Methods 84 (2015) 70–75

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

A method for constructing vascularized muscle flap

Yulia Shandalov [a,1], Dana Egozi [b,1], Alina Freiman [a,c], Dekel Rosenfeld [a], Shulamit Levenberg [a,*]

[a] Biomedical Engineering Department, Technion-Israel Institute of Technology, Haifa 32000, Israel
[b] Department of Plastic and Reconstructive Surgery, Kaplan Hospital, Rehovot, Israel
[c] Inter-departmental Program in Biotechnology, Technion-Israel Institute of Technology, Haifa 32000, Israel

ARTICLE INFO

Article history:
Received 12 November 2014
Received in revised form 10 February 2015
Accepted 26 March 2015
Available online 2 April 2015

Keywords:
Flap
Tissue engineering
Scaffold
Vascularization
Vascularized tissue
Muscle

ABSTRACT

Abdominal wall reconstruction following extensive tissue loss is essential and can be achieved using autologous flaps. However, their use is limited due to their inadequate availability and due to post-operative donor site scarification. This work presents a step-by-step technique for fabrication of a vascularized muscle flap, to be applied in full-thickness abdominal wall defect reconstruction. Poly L-lactic acid/poly lactic-co-glycolic acid scaffolds, prepared using a salt leaching technique, were used as the supporting matrix *in vitro* for simultaneously seeded endothelial cells, fibroblasts and myoblasts. The cell-embedded graft was then implanted around femoral artery and vein vessels, which provided a central blood supply. Vascularization and perfusion were achieved by capillary sprouting from the main host vessel into the graft. A thick and vascularized tissue was formed within one week, and was then transferred as an autologous flap together with its main vessels, to a full-thickness abdominal wall defect. The flap remained viable after transfer and featured sufficient mechanical strength to support the abdominal viscera. Thus, this engineered muscle flap can be used as an alternative source for autologous flaps to reconstruct full-thickness abdominal wall defects.

1. Introduction

Abdominal wall defects caused by trauma, infection or tumor ablation, involve significant tissue loss [1]. Despite recent progress in regenerative medicine and the introduction of new technologies and materials, reconstruction of large full-thickness defects remains a surgical challenge [2]. Conventional methods, including the Ramirez components separation technique, and synthetic mesh (such as Dexon (polyglycolic acid) and Vicryl (polyglactin 910)) [3–5] and biomaterials (such as Alloderm and Surgisis) reconstruction [6–8], all pose technical and/or clinical limitations. The abdominal wall is composed of skin, subcutis, fascia and muscle tissue, each displaying different textile characteristics and physiologic elasticity. The intra-abdominal pressure is mainly determined by the activity of the transverse muscles. The abdominal fascia withstands forces of 60–80 N/cm in horizontal and 20–30 N/cm in vertical directions [9]. Component separation may not be suitable in cases where the defect is too large and there is an insufficient amount of tissue around the defects, mesh implantation can lead to inflammation, scarring and adhesions, associated with lengthy healing times and with poor aesthetic outcomes, while acellular dermis reconstruction offers some advantages, but is expensive and unsuitable due to its failure to provide sufficient strength over time [5,10,11]. The applicability of grafts and autologous flaps has been investigated. While, grafts do not bear their own vasculature and are consequently ineffective in repairing large defects [12–15], flaps are autologous tissues which can be transferred with their own blood supply, and are therefore advantageous in repair of large defects. However, the scant availability of high-quality vascularized flaps, and donor site morbidity often limit their use [16]. Despite the continuous progress in tissue engineering [17], fabrication of thick engineered tissue with its own blood supply remains a challenge.

Herein, we describe a method for creating a thick, well-vascularized tissue flap to be exploited for reconstruction of full-thickness abdominal wall defects [1]. The flap was constructed by seeding endothelial cells (ECs) that have been demonstrated to form vascular networks similar to that formed by human embryonic stem cell-derived endothelial cells [18], myoblasts, capable of differentiating into multi-nuclear myotubes [19] and fibroblasts, as supporting cells, within a biodegradable PLLA/PLGA scaffold, which was then cultured *in vitro* to allow the cells to self-assemble. PLLA and PLGA are both approved by US Food and Drug Administration (FDA) for clinical use. Previous work has shown that Surgisis scaffolds (SIS) can also be used to engineer

* Corresponding author.
E-mail address: Shulamit@bm.technion.ac.il (S. Levenberg).
[1] Equally contributed.

http://dx.doi.org/10.1016/j.ymeth.2015.03.021
1046-2023/

vascular networks *in vitro* and *in vivo* [20], however, in this study, the PLLA/PLGA co-polymer was chosen due to the contribution of PLLA on scaffold stiffness and of PLGA to scaffold flexibility [21]. Moreover, the PLLA/PLGA scaffold is more porous than the Surgisis scaffold, and thus, the cells can spread homogenously throughout all scaffold layers. A 1:1 ratio was shown to provide the mechanical properties most desirable for the planned application [21].

The engineered tissue was then implanted around the femoral artery and vein (AV) vessels of the mouse, where is underwent anastomosis with host vessels and became highly vascularized. The graft was then transferred as a pedicled flap, to a full-thickness abdominal wall defect, where it proved viable and well vascularized, provided mechanical support to the abdominal wall, and became well integrated in the surrounding tissue.

2. Description of methods

2.1. Preparation of biocompatible scaffold

Scaffolds were prepared with poly-L-lactic-acid (PLLA; Polysciences Inc.) and polylactic-co-glycolic-acid (PLGA; Boehringer-Ingelheim). A 5% (w/v) polymer solution of each was prepared separately, by dissolving 0.5 g polymer in 10 ml chloroform, in a small glass tube which was placed on a magnetic stirring plate over-night (ON) (Fig. 1A). The polymers were then mixed together in a 1:1 ratio, to create a PLLA/PLGA solution (Fig. 1B). NaCl was sieved to 212–600 μm particles and 0.4 g was poured into Teflon cylinder molds (18 mm internal diameter) and dissolved in 0.24 ml PLLA/PLGA solution (Fig. 1C). This range of NaCl particle size was used, as they form a sufficiently large interconnected pore network that allows cells to associate with each other. On the other hand, the pores are not too big, which is critical toward maintenance of the mechanical strength optimal for *in vivo* implantation. The Teflon molds were left ON to allow for chloroform evaporation, after which, the scaffolds were gently removed from the molds and placed into histology cassettes (Fig. 1D). The salt was leached by placing the histology cassettes in a beaker filled with distilled water, on a magnetic stirring plate; the water were exchanged every hour for 6–8 h (Fig. 1E). The scaffolds were then removed from the histology cassettes, dried on a Kimwipe and frozen ON at −80 °C, in a 50 ml tube. Finally, the scaffolds were lyophilized ON and kept dry under vacuum until use (Fig. 1F).

2.2. Cell seeding and graft preparation

C2C12 myoblasts (American Type Culture Collection, VA, USA), human umbilical vein endothelial cells (HUVECs, Lonza) and normal human dermal fibroblasts (NHDF, Lonza) were prepared for seeding by preparing a tri-culture of 0.5×10^6 C2C12, 0.9×10^6 HUVECs and 0.2×10^6 NHDFs in 1:1 endothelial cell medium (EGM-2 supplemented with the components of its bullet kit (Lonza, USA)) and muscle cell medium (Dulbecco's minimal essential medium (DMEM; Gibco® Life Technologies), supplemented with 10% fetal bovine serum (FBS; HyClone, Thermo Fisher Scientific, USA), 2.5% HEPES buffer (Biological Industries, Israel), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Pen-Strep Solution, Biological Industries, Israel) (hereafter, cell medium). The cells were mixed together in an Eppendorf tube and centrifuged at 200*g* for 4 min. The medium was then aspirated and the pellet was re-suspended in a mixture of 4 μl ice cold Matrigel and 4 μl cell medium (Fig. 1H). The cell suspension was then seeded on a piece of 10 mm × 7 mm × 1 mm (length × width × thickness) PLLA/PLGA scaffold and allowed to solidify (30 min, 37 °C, 5% CO_2) in a 6-well plate, before 4 ml cell medium were added. The scaffolds were then incubated for ten days before implantation and the medium was replaced every other day. All incubations were performed in a 5% CO_2-humidified atmosphere, at 37 °C. Scaffolds were cultured *in vitro* for ten days, until optimal vascularization within the scaffold was achieved [18].

2.3. Flap construction (graft implantation)

All animal studies were approved by the Committee on the Ethics of Animal Experiments of the Technion. Athymic nude male mice (7–9-week-old, Harlan Laboratories Inc., Israel) were used.

Prior to graft implantation, the mouse was anesthetized by an intraperitoneal injection (i.p) (35 μL/20 g) of a ketamine:xylazine (6:1) mixture. The mouse was then placed on a heated stage (37 °C), to maintain body temperature, and then secured using an adhesive bandage. The incision site was cleaned with iodine and then alcohol, to establish an aseptic working field. An incision was made, using a small scissor, through the skin and the femoral artery and vein vessels were then exposed from the level of the inguinal ligament to the knee area (Fig. 2A). The profunda was left untouched, in order to preserve blood flow to the leg and to prevent ischemia. The femoral artery and vein were used as an arteriovenous (AV) bundle. The skin was held down with clips or a needle, to provide better access to the tissues. The femoral artery and vein vessels were carefully isolated from the surrounding tissue, from the level of the profunda bifurcation to the knee area, proximal to the popliteal vessels. The graft was folded around the exposed femoral AV and its ends were sutured using 8-0 silk sutures (Fig. 2B). To ensure implant vascularization by the capillaries sprouting solely from the femoral AV bundle, and not from capillaries of the surrounding tissues, a piece of sterilized latex was wrapped around the graft, and sutured with 8-0 silk sutures (Fig. 2C). The overlying skin was then closed, using 4-0 silk sutures (Fig. 2D). A subcutaneous injection of buprenorphine was administered immediately after the operation and twice a day for 2–3 days thereafter. All mice were closely monitored until they recovered from the anesthesia and every day thereafter, for one week, until grafts were transferred as flaps.

2.4. Graft analysis-determination of graft vascularization

2.4.1. Confocal imaging of graft vascularization

One week post-implantation, the mouse was anesthetized by an i.p injection (35 μL/20 g) of a ketamine:xylazine (6:1) mixture and 10 mg/ml fluorescein isothiocyanate-conjugated dextran (FITC-Dextran, Sigma–Aldrich) was intravenously injected into the tail vein. Upon completion of the injection, the mice were euthanized, and the leg of the mouse was opened, exposing the graft, which was then imaged using confocal microscopy. The grafts were then excised and transferred to 10% buffered formalin (Sigma–Aldrich) for histological or immunohistological analysis, and embedded in paraffin using standard fixation and embedding procedures.

2.4.2. Masson's trichrome staining of the graft

2.4.2.1. Preparation of the trichrome solution. The following ingredients were dissolved: 0.3 g Fast green FCF (Sigma), 0.4 g Chtomotrope 2R (Sigma), 0.6 g Phosphotungstic Acid (Sigma–aldrich) in 100 ml distilled water containing 1 ml of acetic acid (glacial; Frutarom). The pH was then adjusted to 3.4, using NaOH.

2.4.2.2. Preparation of Mayer's hematoxylin solution. 50 g of aluminum potassium sulfate (Sigma) were dissolved in 1 L distilled water before adding 1 g hematoxylin (Fluka). 0.2 g sodium iodate (Riedel-de-Haen) and 20 ml acetic acid were then added and the