the remodeled area, where the scaffold was degraded and partially replaced by regenerated ECM tissue. On the other hand, CD80+ (M1) macrophages were located mostly within the scaffold area, where the cellular infiltration was still in the inflammatory stage. At 12 weeks, the scaffold+VEGF microspheres+hASCs group had significantly more CD163+ macrophages and a significantly higher M2:M1 ratio than in the other groups did (Figure 7B). These data suggest that in our model, VEGF+hASCs modulate macrophage polarization toward the M2 profile; thus, there is a close relationship between macrophage phenotypes and bio-scaffold constructive remodeling.

The pedicle patency of the tissue constructs was determined on the basis of bleeding from both ends of the chamber construct during harvesting and histological assessment. Most of the tissue constructs maintained vascular pedicle patency at each of the times examined. Angiogenesis and microvascular ingrowth around the pedicle increased drastically for all groups early on, as confirmed by CD31 immunohistochemical staining (Figure 8A). The scaffold+VEGF microspheres+hASCs group had significantly more vessels than the control groups did from 2 weeks to 12 weeks (Figure 8B). In addition, the VEGF+hASC scaffolds exhibited a propensity to vascularize areas of the construct farther away from the pedicle. At 8 to 12 weeks, VEGF-containing scaffolds continued to show persistent angiogenesis in areas remote from the pedicle, whereas angiogenesis in the non-VEGF-containing scaffolds remained centralized around the pedicle. Comparisons of the perimeters of the main arteries and veins indicated that the pedicle perimeter and patency were greater in the presence of VEGF, but this difference was not significant (data not shown). There was an association between fat growth and microvasculature structures at weeks 2 and 4 and between adipose persistence and microvasculature structures at weeks 8 and 12. These data indicate that angiogenesis is crucial to adipogenesis. This model yielded well-vascularized engineered soft tissue along a predominant vascular pedicle that could be transferred as a vascularized soft tissue free flap to a recipient site using microsurgery techniques (Figure 8C).

4. Discussion

We created a promising, clinically translatable platform for engineering vascularized soft tissue flaps. Our model has several important elements, including a collagen-chitosan scaffold, VEGF+PLGA/PEG microspheres, and hASCs, which are incorporated together inside a silicone chamber and supported by a flow-through vascular pedicle. The results of the present study show that substantial amounts of vascularized adipose and soft tissue were formed and persisted for up to 12 weeks in this model.

Our *in vitro* and *in vivo* data suggest that the soft stiffness of collagen-chitosan scaffolds matches that of the natural ECM to provide a supportive environment for the adhesion and proliferation of hASCs, which require a niche to preserve their normal function. Unlike Matrigel, a sarcoma tissue extract not suitable for use in humans, collagen-chitosan scaffolds have been used widely for tissue engineering and have the potential to be used clinically. Although synthetic scaffolds can be controlled for physical properties such as shape, they often lack biological functionality to support and guide hASC differentiation into angiogenic and adipogenic pathways. One way to overcome the limitations of these natural polymers is to add bioactive factors into the scaffold architecture. We found that PLGA/PEG microspheres, with or without VEGF, are biocompatible with hASCs and have no negative effects on hASC viability. The release of VEGF from these microspheres was linear over time, and VEGF microspheres were the major contributor to VEGF levels in the hASCs +scaffold culture system. An aortic ring assay confirmed the effectiveness of hASCs combined with VEGF microspheres in inducing angiogenesis *in vitro*. This evidence supports the use of VEGF to more quickly induce a hypoxic angiogenesis response *in vitro*, which may ultimately increase vascular support *in vivo*.

Many studies have employed basic fibroblast growth factor and VEGF in constructs designed to increase angiogenesis [1, 13, 15]. In the present study, the increases in construct remodeling with connective tissues and inflammatory cell infiltrate as early as 2 weeks after implantation suggest that combination of hASCs and VEGF microspheres have a stimulatory effect on angiogenesis and early tissue proliferation. Fibroblasts, macrophages, and pericytes have all been implicated in angiogenesis and adipogenesis [31]. In the present study, we observed cellular infiltration and hASCs around the capillary branches of the vascular pedicle, which was where immature adipocytes manifested at 4 weeks, a phenomenon that others have observed [15]. Although ASCs are capable of producing VEGF in hypoxic environments, however, the negative feedback regulation may limit the secretion. In the present study, the presence of the VEGF microspheres contributed to the constructs' earlier remodeling, which increased the constructs' probability of continued persistence. One unique aspect of the present study was the combination of two main approaches for promoting neovascularization: incorporating a discrete vascular pedicle into the graft and adding angiogenic growth factors into the grafted milieu. These two approaches have both been studied in detail, albeit conventionally as separate methodologies. Evidence from our *in vitro* and *in vivo* models suggests a synergistic relationship between the scaffold and VEGF microspheres and hASCs that promotes the successful remodeling of engineered scaffold material and the growth and persistence of soft

tissue. Results using our system confirm previous reports of increases in graft survivability achieved through the promotion of vascularization [13].

In the present study, adipose tissue was noticeable at as early as 2 weeks and showed pronounced growth by 4 weeks in hASC-seeded scaffolds, and there was significantly more adipose tissue at 8 and 12 weeks in the VEGF+hASC-containing scaffolds than in the scaffolds that did not contain VEGF or hASCs. hASCs were detected in engineered adipose structures at weeks 2 and 4, indicating that pre-seeded hASCs contributed to adipose regeneration within the engineered tissue in our model; we previously reported a similar finding using our other adipose tissue engineering model [30]. An additional source of adipose tissue could be circulating preadipocytes. Blood-borne undifferentiated mesenchymal stem cells have been implicated as the source of new adipose tissue within a closed tissue-engineering chamber [32]. In the present study, the free-standing construct model of angiogenic and adipogenic formation confines the cells inside a protected and isolated silicone shell capped with fibrin sealant. This limits host infiltration to primarily blood migration and soluble factors, so any regeneration of soft tissue is likely to be caused by the complicated reaction between the hASC+bioscaffold system within the chamber and the external bone marrow-derived host cellular system. We found that during construct remodeling, the number of hASCs decreased gradually while the number of host cells increased, suggesting that implanted hASCs play an important role in adipogenesis early on, whereas host bone marrow-derived adipocyte precursors greatly contribute to adipogenesis later on.

Local or systemic host immunological responses toward the implanted cell-biomaterial constructs also play a major role in determining the outcome of the therapeutic biomaterials used. Macrophages are immune cells that play particularly important roles in foreign-body reactions to bioscaffold implants. The dynamic switch of macrophage phenotypes from M1 to M2 is an essential determinant of the tissue remodeling process [33]. In the present study, CD68+ macrophage infiltration within the engineered constructs *in vivo* indicated that the implantation of the constructs had elicited a foreign-body response in the host. Among these macrophages, abundant CD163+ M2 macrophages were distributed around the remodeling area, (indicating the regeneration stage), whereas CD80+ M1 macrophages were mostly located in scaffold-rich areas (indicating the inflammation stage). Interestingly, hASCs +VEGF induced M2 macrophage involvement in the largest portion of the implant; in the scaffold+VEGF microspheres+hASCs group, modulation of the host response toward an M2 phenotype may have contributed to constructive remodeling such as soft tissue regeneration. This effect may have been due to the hASCs' anti-inflammatory and immunomodulatory properties in our model. hASCs and macrophages in a 3-dimensional hydrogel *in vitro* model have been shown to increase the M2 phenotype [34, 35]. A recent study further demonstrated that 3-dimensional topography cues are the key to mesenchymal stem cells' immunomodulatory effects that direct macrophages towards an anti-inflammatory phenotype [36]. Clearly, our *in vivo* results are consistent with those observations, because the collagen-chitosan scaffold provides 3-dimensional topography cues for hASC integration, which may promote hASCs' immunomodulatory effects on infiltrated macrophages. However, the underlying molecular mechanism by which collagen-chitosan

scaffolds combined with VEGF microspheres and hASCs regulate macrophage polarization is unknown. Given that this mechanism potentially has a tremendous impact on stem cell +bioscaffold-based tissue engineering and regeneration, it warrants further research.

Our study's findings demonstrate that a collagen-chitosan scaffold incorporated with VEGF+ PLGA/PEG microspheres provides a niche for hASCs. Supported by a suitable microenvironment, hASCs have unique immunomodulatory properties; they not only directly participate in soft tissue regeneration but also modulate the host's foreign-body reaction to the engineered constructs. Our findings also show that engineered constructs require a proper blood supply to produce viable engineered tissue grafts and that multifaceted models such as ours have possible therapeutic advantages in the field of soft tissue engineering and regenerative medicine. In our model, the artery and vein vascular pedicle, aided by the slow release of VEGF from the microspheres, grows numerous microvascular branches (Figure 8C) that not only support the pre-seeded hASCs-scaffold construct but also provide a microenvironment conducive to the recruitment of endogenous stem/progenitor cells and inflammatory cells for constructive tissue remodeling. Moreover, our model yielded vascularized engineered soft tissue with a dominant vascular pedicle that could conceivably be transferred as a soft tissue flap to another recipient site by vascular pedicle anastomosis using microsurgery techniques. This could have a tremendous clinical impact on soft tissue repair and reconstruction. In moving this translational research from the bench to the bedside, the success of this proof-of-concept study in a small animal model encourages us to test our hypothesis in a pre-clinical, large animal model (e.g., porcine) to fabricate large-scale soft tissue flaps with large constructs studied over longer periods.

5. Conclusions

Incorporating VEGF-releasing microspheres into an engineered construct *in vivo* with a well-established vascular pedicle significantly increased neovascularization, which in turn increased blood flow and nutrient availability throughout the construct. By 8 weeks, the experimental constructs had significantly increased the amount of microvasculature and persistent adipose tissue and ECM deposits in rats. hASCs were shown to persist inside the construct and to differentiate constructively *in vivo* within these conditions. One of our system's strengths is that the scaffold and microspheres are biocompatible in all aspects. No animals had negative reactions to the implanted constructs aside from typical surgical complications. In conclusion, our model—hASCs integrated with a collagen-chitosan scaffold incorporated with VEGF+PLGA/PEG slow-release microspheres supported by a predominant vascular pedicle inside a chamber—provides a promising, clinically translatable platform for vascularized soft tissue flap engineering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Scaffold preparation and hASC seeding on scaffold. (A) Microsphere morphology was visualized with bright-field microscopy at 200 $\times$ magnification. (B) A representative particle size distribution revealed that virtually all microspheres (95%) were smaller than 50 μm and larger than 10 μm in diameter. (C) Microspheres cross-linked with scaffold material exhibited a smooth spherical morphology similar to that of unbound microspheres. (D) After 14 days, the microspheres began to swell and degrade, appearing to “deflate” and lose their spherical morphology. (E) & (F) hASCs integrated and migrated into the porous scaffold. The overall morphology of the scaffold was largely fibrous and had heterogeneous fiber sizes and small areas with flat sheet-like appearance consistent with cross-linked type I collagen. Both microspheres and hASCs integrated in the scaffold (black arrow: microsphere; white arrow: hASC).

Figure 2.

(A) Fluorescence imaging showed that hASCs (stained with calcein AM; green) proliferated on scaffold and on two-dimensional glass on days 2 and 4 of culture. (B) MTT assay showed that hASCs proliferated on scaffold. *: $P < 0.05$ vs. cells without scaffold control at the same time, equal seeding density. (C) Enzyme-linked immunosorbent assay revealed that the VEGF levels in the medium of groups with VEGF microspheres was significantly higher than those in groups without VEGF microspheres at day 7 and 10. #: $P < 0.05$ vs. group 3, 4, 6, 7 at the same time. *: $P < 0.05$ vs. group 3, 4, 6, 7 at the same time.

Figure 3.

Aortic ring assay with VEGF+hASCs-conditioned media. (A) Phase contrast images of aortic rings on day 7 of *in vitro* cultures treated with control medium (EGM), VEGF medium (released VEGF added to EGM), hASCs-conditioned medium (hASCs-cultured medium added to EGM), and VEGF+hASCs-conditioned medium. Samples were also stained with hematoxylin and imaged. (B) Confocal images showing endothelial cell migration (white arrow) and vessel branch outgrowth (red arrow) from the aortic ring (yellow arrow) in 3-dimensional collagen gel on day 7 of *in vitro* culture treated with VEGF+hASCs-conditioned medium. Samples were stained for α-smooth muscle actin (α-SMA, red), CD31 (green), and DAPI (blue). (C) & (D) Cell density and migration distance from the aortic ring measured on day 7 of *in vitro* cultures treated with different media. *: $P < 0.05$ vs. group 2-4. #: $P < 0.05$ vs. group 1-3.

Figure 4.

Animal model of vascularized soft tissue engineering. (A) A silicon chamber was carefully placed around femoral artery-vein pedicles (AVPs) on the left and right side of the animal. Four types of composite scaffold materials were packed within the construct around the AVP. (B) The chamber was closed lengthwise with a polypropylene suture, and a small amount of fibrin gel sealant was applied at either end to help ensure proper system isolation from local tissues (blue arrow: silicone chamber; black arrow: scaffold; white arrow: pedicle). (C) Surgical field appearance at 12 weeks after placement of the chamber. (D) Chamber explantation at 12 weeks. (E) Appearance of engineered soft tissue at different times. Scale bar=1 cm.

B

Figure 5.

Evaluation of soft tissue formation. (A) Hematoxylin and eosin staining showed that all graft samples were encapsulated by a fibrous layer (asterisk) at 12 weeks. There was a patent vascular pedicle (artery and accompanying vein) at the center of the engineered construct. The group with hASCs and VEGF retained significant amounts of adipose tissue (blue arrow), which was distributed throughout the sample from the capsular area to the center pedicle area, whereas the other groups had mostly scaffold material only (black arrows). (B) Oil Red O staining revealed that adipose tissue (blue arrows) had formed along the scaffold (black arrows) at 12 weeks. (C) The levels of adipose tissue formation in the scaffold +VEGF microspheres+hASCs group were significantly higher than those in other groups. * : $P < 0.05$ vs. scaffold group at week 4; \$: $P < 0.05$ vs. other three groups at weeks 8 and 12. (D) The presence of hASCs was evaluated by immunohistochemical staining for an anti-GFP antibody. GFP+ hASCs were distinguishable and persistent from at 4 weeks. hASCs were closely located around the vascular structures (red arrows) and adipose structures (black arrows) in the scaffold+VEGF microspheres+hASCs group.

Figure 6. Evaluation of tissue remodeling. (A) Masson Trichrome staining showed that dense soft tissue formed in the scaffold+VEGF microspheres+hASCs group; this connective tissue was observed throughout the graft, from the capsular area to the pedicle area. However, in the

other groups, cellular infiltration and tissue remodeling were seen around the pedicle area only; scaffold itself remained the majority in the other groups at week 12 (black arrow: collagen; asterisk: capsule layer). (B) Collagen type I and III immunohistochemical staining for the same area of the engineered soft tissue in the series sections showed a significantly high level of collagen type III production (red arrows) along with collagen type I production (black arrows) in the remodeling soft tissue in the scaffold+VEGF microspheres+hASCs group, suggesting a large amount of new ECM deposits in this group at week 12 (*: capsule layer). (C) The levels of remodeled area in the scaffold+VEGF microspheres+hASCs group were significantly higher than those in other groups. *: $p < 0.05$ vs. scaffold group at week 4; #: $p < 0.05$ vs. scaffold+VEGF microspheres group at week 4; \$: $p < 0.05$ vs. other three groups at weeks 2, 8, and 12.

Figure 7.

Evaluation of macrophage infiltration. (A) Immunohistochemical staining showed different distributions of macrophage phenotypes in engineered soft tissue at week 12. CD163+ (M2) macrophages (black arrows) were located predominantly at the remodeling area where scaffold was mostly degraded and replaced by regenerated ECM tissue (cellular infiltration was in the regenerative stage). On the other hand, CD80+ (M1) macrophages (red arrows) were located mostly within the scaffold area where the cellular infiltration was still in the inflammatory stage (blue arrows: CD68+ macrophages). (B) The M2/M1 ratio in the scaffold+VEGF microspheres+hASCs group was significantly higher than that in other groups. *: $p < 0.05$ vs. other three groups. $M2/M1 = (CD163^+ \text{ cells}/CD68^+ \text{ cells})/(CD80^+ \text{ cells}/CD68^+ \text{ cells})$.

Figure 8.

Evaluation of vascularization. (A) CD31 immunohistochemical staining showed that numerous microvascular vessels formed throughout the soft tissue in the scaffold+VEGF microspheres+hASCs group at week 12. There were more vessels around the pedicle area and capsule layer area in the samples of all groups (black arrow: main vascular pedicle; red arrows: microvascular vessel; asterisk: capsule layer). (B) There were significantly more blood vessels in the scaffold+VEGF microspheres+hASCs group than in the other groups. *:

$p < 0.05$ vs. other three groups at weeks 4 and 12; #: $p < 0.05$ vs. scaffold group at weeks 2 and 8. (C) Diagram of the concept of fabricating a vascularized soft tissue flap by using an *in vivo* scenario as the bioreactor. The implantation of the chamber construct initiated a foreign body reaction. Cellular infiltration through the main vascular pedicle resulted in inflammation, proliferation, and tissue remodeling within the scaffold-cells construct. Simultaneously, the artery and vein vascular pedicle grew numerous microvascular branches to support the engineered tissue. Finally, the capsule formation served as a support structure for the engineered soft tissue flap. Throughout the soft tissue engineering process, there was a close relationship between the constructive tissue remodeling and neovascularization and the M2 macrophage infiltration.

Table 1
Summarization of cellular morphology on 3D scaffold and 2D glass slides on days 2 and 4 in culture.

	3D Scaffold			2D glass		
	Area (µm ²)	Perimeter (µm)	Roundness	Area (µm ²)	Perimeter (µm)	Roundness
Day 2	7.7×10 ² ± 3.2×10 ² (n=20)	0.7×10 ² ± 0.2×10 ² (n=20)	0.728±0.122 (n=20)	2.0×10 ³ ± 1.3×10 ³ (n=19)	1.4×10 ² ± 0.7×10 ² (n=19)	0.144±0.063 (n=19)
Day 4	9.2×10 ² ± 3.6×10 ² (n=19)	0.8×10 ² ± 0.2×10 ² (n=19)	0.739±0.106 (n=19)	2.1×10 ³ ± 1.2×10 ³ (n=25)	1.1×10 ² ± 0.3×10 ² (n=25)	0.231±0.106 (n=25)

Values are presented as mean ± standard deviations. *P*<0.05 for all parameters 3D vs 2D at same time point.