Biomaterials 48 (2015) 66–83

Contents lists available at ScienceDirect

Biomaterials

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

A fibrin-supported myocardial organ culture for isolation of cardiac stem cells via the recapitulation of cardiac homeostasis

Jong-Tae Kim [a], Hye Jin Chung [b], Ji-Yeon Seo [a], Young-Il Yang [a, *], Min-Young Choi [a], Hyeong-In Kim [a], Tae-Hyun Yang [c], Won-Jin Lee [a], Young Chul Youn [a], Hye Jung Kim [a], Yeon Mee Kim [a], Hyukjin Lee [b], Yang-Soo Jang [d], Seung-Jin Lee [b, **]

[a] Paik Institute for Clinical Research, Inje University College of Medicine, Busan 614-735, Republic of Korea
[b] Department of Pharmacy, Ewha Womans University, Seoul 120-750, Republic of Korea
[c] Division of Cardiology, Inje University College of Medicine, Busan 614-735, Republic of Korea
[d] Division of Cardiology, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea

ARTICLE INFO

Article history:
Received 8 October 2014
Accepted 20 January 2015
Available online 10 February 2015

Keywords:
Organ culture
Cardiac homeostasis
Cardiac stem cells
Fibrin
Myocardial infarction
Stem cell niche

ABSTRACT

There is great interest in the development of cardiac stem cells (CSCs) cell-based therapeutics; thus, clinical translation requires an efficient method for attaining therapeutic quantities of these cells. Furthermore, an *in vitro* model to investigate the mechanisms regulating the cardiac homeostasis is crucial. We sought to develop a simple myocardial culture method for enabling both the recapitulation of myocardial homeostasis and the simultaneous isolation of CSCs. The intact myocardial fragments were encapsulated 3-dimensionally into the fibrin and cultured under dynamic conditions. The fibrin provided secure physical support and substratum to the myocardium, which mediated integrin-mediated cell signaling that allowed *in situ* renewal, outgrowth and cardiomyogenic differentiation of CSCs, mimicking myocardial homeostasis. Since our culture maintained the myocardial CSCs niches, it was possible to define the identity of *in vitro* renewed CSCs that situated in the interstitium between cardiomyocytes and microvessels. Lastly, the use of matrix-restricted fibrinolysis enabled the selective isolation of outgrown CSCs that retained the clonogenicity, long-term growth competency and cardiovascular commitment potential. Collectively, this myocardial culture might be used as an alternative tool for studying cardiac biology and developing cell-based therapeutics.

1. Introduction

All mammalian tissues need to constantly replace damaged or dead cells throughout the life of the animal. This process of continual cell replacement is critical for the maintenance of adult tissues and is defined as tissue homeostasis [1]. A growing body of evidence indicates that tissue-resident stem cells that distribute in virtually all tissues are key effectors that mediate the tissue-specific homeostasis in response to perturbations [2,3]. The replacement of damaged cells varies substantially among different tissues, which relies on the regenerative activity of tissue-specific stem cells [4]. The adult heart has been previously considered a terminally differentiated organ, with a very low intrinsic renewal capacity. However, the identification of endogenous cardiac stem cells (CSCs) residing in the adult heart raises questions about this dogma [5–17]. Convincing evidence has been reported that the cardiac homeostatic process is regulated by CSCs, which includes CSCs renewal, proliferation, differentiation and the replacement of cardiac cells in response to the myocardial loss [5,13–15].

CSCs have been defined by the following characteristics: self-renewal, clonogenicity, long-term growth and multipotency into cardiomyocytes (CMCs), endothelial cells (ECs), vascular smooth muscle cells (vSMCs) and cardiac fibroblasts [13,15]. In the adult mammalian heart, there are at least seven CSC populations identified, so far, by the expression of stem cell-associated markers [5,6,9,14] and the ability to form cardiosphere [7,8], to efflux

* Corresponding author. Paik Institute for Clinical Research, Inje University College of Medicine, 633-165 Gae-Gum-dong, Busan-Jin-gu, Busan 614-735, Republic of Korea. Tel.: +82 51 890 6630; fax: +82 51 894 6709.
** Corresponding author. Department of Pharmacy, Ewha Womans University, 11-1 Daehyun-dong, Seodaemun-gu, Seoul 120-750, Republic of Korea. Tel.: +82 2 3277 3043; fax: +82 2 3277 3051.
E-mail addresses: pathyang@inje.ac.kr (Y.-I. Yang), sjlee@ewha.ac.kr (S.-J. Lee).

http://dx.doi.org/10.1016/j.biomaterials.2015.01.041
0142-9612/

Hoechst dye [16] and to form colony forming unit-fibroblasts (CFU-F) [17]. Aside from CSCs that are derived from the embryonic heart, accumulating studies reported that most adult CSC populations are widely distributed in the myocardium [5,6,10,14]. Although independent works have identified these myocardium-resident CSC populations, questions still remain regarding their role in cardiac homeostasis, *in situ* identity and localization due to lack of a suitable *in vitro* method. Therefore, most studies have been largely dependent on *in vivo* approaches for determining the *in situ* identity and role of CSCs.

Since CSCs intrinsically mediate cardiomyogenesis and angiogenesis, they could provide a promising avenue for preventing and restoring heart failure [13,15]. Clinical translation of the therapeutic potential of CSCs demands an efficient isolation method to attain a therapeutic amount of CSCs from the limited myocardial biopsy samples [12,15]. While several approaches have been reported based on the immunoselection using CSCs-associated antibodies [5,6,9,10,14] or cardiac explants-derived cardiospheres [7,8], these methods demonstrated critical drawbacks such as absence of a CSC-specific marker, low cell yield, heterogeneous cell populations and/or a lack of consistency [12,13,15]. Therefore, one of the primary challenges in CSCs-based strategies is developing a reproducible culture method to isolate CSCs.

Limited studies have succeeded to establish organ culture methods that recapitulate tissue-specific homeostatic process *ex vivo* mediated by tissue-resident stem cells [18–20], while, to date, no study has described an *in vitro* method to recapitulate cardiac homeostasis. In addition, while previous approaches based on organ culture methods have been successful to mobilize tissue-specific stem cells, they failed to provide a way for isolating these *in vitro* renewed and outgrown cells. We have recently reported an organ culture method that enables to renew *in situ*, mobilize and enrich tissue-resident stem cells into the fibrin [21–23]. The fibrin employed in this culture system functions as an engineered 3-dimensional (3D) provisional matrix that provides the secure physical support, as well as a larger surface area, for cell adhesion and outgrowth. By combining matrix-restricted proteolysis, outgrown tissue-resident stem cells could successfully isolate from the fibrin. Our unique approach ensures both the selective isolation of tissue-resident stem cells along with preservation of their niches *ex vivo*.

Similar to tissue-resident stem cells residing in various types of tissues, certain populations of CSCs function as myocardial-resident stem cells that might be primarily responsible for cardiac homeostasis [24]. Based on this hypothesis, we tried to establish a fibrin-supported 3D myocardial organ culture that recapitulates a cardiac homeostatic mechanism *ex vivo* regulated by CSCs. This culture enables CSCs to renew *in situ*, outgrow into the fibrin, proliferate and spontaneously differentiate into CMCs. After enrichment of CSCs that have outgrown into the fibrin, we provided a simple strategy to selectively isolate these cells from the fibrin by matrix-restricted fibrinolysis without any sophisticated tissue dissociation and purification steps. Moreover, since our culture preserves the structural and functional integrity of CSCs niches, the tracking and identifying of those *in vitro* renewed cells could elucidate the *in situ* identity of CSCs.

2. Materials and methods

2.1. Animals and reagents

The following animals were purchased from Charles River Laboratories (Seoul, Korea): Balb/c (6–28 weeks), C57BL/6 (6–28 weeks), ICR (6–28 weeks), Sprague Dawley (6–32 weeks), Fisher 344 (6–32 weeks) and New Zealand White rabbit (6–48 weeks). The Institutional Animal Care and Use Committee at the Inje University School of Medicine approved all animal experimental protocols. Plasminogen-free fibrinogen (Sigma–Aldrich, Seoul, Korea) was dissolved in Dulbecco's Eagle Modified Medium (DMEM) containing 100 μg/mL of aminomethylbenzoic acid (Sigma–Aldrich) to make a 5 mg/mL fibrinogen solution. Thrombin (Sigma–Aldrich) solution was prepared by dissolving thrombin in DMEM containing 40 mmol/L $CaCl_2$ at a concentration of 1 unit/mL. All culture media, animal serum and reagents were purchased from Gibco (Seoul, Korea) unless otherwise specified. Growth factors and cytokines were purchased from Peprotech (Seoul, Korea) unless otherwise specified.

2.2. Fibrin-supported 3D myocardial organ culture

All myocardial organ cultures were obtained from both the right and left atrial free wall and septum unless otherwise specified. For removing epicardium and endocardium, a small incision was made on the heart base using a scalpel. The exposed epicardium was gently pulled out using microtweezers and separated from the heart. After selective dissection of atrial walls and septum, the endocardium was then removed using microscissors. All procedures were performed under dissection microscope guidance. More than 95% of the myocardial fraction was collected and used for organ culture. The myocardium was minced into 2–3 mm^3 fragments and washed with phosphate-buffered saline (PBS). The myocardial fragments suspended in thrombin solution were mixed with the same volume of fibrinogen solution, and then 10 mL of a mixture containing approximately 200 mg of the myocardial fragments was placed in a 100-mm tissue culture dish.

By incubating a culture dish in a humidified chamber at 37 °C for 2 h, the fibrin hydrogel composed of 0.25% fibrinogen, 0.5 unit/mL of thrombin, and 100 μg/mL of aminomethylbenzoic acid was completely polymerized. After then, 10 mL of growth culture media (90% DMEM-Ham's F12, 10% fetal bovine serum), 10 ng/mL epidermal growth factor (EGF), 2 ng/mL basic fibroblast growth factor (bFGF), 10 ng/mL insulin-like growth factor (IGF) and 10 μg/mL gentamicin) was added.

Organ culture was performed under dynamic conditions at 15 rpm or static conditions. The medium was replenished three times per week. The procedure of fibrin-supported myocardial organ culture was illustrated schematically in the diagram in Fig. 1A.

For evaluating interspecies and inter-strain differences in cell outgrowth, the atrial myocardium obtained from mouse (Balb/c, n = 15; ICR, n = 13; C57BL/6, n = 9), rat (Sprague Dawley, n = 18; Fisher 344, n = 8) and rabbit (New Zealand White, n = 7) were cultured with the fibrin support. To determine the effect of entrapped blood cells in the myocardium on cell outgrowth, rat hearts (Sprague Dawley, male, 8-week old) were perfused (n = 5) with 10 mL of PBS containing 1 unit/mL of heparin (Sigma-Aldrich). Before euthanasia, under anesthesia, a 24-gauge angiocath was inserted into the LV followed by retrograde infusion of PBS containing 1 unit/mL of heparin using a syringe pump at a flow rate of 1 mL/min. After continuous perfusion of 10 mL of heparinized PBS, circulating blood cells in the heart were completely removed. And then, the atrial myocardium was selectively harvested and cultured with the fibrin support. For determining the electrophysiologic integrity, the sinoatrial node and surrounding atrial myocardium were obtained from rabbits (New Zealand White, male, 8-week old, n = 5) and then cultured with the fibrin support. The atrial myocardium of rats (Sprague Dawley, 8-week old, male) was used to isolate allogeneic cells for intramyocardial cell injection into the infarcted heart of rats.

2.3. Isolation and propagation of outgrown cells

After 7 days of culture, outgrown cells (OCs) were recovered by the selective degradation of the fibrin using 5000 units of urokinase (Sigma-Aldrich) for 30 min in the presence of 30% calf serum in DMEM. When the fibrin was degraded, the released OCs from the fibrin were collected after centrifugation at 150 *g* for 5 min as described in our previous work [21–23]. These collected OCs were suspended in growth culture media, plated into a 150-mm tissue culture dish and then cultured under a conventional monolayer culture condition. When cells reached 80% confluence, OCs were detached, seeded at a 10,000 cells per cm^2 and subcultured. This initial passage (P) was referred to as P1. All *in vitro* assays used 5 different batches of subcultured OCs at P3 that derived from the atrial myocardium of 3 different mouse donors (Balb/c, male, 8-week old) unless otherwise specified.

2.4. Assessment of cell outgrowth, isolation yield and cell number

To assess cell outgrowth, photoplanimetric method was used as described previously [22]. Briefly, 10 random fields per each culture were captured following 7 days of culture and the area occupied by OCs was calculated using Image J (NIH, Bethesda, MD).

Initial collected OCs from the fibrin were aggregated with each other, which results in difficulty in cell counting using a haemocytometer or flow cytometry. Assay based on DNA quantification using flurophores, such as Hoechst and PicoGreen, has been well established for the indirect assessment of cell numbers [24]. Therefore, in this study, we used fluorometric DNA quantitation to quantify cell numbers. To generate a DNA standard curve, the standard DNA samples at concentrations of 10 ng, 25 ng, 50 ng, 100 ng and 500 ng/mL of calf thymus DNA (Sigma–Aldrich) were prepared by serial dilution with deionized water, incubated with PicoGreen fluorophore (Invitrogen, Carlsbad, CA) and then measured fluorescent intensity by a microplate fluorometric reader (Gemini XPS, Molecular Devices, Sunnyvale, CA). To prepare a cell number standard curve, samples containing 1,000,