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Full Length Article

Bioengineering vascularized tissue constructs using an injectable cell-laden enzymatically crosslinked collagen hydrogel derived from dermal extracellular matrix

Kuan-Chih Kuo [a], Ruei-Zeng Lin [b], Han-Wen Tien [a], Pei-Yun Wu [a], Yen-Cheng Li [c], Juan M. Melero-Martin [b], Ying-Chieh Chen [a,*]

[a] Department of Applied Science, National Hsinchu University of Education, Hsinchu 30014, Taiwan, ROC
[b] Department of Cardiac Surgery, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA
[c] Material and Chemical Research Laboratories, Industrial Technology Research Institute, Hsinchu 31040, Taiwan, ROC

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ABSTRACT

Tissue engineering promises to restore or replace diseased or damaged tissue by creating functional and transplantable artificial tissues. The development of artificial tissues with large dimensions that exceed the diffusion limitation will require nutrients and oxygen to be delivered via perfusion instead of diffusion alone over a short time period. One approach to perfusion is to vascularize engineered tissues, creating a *de novo* three-dimensional (3D) microvascular network within the tissue construct. This significantly shortens the time of *in vivo* anastomosis, perfusion and graft integration with the host. In this study, we aimed to develop injectable allogeneic collagen-phenolic hydroxyl (collagen-Ph) hydrogels that are capable of controlling a wide range of physicochemical properties, including stiffness, water absorption and degradability. We tested whether collagen-Ph hydrogels could support the formation of vascularized engineered tissue graft by human blood-derived endothelial colony-forming cells (ECFCs) and bone marrow-derived mesenchymal stem cells (MSC) *in vivo*. First, we studied the growth of adherent ECFCs and MSCs on or in the hydrogels. To examine the potential formation of functional vascular networks *in vivo*, a liquid pre-polymer solution of collagen-Ph containing human ECFCs and MSCs, horseradish peroxidase and hydrogen peroxide was injected into the subcutaneous space or abdominal muscle defect of an immunodeficient mouse before gelation, to form a 3D cell-laden polymerized construct. These results showed that extensive human ECFC-lined vascular networks can be generated within 7 days, the engineered vascular density inside collagen-Ph hydrogel constructs can be manipulated through refinable mechanical properties and proteolytic degradability, and these networks can form functional anastomoses with the existing vasculature to further support the survival of host muscle tissues. Finally, optimized conditions of the cell-laden collagen-Ph hydrogel resulted in not only improving the long-term differentiation of transplanted MSCs into mineralized osteoblasts, but the collagen-Ph hydrogel also improved an increased of adipocytes within the vascularized bioengineered tissue in a mouse after 1 month of implantation.

Statement of Significance

We reported a method for preparing autologous extracellular matrix scaffolds, murine collagen-Ph hydrogels, and demonstrated its suitability for use in supporting human progenitor cell-based formation of 3D vascular networks *in vitro* and *in vivo*. Results showed extensive human vascular networks can be generated within 7 days, engineered vascular density inside collagen-Ph constructs can be manipulated through refinable mechanical properties and proteolytic degradability, and these networks can form functional anastomoses with existing vasculature to further support the survival of host muscle tissues. Moreover, optimized conditions of cell-laden collagen-Ph hydrogel resulted in not only improving the long-term differentiation of transplanted MSCs into mineralized osteoblasts, but the collagen-Ph hydrogel also improved an increased of adipocytes within the vascularized bioengineered tissue in a mouse.

* Corresponding author at: Department of Applied Science, National Hsinchu University of Education, No. 521, Nanda Rd., Hsinchu City 30014, Taiwan, ROC.
E-mail address: yingchiehchen@gmail.com (Y.-C. Chen).

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1. Introduction

Creating large functional and transplantable tissues for organ replacement have failed in the past because of the limited diffusion distance of oxygen and nutrients to a few hundred micrometers. This can lead to necrosis at the central region of the large-sized tissue-engineered or highly vascularized tissues [1–3]. Host blood vessels usually take days or weeks to invade the centre of engineered tissue, so during this process an insufficient vascular supply leads the tissue to nutrient depletion and ischemia, which can compromise cell viability and function [2,4,5]. To overcome this problem, nutrients and oxygen need to be delivered via perfusion instead of diffusion alone [3]. Aside from current strategies promoting angiogenesis from the host, an alternative concept is to pre-vascularize a tissue that creates a vascular network within the tissue construct prior to implantation [6]. Vascularization generally refers to the formation of an *in vitro* well-connected microvessel network within an implantable tissue construct through vasculogenesis. Following implantation, this vasculature can rapidly anastomose with the host and enhances tissue survival and function [1,3]. Furthermore, microfabrication techniques have been applied as a means to control space and direct the growth of vascular networks *in vitro* [7–10]; however, these pre-engineered networks are too simple to grow and reform in response to specific physiological demands from the organs they are supporting in the host [1,11]. Therefore, we have demonstrated the formation of vascular networks, *de novo*, from encapsulating endothelial colony-forming cells (ECFCs) and mesenchymal stem cells (MSC) in the liquid matrix prior to gelation, and injected or implanted subcutaneously in immune-deficient mice to form a 3D cell-laden vascularized construct within one week [12–14]. ECFCs circulating in peripheral blood participate in the formation of new blood vasculature and have been a promising source in producing non-invasive large quantities of autologous endothelial cells for clinical use [13,15]. Together with suitable support from scaffolds, MSCs can function as pericytes to promote vessel formation and maturation through secretion of specific pro-angiogenic cytokines [14,16]. Meanwhile, transplanted ECFCs provided critical angiocrine factors needed to preserve MSC as viable and further support ultimately long-term differentiation of transplanted MSCs to osteoblasts to form vascularized engineered bone tissue constructs by inducing specific stimulants of BMP-2 [12].

A critical requirement for engineering a tissue is the use of a suitable scaffold to mimic structural and functional properties of the natural extracellular matrix (ECM) that includes providing appropriate binding sites for cell–material interactions, mechanical properties to maintain cell function prior to host remodeling without negatively impacting the development of the capillary network, and biodegradation that matches the deposition rate of new extracellular matrix protein by the host [6]. Over the last few years, a variety of natural-based hydrogels [17] have been shown to be compatible with endothelial cell-mediated vascular morphogenesis, including natural materials (type I collagen gel [18–22], fibrin gels [19,23], Matrigel [12,24]), semi-synthetic materials, i.e. modified natural material (photo-crosslinkable methacrylated gelatin [10,14,16] and enzymatic-crosslinkable tyramine-modified gelatin hydrogels [25,26]). However, there still remains a significant discrepancy in how physicochemical properties of scaffolds, such as mechanical properties, density of cell-adhesive ligand (RGD) or degradable sites (MMP) of scaffolding affect the angiogenic potential of endothelial cells (EC) to form functional blood vessels *in vivo*. RGD-mediated and integrin-dependent bindings on native collagen nanofibrins support the development of sufficient matrix-transduced tensional forces necessary for EC sprouting and tube formation [27]. The stiffness of self-assembled collagen matrices affects cell spreading, migration, growth and matrix remodeling, all of which are critical components of vascular formation [20,28]. Varying collagen concentration of polymerized 3D collagen gels that alter matrix stiffness and fibril density has been shown to alter EC lumen size and tube length [18–20,28,29], but in this case the RGD is dependent on collagen concentrations. These issues raised the difficulties of discerning which matrix properties induce EC responses. The aforementioned results provide initial evidence that specific collagen matrix physical properties are important parameters in determining the ability of matrices to guide vessel formation. However, most studies [19,26,28,29] have not been followed up with investigations addressing whether physicochemical properties of matrices impact upon vessel formation and if they further influence the success of creating vascularized tissue construct *in vivo*, which would lead to more clinically relevant cell-based therapies.

So far, there have only been a few successful thick vascularized engineered tissue constructs, because the lack of proper scaffolding material that cannot only support vascularization in a short time period, but also directs transplanted MSCs differentiation into a specific lineage, remains a major challenge [4,5,11]. Although collagen offers an ideal injectable gel system in vascular tissue engineering, the extensive contraction, poor stiffness, rapid degradation and temperature instability of collagen gels limit their practical applications in regenerating and engineering living tissues, such as adipose or bone tissue grafts [22,30]. Thus, the development of collagen-based scaffolds that can maintain the mechanical integrity of the tissue, support the development and growth of complex microvessel networks, while simultaneously improving the mature differentiation of stem cells to meet the functional requirements of specific tissue, is more desirable. In response to these limitations, we aimed to develop chemical functionalization of natural-derived ECM proteins, i.e. injectable collagen-Ph hydrogels, which are capable of having controlled physicochemical properties over a wide range. To test the biocompatibility and potential for applications in tissue engineering, the ECFCs and MSCs were seeded on or inside collagen-Ph hydrogels to study the cell adhesion, proliferation and function *in vitro*. Pre-clinical studies with immunodeficient mice were carried out and demonstrated that functional human vascular networks can be generated *in situ* by means of post-injectable enzymatic polymerization that enables tuning of the final vascular density inside the collagen-Ph hydrogel constructs. We hypothesized that this cell-laden collagen-Ph construct would rapidly anastomose with host vasculature and improve vascularization and survival of the host abdominal muscle defect with the formation of ECFC-lined vascular networks throughout and in-between skeletal muscle fibers in the host. Finally, feasibility studies toward an ultimate goal of bioengineered 3D vascularized tissue grafts were developed and characterized. Our studies importantly suggest the capacity of ECM-based cell delivery hydrogel systems to incorporate physicochemical cues to modulate vessel formation and further evaluate the feasibility to engineer vascularized transplantable artificial tissues *in vivo*.

2. Materials and methods

2.1. Extraction of murine collagen-Ph hydrogels from epidermis

Before collagen extraction, murine epidermal tissue was cut into small pieces (<25 mm^2) and soaked into 70% ethanol for 2 min at a skin/solution ratio of 1:4 (w/v) to remove debris and sterile murine epidermis at room temperature. Collagen was extracted by incubating small pieces of tissue with a skin/pepsin

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