Journal of Controlled Release 205 (2015) 83–88

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Construction of three-dimensional vascularized cardiac tissue with cell sheet engineering

Katsuhisa Sakaguchi [a], Tatsuya Shimizu [b], Teruo Okano [b,*]

[a] Faculty of Science and Engineering, TWIns, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan
[b] Institute of Advanced Biomedical Engineering and Science, TWIns, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

ARTICLE INFO

Article history:
Received 20 October 2014
Received in revised form 10 December 2014
Accepted 14 December 2014
Available online 16 December 2014

Keywords:
Cell sheet
Tissue engineering
Vascularization
Bioreactor

ABSTRACT

Construction of three-dimensional (3D) tissues with pre-isolated cells is a promising achievement for novel medicine and drug-discovery research. Our laboratory constructs 3D tissues with an innovative and unique method for layering multiple cell sheets. Cell sheets maintain a high-efficiently regenerating function, because of the higher cell density and higher transplantation efficiency, compared to other cell-delivery methods. Cell sheets have already been applied in clinical applications for regenerative medicine in treating patients with various diseases. Therefore, in our search to develop a more efficient treatment with cell sheets, we are constructing 3D tissues by layering cell sheets. Native animal tissues and organs have an abundance of capillaries to supply oxygen and nutrients, and to remove waste molecules. In our investigation of vascularized cardiac cell sheets, we have found that endothelial cells within cell sheets spontaneously form blood vessel networks as *in vivo* capillaries. To construct even thicker 3D tissues by layering multiple cell sheets, it is critical to have a medium or blood flow within the vascular networks of the cell sheets. Therefore, to perfuse medium or blood in the cell sheet vascular network to maintain the viability of all cells, we developed two types of vascular beds; (1) a femoral muscle-based vascular bed, and (2) a synthetic collagen gel-based vascular bed. Both vascular beds successfully provide the critical flow of culture medium, which allows 12-layer cell sheets to survive. Such bioreactor systems, when combined with cell sheet engineering techniques, have produced functional vascularized 3D tissues. Here we explain and discuss the various processes to obtain vascular networks by properly connecting cell sheets and the engineering of 3D tissues.

1. Introduction

Rapid progress has been made recently in tissue engineering toward new medical treatments and drug screening procedures since the discovery of embryo stem cells and induced pluripotent stem cells. Various tissue engineering technologies are currently being used, such as "top down" approaches where cells are seeded to biodegradable scaffolds [1,2], and "bottom up" approaches where cells are shaped into fiber and spheroid forms to construct organs [3–5]. In our laboratory, cells are shaped into a sheet, which has a highly efficient regenerating function because of its higher cell density and transplantation efficiency, compared with other cell-delivery methods [6–8]. Consequently, cell sheet engineering has attracted the attention of researchers around the world as a novel bottom-up approach.

Cell sheet engineering has only become possible since the development of new culture dishes. The surface of these new culture dishes alters the surface characteristics from hydrophobic to hydrophilic by simply changing the temperature, which allows cells to attach or detach from the surface. The surface of temperature-responsive culture dishes is grafted with a nanoscale-thick polymer layer (~20 nm) of poly (*N*-isopropylyacrylamide) by electron beam irradiation. At 37 °C, the dish surface becomes hydrophobic and allows cells to attach; in contrast, under 32 °C, the surface becomes hydrophilic and allows cells to detach from the surface while preserving the cell membrane connections and cell–cell communication [9].

Cell sheet engineering has already been successfully used in clinical applications of regenerative medicine for the cornea, heart, esophagus, periodontal membrane, and cartilage [10–15]. Future progress of cell-sheet treatments for serious injury and tissue deficiency, such as organ transplantation therapy, now depends on the development of 3D tissues. Therefore, we are now constructing not only single-layer cell sheets, but layering many cell sheets to fabricate highly functional three-dimensional and multi-layered cell-sheet tissues. However, the thickness of a multi-layered cell sheet has limitations, because of the possible necrosis inside the cell sheet due to the lack of capillaries to supply oxygen and nutrients [16,17]. Our laboratory found that endothelial cells spontaneously form blood vessel networks as capillaries in a single-layer cell sheet, as well as between sandwiched cell sheets. Furthermore, methods to optimize endothelial network formation are also

* Corresponding author.
E-mail address: tokano@abmes.twmu.ac.jp (T. Okano).

http://dx.doi.org/10.1016/j.jconrel.2014.12.016
0168-3659/

being investigated by sandwiching oriented cell sheets. In this article, we explain and discuss the processes we use for constructing 3D tissues using cell sheet engineering and vascular bed bioreactors.

2. Vascular network formation in cell sheets

Since native *in vivo* capillaries have multiple functions such as supplying oxygen and nutrients for maintaining tissue viability, engineered 3D tissues also need capillaries to maintain their functions and viability. We have developed a functional cardiomyocyte cell sheet with the ability to contract, because the cell membrane functions were preserved [18]. The challenge to create thicker cardiac tissues, with high-functionality and stronger contractile ability, is entirely dependent on the number of capillaries created in the tissues. The human adult heart has ~10% of the entire body's capillaries and has an extremely high density of capillaries where separation distances are in the order of ~15 μm [19]. In a conventional culture dish, thin cardiac cell sheets with a thickness of approximately 20 μm, which is comparable to that of a single cell, can survive by simple diffusion of oxygen and nutrients in a culture medium, without capillaries. An innovative technology is required to break through this diffusion limit in 3D tissue engineering. Endothelial cells co-cultured with cardiac cells spontaneously formed a network structure (Fig. 1A) [20]. Cell sheets co-cultured with endothelial cells released more cytokines, such as vascular endothelial growth factor (VEGF), than cell sheets without endothelial cells (Fig. 1B) [21]. When a vascularized cardiac cell sheet was transplanted to the back of a nude rat, the cell sheet attached to the host tissue after 1–2 days, and the newly-created vascular network in the sheet has been confirmed to connect to the host capillaries. In contrast, transplanted cell sheets without endothelial cells did not produce a vascular network and subsequently showed a marked decrease in survival [21]. Furthermore, after transplantation to the infarcted myocardial tissue of an adult rat, a vascularized cell sheet was found to improve function of the infarcted heart. Fig. 1C demonstrates that vascular networks in a cell sheet connected to the host capillaries [22]. The green fluorescence color indicates the vascular network of the cell sheet and the red blood cells were from the host (the lower right photograph of Fig. 1C). Moreover, co-cultures of fibroblasts and endothelial cells, and myoblast and endothelial cells have also been found to offer sufficient vascularization and were highly effective for transplantation [23]. These results demonstrated how critical vascularization was to successful transplantation of highly functional 3D tissue.

3. Controlled orientation of vascular cells in sandwiched cell sheets

The spontaneous network formation of endothelial cells in a co-culture with cardiomyocytes, fibroblasts, and myoblasts has been reported. However, the cause of *in vitro* vascular formations is still poorly understood, and the formation in cell sheets is known to be uncontrollable. Therefore, we have investigated the possibility of controlling the shape of endothelial cell networks by using various types of patterned cell sheets having a single orientation. A culture dish grafted with a

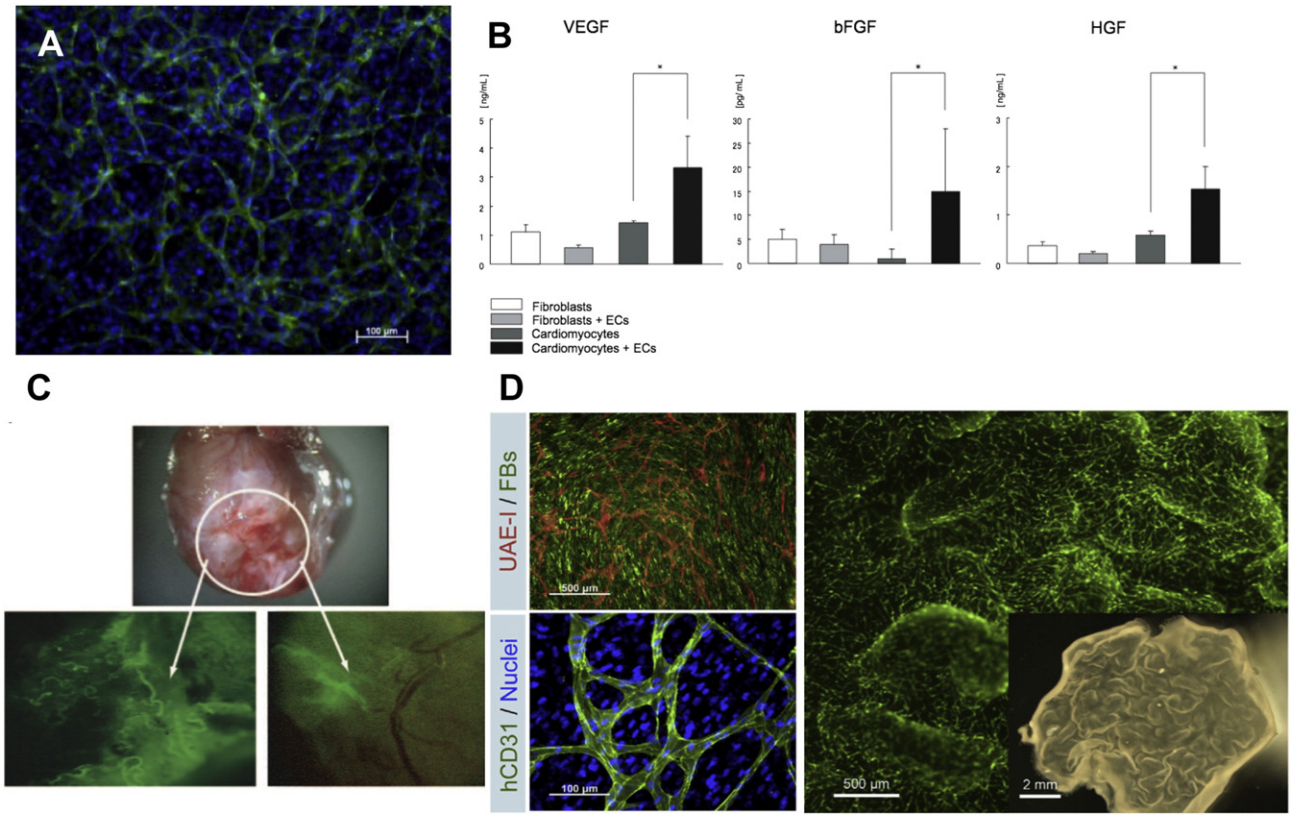

Fig. 1. Vascular networks in cell sheets. (A) Vascular network of endothelial cells in cardiac cell sheets treated with 10 mol/L rat recombinant vascular endothelial growth factor after 4 days. (B) Secretions of vascular growth factors from cardiac cell sheets. Amounts of cytokines secreted from cardiomyocyte and fibroblast sheets co-cultured with endothelial cells are measured by enzyme-linked immunosorbent assay. Protein expressions for vascular endothelial growth factor, basic fibroblast growth factor, and hepatocyte growth factor are demonstrated. Cardiac cell sheets containing endothelial cells secrete a significantly larger amount of all three angiogenesis growth factors than the sheet without endothelial cells ($n = 12$). Error bars indicate SD (*, $P = 0.05$). (C) Contribution of cell sheet with a vascular network to the neovascularization of ischemic hearts. Macroscopic view of capillary formation is shown. Green fluorescent protein-expressing vessels migrate into the host myocardium and connect to the capillaries of the host heart. White circle indicates the position of the transplanted cardiac cell sheets. (D) Fabrication of double-layered fibroblast sheet with vascular human aortic endothelial cells (HAECs). HAECs and fibroblast sheets strained with Ulex Europaeus Agglutinin 1 and anti-prolyl-4-hydroxylase antibody, respectively (upper left photograph). The networked cells are stained with anti-human CD31 antibody, and the nuclei are counterstained with Hoechst 33342 (lower left photograph). The photograph on the right shows harvested cell sheets consisting of a double-layered fibroblast sheet with HAECs.