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Engineered composite tissue as a bioartificial limb graft

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ABSTRACT

The loss of an extremity is a disastrous injury with tremendous impact on a patient's life. Current mechanical prostheses are technically highly sophisticated, but only partially replace physiologic function and aesthetic appearance. As a biologic alternative, approximately 70 patients have undergone allogeneic hand transplantation to date worldwide. While outcomes are favorable, risks and side effects of transplantation and long-term immunosuppression pose a significant ethical dilemma. An autologous, bioartificial graft based on native extracellular matrix and patient derived cells could be produced on demand and would not require immunosuppression after transplantation. To create such a graft, we decellularized rat and primate forearms by detergent perfusion and yielded acellular scaffolds with preserved composite architecture. We then repopulated muscle and vasculature with cells of appropriate phenotypes, and matured the composite tissue in a perfusion bioreactor under electrical stimulation *in vitro*. After confirmation of composite tissue formation, we transplanted the resulting bio-composite grafts to confirm perfusion *in vivo*.

1. Introduction

In the United States, over 1.5 million people live with limb loss [1]. Amputation is a severe socioeconomic challenge for most patients, causing emotional trauma equivalent to the loss a family member [2–4]. Therapeutic options after limb loss include reconstructive surgery using autologous tissue, or the use of prosthetic devices ranging from purely aesthetic prostheses to those with a focus on function [5]. Although current prostheses are technically highly sophisticated devices, they only fulfill a minimum of physiologic function and many offer less than satisfactory aesthetics [5]. The vast majority of patients consider the option of prosthesis, but amputees who suffer from large defects such as bilateral above elbow amputations adapt poorly and are usually dependent on others for personal care and hygiene [6]. As a new approach, worldwide about 70 patients have received allogeneic hand transplants since 1998 [7]. Hand transplantation significantly improved the quality of life of upper limb amputees and eventually demonstrated hand function superior to that obtained with prosthetics [6,8,9]. However, side effects and potentially life-threatening complications of long-term immunosuppression pose a significant ethical dilemma regarding this non life-saving reconstructive procedure [5,9–11]. A reduction of donor related risk factors, and elimination of long term immunosuppression would allow wider application of such reconstructive treatment options [6]. Creation of an autologous, bioartificial forearm graft from patient derived cells would therefore be a valid alternative to allogeneic grafts. Cellular candidates to regenerate the required tissues such as muscle progenitor cells, endothelial progenitor cells, and mesenchymal stem cells can be isolated from patients [12–14]. However, engineering of a composite tissue graft of the complexity of a hand or a forearm has been impossible to date due to the lack of appropriate scaffold materials to support the engraftment of several cell phenotypes and the formation of viable and functional tissue in its physiologic three dimensional context. A recent report

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of successful clinical implantation of acellular biological scaffolds into patients suffering from volumetric muscle loss underlines the huge potential of this principle for reconstructive surgery [15].

Using perfusion decellularization, we have shown that complex cadaveric organs can be rendered acellular, resulting in native extracellular matrix (ECM) scaffolds with intact tissue architecture that can be repopulated with cells to engineer functional tissue [16,17]. To investigate if these methods can be applied to complex composite tissues such as limb grafts, we isolated rodent and primate upper limbs, and perfused these with a sequence of detergent and washing solutions via the native vascular system. Perfusion decellularization led to the removal of cellular material in all respective tissue compartments, while retaining the mechanical properties of the musculoskeletal system. Repopulation of acellular composite tissue grafts with muscle progenitor, endothelial and mesenchymal cells resulted in formation of vascularized, muscle-like tissue within its native histological compartment. To enhance the formation of functional muscle-like tissue, we cultivated repopulated limb grafts in a biomimetic bioreactor system, including vascular perfusion and electrical stimulation. Finally, we tested functionality of engineered muscle in terms of isometric force measurement and patency of the vascular system by orthotopic limb transplantation.

2. Materials and methods

2.1. Perfusion decellularization

Research animals were cared for in accordance with the guidelines set by the Committee on Laboratory Resources, US National Institutes of Health, and Subcommittee on Research Animal Care and Laboratory Animal Resources of Massachusetts General Hospital. Male Sprague Dawley rats (Charles River Laboratories) were euthanized with 100 mg/kg ketamine (Phoenix Pharmaceutical) and 10 mg/kg xylazine (Phoenix Pharmaceutical) injected intraperitoneally. After systemic heparinization (American Pharmaceutical Partners) through the IVC, the dissection of the skin of the whole upper limb allowed us to identify the brachial artery, the brachial vein and the nerve plexus. After dissecting the upper limb from the shoulder the brachial artery was cannulated with a prefilled 25G cannula (Luer Stubs, Harvard/Instech) using a surgical microscope. Fasciotomies were performed before flushing the forearm with phosphate buffered saline (PBS). After flushing with 5 ml PBS the isolated forearm was mounted into the organ chamber and perfusion was started with 1% SDS (Sigma) for up to 50 h at a constant flow perfusion of 1 ml/min. This was followed by deionized water for 1 h and 1 h of perfusion with 1% Triton-X100 (Sigma). To wash out all debris, antibiotic-containing PBS (100 U/ml penicillin-G; Sigma, 0.25 mg/ml streptomycin; Sigma and amphotericin B; Sigma) was used to perfuse the forearm for 124 h.

2.2. Recellularization of decellularized forearms

After washing with PBS for 124 h, decellularized rat forearms were removed from the decellularization chamber and mounted in a biomimetic stimulation bioreactor system under sterile conditions. Prior to cell seeding, we perfused forearm matrixes with 37 °C oxygenated C_2C_{12} growth medium for at least 1 h at constant flow perfusion of 1 ml/min under standard culture conditions (37 °C in 5% CO_2). The biomimetic simulation bioreactor contains an organ chamber, which also serves as the main reservoir, in which the decellularized forearm is mounted. The bioreactor works as a closed-circuit system in which medium is perfused into the brachial artery by a constant flow pump (Ismatec). At day 0 we seeded the forearm matrix with 5×10^6 HUVECs by gravity infusion (100 cm H_2O) into the brachial artery suspended in 75–100 ml Endothelial Cell Growth media (EGM-2 Bulletkit; Lonza). After a 60-min static period to allow for cell attachment, we restarted perfusion. For regeneration of muscle tissue we injected a cell mixture of 10×10^6 C_2C_{12} cells, 0.5×10^6 mouse embryonic fibroblasts and $X \times 10^6$ HUVECs suspended in 1 ml of growth medium trough twenty injections in the compartments of the decellularized forearm with a 27-G needle and a 1-cc tuberculin syringe. After cell seeding we mounted the forearm in the biomimetic stimulation bioreactor. We applied no electrical stimulation in the first 5 days. We perfused the forearm with C_2C_{12} growth media from day 0 to day 3. We changed the medium every 48 h. On day 3, we switched the medium to C_2C_{12} differentiation medium supplemented with 3% horse serum and EGM2 bulletkit (Lonza). At day 6 we started electrical stimulation by applying 6-ms pulses of 20 V and 1 Hz with a Grass S48 square pulse stimulator (Grass Technologies). Skin transplantation was performed as follows. Full thickness skin grafts were harvested from the upper extremities of deceased Sprague Dawley rats. After removal of access tissue, full thickness skin grafts were meshed to allow for better attachment and perfusion. After washing in PBS, full thickness mesh grafts were transplanted onto the engineered constructs using sutures and fibrin glue (Tisseel, Baxter). We maintained the matrix in culture for up 21 days.

2.3. Cell culture

2.3.1. Myoblasts

Mouse skeletal myoblasts (C_2C_{12}) were purchased from ATCC and expanded in DMEM (Gibco) supplemented with 10% FBS, and 1% HyClone Antibiotic Antimycotic solution (Thermo Scientific) on gelatin coated cell-culture plastic (BD Biosciences). Cells were cultured under standard culture conditions (37 °C in 5% CO_2). Medium changed every other day. To avoid myotube formation, cells were passaged with 0.05% trypsin/EDTA (cellgro-25052CI) at 70–80% confluency. For C_2C_{12} differentiation, we supplemented DMEM (Gibco) with 2% horse serum (Gibco) and 1% antibiotic antimycotic solution (10,000 units/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml Amphotericin B; HyClone). C_2C_{12} were expanded until passage 6–10.

2.3.2. MEFs

Mouse embryonic fibroblasts were purchased from ATCC and cultured in DMEM, (Gibco) supplemented with 10% FBS and 1% Pen/Strep, (Sigma) until passage 4–6 on gelatin coated cell-culture plastic (BD Biosciences).

2.3.3. HUVECs

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and expanded in EBM2 endothelial cell media (Lonza) supplemented with EGM-2 bulletkit until passage 6–10 on gelatin coated cell-culture plastic (BD Biosciences).

2.4. Electrical stimulation bioreactor

We designed and custom built an electrical stimulation bioreactor based on our perfusion bioreactors. Decellularized forearms were mounted into the bioreactor by clamping the humeral head into a tissue clamp (Mueller, Germany), stabilizing the whole limb. Carbon rods were submerged into the culture medium for electrical field stimulation of the engineered graft. Electrical pulses were generated with a square pulse stimulator (Grass S48, Grass Technologies).