TISSUE ENGINEERED PREFABRICATED VASCULARIZED FLAPS

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Abstract: Background. Microvascular free tissue transfer has become increasingly popular in the reconstruction of head and neck defects, but it also has its disadvantages. Tissue engineering allows the generation of neo-tissue for implantation, but these tissues are often avascular. We propose to combine tissue-engineering techniques together with flap prefabrication techniques to generate a prefabricated vascularized soft tissue flap. Methods. Human dermal fibroblasts (HDFs) labeled with fluorescein diacetate were static seeded onto polylactic-co-glycolic acid-collagen (PLGA-c) mesh. Controls were plain PLGA-c mesh. The femoral artery and vein of the nude rat was ligated and used as a vascular carrier for the constructs. After 4 weeks of implantation, the constructs were assessed by gross morphology, routine histology, Masson trichrome, and cell viability determined by green fluorescence.

Results. All the constructs maintained their initial shape and dimensions. Angiogenesis was evident in all the constructs with neo-capillary formation within the PLGA-c mesh seen. HDFs proliferated and filled the interyarn spaces of the PLGA-c mesh, while unseeded PLGA-c mesh remained relatively acellular. Cell tracer study indicated that the seeded HDFs remained viable and closely associated to remaining PLGA-c fibers. Collagen formation was more abundant in the constructs seeded with HDFs.

Conclusions. PLGA-c, enveloped by a cell sheet composed of fibroblasts, can serve as a suitable scaffold for generation of a soft tissue flap. A ligated arteriovenous pedicle can serve as a vascular carrier for the generation of a tissue engineered vascularized flap.

Keywords: tissue engineering; prefabricated; vascularized; polylactic-co-glycolic acid (PLGA)

In current clinical practice, there is a constant need for autologous tissue for reconstruction. The pectoralis major flap,1,2 radial forearm flap,3,4 and osteocutaneous fibula flap5 are some of the more commonly used flaps for head and neck reconstruction. The drive by reconstructive surgeons to provide the best functional and aesthetic outcome for their patients has made microvascular free tissue transfer the preferred reconstructive method.6 These flaps have superior versatility when compared with regional pedicled flaps, and their reliability7 makes them increasingly popular. Advances in microsurgical techniques have allowed a wider repertoire of autologous tissue transfer. In recent years, perforator flaps like the anterolateral thigh flap8 have gained popular...
ity. However, a major drawback of using these autologous tissues is donor site morbidity. A steep learning curve often limits its use, especially in the case of perforator flaps. Furthermore, the harvested flap often requires some surgical manipulation and adjustments in spatial positioning in order to obtain the desired shape to fit the defect. Prefabrication can address this problem by creating a 3-dimensional (3D) construct that matches the defect before it is implanted.

Currently, in vitro engineered tissue that has been re-implanted in vivo is either avascular or thin enough to obtain sufficient nutrients by diffusion from surrounding vessels. Tissues that need to be transferred from 1 part of the body to another as 3-dimensional constructs with their own vascular supply need to be prefabricated by either wrapping vascularized soft tissue around the construct or placing a vascular pedicle within the tissue to be transferred so that angiogenic outgrowth from surrounding vessels may allow successful microvascular transfer subsequently. Therefore, a distinct alternative would be to use tissue engineering techniques consisting of a scaffold and mesenchymal stem cells, with a vascular supply. The generated prefabricated flap would address the many pressing problems that harvesting autologous tissue entails and exploit the potential of tissue engineering. Current techniques have shown that generating vascularized soft tissue is possible by either using an arteriovenous (AV) shunt loop or an arteriovenous bundle as a vascular carrier. Polyactic-co-glycolic acid (PLGA) has been shown to be a suitable matrix for seeding human dermal fibroblasts (HDFs) in in vitro studies carried out in our laboratory. PLGA, in the form of Dermagraft (Advanced Tissue Sciences, La Jolla, CA), has also been used clinically to treat diabetic foot ulcers. Poly lactide-co-glycolide (71.2 mg/mL) in the ratio of 100:9. PLGA-collagen (PLGA-c) meshes were left at room temperature for 30 minutes for complete polymerization, deep frozen at −80°C, and freeze dried overnight. PLGA-c was stored in a dry cabinet at 17% relative humidity and sterilized under UV irradiation prior to use. The meshes were flexible and highly porous, with loosely bundled fibers not more than 100 μm apart. Interyarn spaces ranged from 300 to 800 μm wide. Pieces of mesh (8 × 10 mm²) were used for the study.

**MATERIALS AND METHODS**

**Scaffold Preparation.** The PLGA-10/90 fiber was supplied by Shanghai Tianqing Biomaterials Company, Shanghai, China. The PLGA mesh was knitted from continuous fiber yarns of PLGA as described: 24 continuous PLGA single fibers of 20 μm diameter were combined to form a PLGA yarn, which was knitted into a mesh using a Silver Reed SK270 knitting machine. The mesh was folded and fused by heat along the edges to prevent fraying. The double-layered mesh was approximately 2 mm thick. They were stored in a dry cabinet at 17% relative humidity prior to use. Rat tail collagen was extracted from fresh animal carcasses and stored in lyophilized form until use. The collagen is reconstituted (1.3 mg/mL) in 0.05% acetic acid and polymerized onto the PLGA meshes by neutralizing with sodium bicarbonate in 100:9. PLGA-collagen (PLGA-c) meshes were left at room temperature for 30 minutes for complete polymerization, deep frozen at −80°C, and freeze dried overnight. PLGA-c was stored in a dry cabinet at 17% relative humidity and sterilized under UV irradiation prior to use. The meshes were flexible and highly porous, with loosely bundled fibers not more than 100 μm apart. Interyarn spaces ranged from 300 to 800 μm wide. Pieces of mesh (8 × 10 mm²) were used for the study.

**Polycaprolactone Film Preparation.** Polycaprolactone (PCL) was purchased from Sigma-Aldrich. It is a semi-crystalline and biodegradable polymer belonging to a group of aliphatic ester. PCL film was first processed using standard solution casting method, which gave a thickness of approximately 80 to 100 μm. The film was then heat pressed and quenched in ice water and biaxially stretched in a temperature controlled environment, as described by Ng et al. This ultimately produced a relatively thin impervious membrane with a thickness of approximately 10 to 15 μm. The PCL film was cut into 1 cm × 2 cm strips and sterilized in 70% ethanol for 1 hour prior to use.

**Cell Culture and Seeding.** HDFs were derived from enzymatic digestion of human skin samples with 2 mg/mL collagenase type I (Roche, Switzerland), overnight at 37°C. Isolated cells were subsequently cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, NY) supplemented with 10% fetal bovine serum (FBS, Hyclone, UT) and 1% penicillin-streptomycin solution (Gibco, NY) in culture flasks. For routine culture and experi-
ments, all cells were maintained in a self sterilizable incubator (WTB Binder, Tuttlingen, Germany) at 37°C in 5% CO₂, 95% air, and 99% relative humidity, with medium changed every 2 days. HDF at their 7th passage were used for the entire study. Cell viability assay using fluorescein diacetate (Molecular probes, OR) was used. Pelleted HDF at their 7th passage were resuspended in prewarmed PBS containing 5 lM of the probe. Cells were then incubated for 15 minutes at 37°C and re-pelleted by centrifugation at 1000 rpm for 10 minutes and resuspended in complete DMEM. As previously described,19,20 the cells were then counted with a hemocytometer and static seeded at 100,000 cells onto each mesh in 6-well plates. Additionally, HDFs were seeded at a density of 50,000 per cm², over the entire well surface. The well plates were transferred into the incubator and left for 90 minutes to allow for cell attachment. Each well was then filled with 3 mL complete DMEM supplemented with 50 µg/mL L-ascorbic acid (Sigma, St. Louis). The samples were cultured for 10 days, with medium change done every 2 days.

On the day of implantation, the confluent HDF sheets were mechanically peeled off using fine forceps and folded over the matrices to form a PLGA-c/HDF construct (Figure 1). Each construct formed half of the “sandwich” scaffold to be implanted.

A total of 5 pairs of constructs were implanted. Three pairs were PLGA-c/HDF constructs, while 2 plain pairs of PLGA-c mesh served as controls.

**In Vivo Implantation.** The experiment was carried out on 14- to 16-week-old nude rats weighing 200 to 250 g. The operative technique is as follows. Each animal is anesthetized and placed supine. Through a skin incision parallel to the inguinal ligament on one side, the femoral vessels are fully exposed and dissected from the inguinal ligament proximally to the origin of the inferior epigastric vessels. The femoral artery and vein were used as an arteriovenous bundle and were ligated proximal to the origin of the inferior epigastric vessels. The PLGA-c/HDF constructs were then used to sandwich the arteriovenous bundle. PCL film was then wrapped around this entire complex to isolate it from surrounding tissue to prevent vascular in-growth from the surrounding tissue (Figure 2), hence ensuring that the arteriovenous bundle remains as the only vascular supply. Entire complex was anchored to the upper thigh using 8/0 ethilon sutures. Control group had unseeded PLGA-c mesh sandwiched around the vascular pedicle as in the experimental group. Four weeks after implantation, the constructs were harvested for analysis.

**Histology.** For histology, specimens were fixed in 10% buffered formaline, dehydrated in ethanol, embedded in paraffin wax, and routinely sectioned and stained. Sections were stained with hematoxylin–eosin and Masson’s Trichrome to demonstrate collagen formation.

![FIGURE 1. Polyactic-co-glycolic acid-collagen/human dermal fibroblast (PLGA-c/HDF) construct prior to implantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]](image1)

![FIGURE 2. Polyactic-co-glycolic acid-collagen (PLGA-c) mesh “sandwiched” over femoral artery and vein. Polycaprolactone film envelopes the entire construct and edges secured with running ethilon suture. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]](image2)
RESULTS

Gross Morphology. After 4 weeks, the implanted scaffolds were examined for external size, shape, and patency of the pedicle. All the implants showed no significant contraction and their square shape remained (Figures 3A and 3B). The PLGA-c/HDF constructs appeared thicker and more opaque when compared with the unseeded PLGA constructs. The PCL sheet surrounding the construct remained intact. All vascular pedicles remained pulsatile and bled upon truncation.

Histology. The PLGA-c/HDF constructs showed a relatively homogeneous distribution of HDFs. Most of the PLGA-c fibers were surrounded by neotissue and had greater number of cells when compared with the controls. These cells had abundant cytoplasm when compared with the control scaffolds. PLGA-c fibers seeded with HDF appeared to undergo greater degradation when compared with controls (Figure 4A).

FIGURE 3. (A) Gross appearance of plain polylactic-co-glycolic acid-collagen (PLGA-c) generated flap. The construct appears thin and similar to its preimplantation dimensions. (B) Gross appearance of the human dermal fibroblast (HDF)/PLGA-c generated flap. It appears thicker and more opaque than the unseeded controls. Dimensions remained unchanged after 4 weeks of implantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 4. (A) Histology slide (hematoxylin-eosin [H&E]) of plain PLGA-c construct. There are fewer cells within the construct and the cells appear to have less cytoplasm than the seeded constructs. PLGA fibers can clearly be seen (original magnification x40). (B) Histology slide (H&E) of human dermal fibroblast (HDF)/PLGA-c construct. A much more cellular appearance is noted with greater degradation of PLGA fibers seen. There appears to be more cytoplasm within these cells as evident by the eosinophilic cytoplasm (original magnification x40). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
In contrast, plain PLGA-c constructs consisted mainly of PLGA-c fibers with little tissue formation or cells in between the fibers. The PLGA-c fibers remained largely intact with little degradation observed (Figure 4B).

Masson Trichrome stains demonstrated a greater amount of collagen formation in the HDF seeded constructs when compared with the unseeded constructs (Figures 5A and 5B).

Angiogenesis was also evident in both the control and PLGA-c/HDF constructs. Neocapillaries were seen sprouting from the lumen of the femoral vein (see Figure 6). These capillaries were noted within the interyarn spaces and neotissue of both the PLGA-c/HDF as well as the control constructs. New capillaries were also found away from the arteriovenous bundle. No new capillaries were seen sprouting from the arterial side of the pedicle.

Cell tracer studies showed that the labeled HDFs that were used to seed the PLGA-c scaffolds at the start of the experiment remained viable and were adjacent to the remaining scaffolds as evident by their fluorescence (Figures 7A and 7B).

DISCUSSION

Tissue engineering provides a prospect of generating tissue to replace or restore defects that result from surgical extirpation, disease, or congenital anomaly. It allows us to alleviate the problem associated with lack of suitable autologous tissue or donor site morbidity. Over the years we have evolved from just filling a defect to replacing like-for-like tissue, allowing for true functional reconstruction. With improvement in microsurgical techniques, prefabrication of flaps for use at a distant site has become a reality. Therefore, this experiment aims to combine the promise of tissue engineering together with flap prefabrication techniques to produce a tissue engineered prefabricated vascularized flap. This tissue engineered flap would require little intrinsic tissue and hence minimal donor site morbidity. The flap has the potential to
be transferred to a distant site through microsurgical techniques and can be designed to a predetermined shape and size to fit the defect.

Prefabrication of a tissue engineered soft tissue flap requires a scaffold that is capable of supporting the development of dermal-like neotissue. The scaffold should also allow the homogenous proliferation of cells and yet be able to withstand cell contraction forces. Previous in vitro studies have demonstrated that PLGA has properties that allow relatively homogenous distribution and proliferation of cells within the interyarn spaces when compared with AlloDerm. Furthermore, contraction and distortion of PLGA was minimal when compared with TissuFleece. In this in vivo model, results after 4 weeks of implantation showed that the PLGA-c/HDF and plain PLGA-c constructs maintained their original shape and size regardless of whether they were seeded with HDF or not. Effects of capsular contraction were avoided possibly because of the duration of implantation (more extensive contraction might have been seen if implantation was for a longer duration), and the “splinting” effect of the PCL film that was used to isolate the construct from the surrounding tissue. This quality is crucial for prefabricated flaps as contraction and distortion would lead to loss of definition, scarring, and contracture. Scaffolds must therefore possess mechanical properties that would be able to resist contraction forces of both the seeded cells within the scaffold as well as cells surrounding the construct. One of the greatest advantages of using synthetic materials as scaffolds is the possibility of using various processing techniques to fabricate matrices of desired form, mechanical strength, and degradation profiles to achieve specific tissue engineering goals. Future studies should assess the performance of this construct in an immunocompetent model as capsular formation and inflammation may affect the results.

The PLGA-c fibers served as suitable scaffold for the continued survival and proliferation of HDFs. These fibroblasts that proliferated within the construct were able to induce more collagen production than the unseeded PLGA-c constructs. The HDFs were also generally homogenously distributed within the construct with high cell counts per high-powered field when compared with unseeded scaffolds. Continued proliferation and survival of HDFs in PLGA-c scaffold makes it a suitable cell carrier and model for a resorbable scaffold that would be replaced by proliferating autologous cells. This generated construct may therefore serve as a dermal substitute. Current in vitro studies in our laboratory have seen some success in adding an epithelial layer over the dermal construct and further studies in an in vitro model will hopefully lead to the development of a more complex soft tissue flap.

Tissue engineered constructs are often limited by their intrinsic lack of blood supply. Most constructs either rely on surrounding tissue for blood supply or are avascular. Recent work suggests that by combining a vascular supply, either in the form of a pedicle or an arteriovenous loop, neotissue can be generated. It is a natural extension, therefore, to try to combine this dermal substitute with a vas-
cular supply such that a potentially transferable tissue engineered prefabricated flap is produced. This experiment demonstrated luminal sprouting and vascularization of the constructs. This phenomenon was also present in the PLGA-c constructs that were unseeded. Hence, it shows that a ligated pedicle is a suitable model for providing a vascular supply to an otherwise avascular construct. More importantly, vascularization would allow for larger and more complex constructs, make these vascularized constructs “transferable” to a distant site, and enhance a construct’s viability by possessing its own blood supply.

CONCLUSION

PLGA-c enveloped by a cell sheet composed of fibroblasts can serve as a suitable scaffold for generating a soft tissue flap. Documented angiogenesis and proliferation of seeded cells within this scaffolds show that a ligated arteriovenous pedicle can serve as a suitable vascular carrier. It is hoped that through such novel techniques, we may move closer to developing a prefabricated vascularized soft tissue flap for clinical use. Ultimately, with advancement in microsurgical techniques and further progress in tissue engineering, these tissue engineered prefabricated vascularized flaps may help overcome donor site morbidity, enhance reconstructive options, and potentially overcome technical difficulties in raising free flaps.

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