Telocytes accompanying cardiomyocyte in primary culture: two- and three-dimensional culture environment

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Abstract

Recently, the presence of telocytes was demonstrated in human and mammalian tissues and organs (digestive and extra-digestive organs, genitourinary organs, heart, placenta, lungs, pleura, striated muscle). Noteworthy, telocytes seem to play a significant role in the normal function and regeneration of myocardium. By cultures of telocytes in two- and three-dimensional environment we aimed to study the typical morphological features as well as functionality of telocytes, which will provide important support to understand their *in vivo* roles. Neonatal rat cardiomyocytes were isolated and cultured as seeding cells *in vitro* in two-dimensional environment. Furthermore, engineered myocardium tissue was constructed from isolated cells in three-dimensional collagen/Matrigel scaffolds. The identification of telocytes was performed by using histological and immunohistochemical methods. The results showed that typical telocytes are distributed among cardiomyocytes, connecting them by long telopodes. Telocytes have a typical fusiform cell body with two or three long moniliform telopodes, as main characteristics. The vital methylene blue staining showed the existence of telocytes in primary culture. Immunohistochemistry demonstrated that some c-kit or CD34 immuno-positive cells in engineered heart tissue had the morphology of telocytes, with a typical fusiform cell body and long moniliform telopodes. Also, a significant number of vimentin⁺ telocytes were present within engineered heart tissue. We suggest that the model of three-dimensional engineered heart tissue could be useful for the ongoing research on the functional relationships of telocytes with cardiomyocytes. Because the heart has the necessary potential of changing the muscle and non-muscle cells during the lifetime, telocytes might play an active role in the heart regeneration process. Moreover, telocytes might be a useful tool for cardiac tissue engineering.

Keywords: telocytes • telopodes • cardiomyocytes • three-dimensional culture • neonatal heart • engineered myocardium • CD34 • c-kit • vimentin

Introduction

Non-muscle cells of heart can produce marked effects on the cardiac structure and function. Among the non-muscle cell types, there is a special type of interstitial cells, called telocytes [1–5]. The morphology of these cells is outstanding: small oval cell body with extremely thin and long moniliform prolongations [1], appearing as an alternation of podomeres and podoms [2]. Such

cells, previously called interstitial Cajal-like cells (ICLC), were found in many tissues and organs [6–21]. They seem similar, but are different from archetypal Cajal cells found in the gastrointestinal tract [1]. In view of their special morphology and difference from Cajal cells, they were named telocytes in 2010 by Popescu [1].

The current study confirmed that telocytes exist in the heart, including myocard, endocardium and epicardium [1, 4, 5, 22–25]. Located between cardiomyocytes, blood capillaries and nerve endings, they appear to create a network with roles for the function and regeneration of heart [1, 4, 23, 25]. Nevertheless, the mechanism has not yet been entirely understood due to very few reports on telocytes *in vitro*. Our study attempts to isolate and culture telocytes from neonatal rat cardiac tissues *in vitro* to study the appearance and characteristics of telocytes and to get more biological insights of the roles of telocytes in heart.

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At the same time, we have successfully constructed engineered heart tissues by using collagen/Matrigel scaffolds. The engineered heart tissues have rhythmic contractions. Their organizational structure is similar to normal myocardial tissue *in vivo* [26]. Considering the positive reports of telocytes on the normal heart structure, function and regeneration *in vivo*, we assumed that telocytes may have important effects to improve the quality of engineered heart tissue and to ensure the normal function of cardiomyocytes. However, there are no reports on telocytes in engineered heart tissue so far.

The purpose of this study was: (1) to culture telocytes *in vitro* in two-dimensional environment and identify their morphology and characteristics; (2) to investigate them in three-dimensional environment combined with the construction of engineered cardiac tissues and (3) to provide an *in vitro* model to investigate the roles of telocytes in heart.

Materials and methods

Newborn rat cardiac myocytes

We used the method as previously described in our lab [26]. In brief, cardiac myocytes were isolated from 1-day-old Wistar rats. The ventricles were minced and digested with 0.1% trypsin (Sigma, St. Louis, MO, USA) solution to release cells. The isolated cells were pre-plated on the tissue culture dishes for 1 hour to purify the cell suspension with cardiac myocytes by allowing fibroblasts attachment. The unattached cells were collected and cultured in medium.

Engineered cardiac tissue construction

In order to investigate telocytes in a three-dimensional environment, we constructed engineered cardiac tissue in collagen/Matrigel scaffolds using a refined method previously described [27]. In brief, 0.5 ml concentrated $2\times$ H-DMEM (Invitrogen, Carlsbad, CA, USA) culture medium containing 20% FBS (Invitrogen) was mixed with 0.5 ml of liquid collagen type I and a basement membrane protein mixture (Matrigel; Becton Dickinson Biosciences, San Jose, CA, USA) in 4:1 (v/v). The pH of the mixture was neutralized immediately by titration with 0.1 mol/l NaOH. A total of 1.0×10^7 freshly isolated cardiomyocytes were mixed with the mixture and pipetted into casting moulds for incubation. After 60 min., 1.0 ml serum-containing culture medium (DMEM, 10% FBS) was added to the dish. The culture medium was changed daily.

Supravital methylene blue staining

We applied the supravital methylene staining method [6]. Cells were washed in pre-warmed phenol red-free DMEM (Sigma Chemical, St. Louis, MO, USA), and incubated in a 0.02% methylene blue solution for 20 min., 37°C (Merck KGaA, Darmstadt, Germany). Quickly washed with phenol red-free DMEM medium for 30 min., pictures were taken within 10 min. to prevent the colour loss immediately.

Fig. 1 Telocytes in primary culture at day 7. **(A)** Typical telocytes with fusiform cell body and two long prolongations (telopodes). **(B)** Telocytes with two or three prolongations were different from cardiac cells. Bar = 50 μ m.

Histology and immunohistochemistry

Haematoxylin and eosin staining was performed to observe telocytes in engineered cardiac tissue. For haematoxylin and eosin staining, the engineered cardiac tissue was fixed in 4% formaldehyde and embedded with paraffin. Sections of 4 μ m thickness were cut. The regular procedure was performed for haematoxylin and eosin staining. For immunohistochemical staining, the primary antibodies used were: c-kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, diluted 1:100), CD34 (Santa Cruz, diluted 1:100), vimentin (Sigma, diluted 1:1000). The sections were incubated with the primary antibodies overnight at 4°C, then biotin-labelled secondary antibodies, and finally detected with Diaminobenzidine (Sigma). Primary antibodies were omitted through negative controls.

Results

Telocytes in two-dimensional cultures

Phase contrast microscopy

In primary culture of cardiomyocytes isolated from myocardial tissues, the typical telocytes were detected. They have a fusiform cell body and two long prolongations (Fig. 1A). The morphology of telocytes *in vitro* is different from cardiac cells. After 7 days of culture, typical morphological features of telocytes appeared under light microscope: a small oval-shaped cell body with extremely long and thin moniliform prolongations (telopodes) extended from the cell body (Figs. 1 and 2). Telocytes often accompanied the cardiomyocytes and their telopodes ended up on cardiomyocytes. However, telocytes with their long prolongations interconnected cardiomyocytes and accelerated their synchronously beating (Figs. 3 and 4 and Movie S1).

Methylene blue staining

Methylene blue staining further confirmed the existence of telocytes in primary culture. We used supravital methylene blue staining to identify telocytes *in vitro*, as recommended in [12–14]. Methylene blue staining showed that telocytes cultured *in vitro* had long and extremely thin prolongations – telopodes (Fig. 5A).

Fig. 2 Telocytes in primary culture at day 7. (A) Extremely long and thin prolongations – telopodes (arrow) extended from the body of telocytes. (B) Typical moniliform prolongations (arrows): alternation of thin segments – podomeres – and dilated segments – podoms. Bar = 50 μ m.

Fig. 4 Telocytes in primary culture at day 7. (A) Telocyte around the beating cardiomyocytes. (B) Telocytes (arrows) connected the cardiomyocytes to form networks. Bar = 50 μ m.

Fig. 3 Telocytes in primary culture at day 7. (A) The prolongation of telocytes ended on cardiomyocytes. (B) Telocytes connected to cardiomyocytes with long prolongations. Bar = 50 μ m.

Fig. 5 Methylene blue staining of cells in primary culture at day 10. (A) Telocytes (arrow) with long and extremely thin prolongations, telopodes. (B) Telocytes (arrows) involved in the consistent beating 'syncytium'. Bar = 50 μ m.

Fig. 6 Haematoxylin and eosin staining of engineered heart tissue at day 14. (A) Cardiac myocytes with rod-shaped body and nuclei centrally located and elongated oriented longitudinally. (B) Telocytes with two or three long cell processes – telopodes. (C) Telocytes with three long cell processes – telopodes. Arrows indicate dense nodular or vacuolar structures along the prolongation. Bar = 200 μ m (A), 20 μ m (B, C).

They connected neighbouring cardiac cells making a consistent beating 'syncytium' (Fig. 5B).

Telocytes in three-dimensional engineered heart tissues

Histology

Conventional histological procedures revealed two type of cells in engineered heart tissue: (i) cardiac myocytes (with rod-shaped body) and nuclei (centrally located and elongated) oriented longitudinally (Fig. 6A); (ii) telocytes, as a distinct cell type with two or three long telopodes. These cells appeared with spindle or triangle shape, depending on the number of the telopodes. There were dense nodular or vacuolar structures along the prolongation (Fig. 6B–C) – podoms [2].

Immunohistochemistry

We used immunohistochemical staining for antigens expressed in telocytes: c-kit, CD34 and vimentin. c-kit or CD34⁺ telocytes existed in engineered heart tissue with long cell processes (Figs 7 and 8). In addition, a number of vimentin⁺ cells were observed in engineered heart tissues (Fig. 9A). Among these cells, telocytes appeared with long and extremely thin telopodes. Vimentin is discontinuously expressed along these telopodes (Fig. 9B), presumably due to the tortuous telopodes' three-dimensional path.

Discussion

We isolated and cultured cardiomyocytes in primary culture *in vitro* and observed typical telocytes among the cardiomyocytes.

Fig. 7 Immunohistochemical staining for c-kit⁺ cells in engineered heart at day 14. Arrow shows a c-kit⁺ telocyte in engineered heart tissue. Bar = 20 μ m.

Fig. 8 Immunohistochemical staining for CD34⁺ cells in engineered heart at day 14. Arrow shows a CD34⁺ telocyte in engineered heart tissue. Bar = 20 μ m.

The typical morphological characteristics of telocytes cells include a fusiform or a triangular cell body and two or three long moniliform prolongations (telopodes). The telocytes distributed among cardiomyocytes seem to connect them to beat synchronously. Furthermore, we have detected telocytes in three-dimensional engineered heart tissues. Histological staining showed that telocytes with two or three long prolongations were present in engineered heart tissue. Immunohistochemistry confirmed that telocytes were immuno-positive for c-kit, CD34 and vimentin in engineered heart tissue. Our study has confirmed that typical telocytes do exist in primary culture *in vitro*. We have also established the model of three-dimensional engineered heart tissues for the ongoing research of the mechanism of telocytes to cardiomyocytes.

Fig. 9 Immunohistochemical staining for vimentin in engineered heart at day 14. (A) Vimentin⁺ cells in engineered heart tissues. (B) Vimentin⁺ telocyte with long and extremely thin prolongation (telopodes) and vimentin discontinuously expressed along the prolongation (arrow). Bar = 50 μ m (A), 20 μ m (B).

We used immunohistochemical identification c-kit, CD34 and vimentin antibodies to identify telocytes in engineered heart tissues. However, telocytes shared some markers with other types of cells [1], *e.g.* cardiac stem cells, which express c-kit [26–30], endothelial cells known for CD34 positivity [1, 31] and fibroblasts, which may express vimentin [16]. In addition, although we used pre-plating for 1 hr to remove fibroblast from primary culture, we failed to completely clear the fibroblasts. Therefore, as a component of seeding cells, fibroblast existed in engineered heart tissues with strong vimentin expression. As an identifying method for telocytes, it will also depend on different markers in bi-labelling or tri-labelling immunohistochemistry to this end. A certainty in identification of telocytes is based upon the results of transmission electron microscopy examination.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

The following supporting information is available online:

Movie S1 Telocytes interconnecting cardiomyocytes with their long prolongations. They seem to accelerate the synchronously beating of cardiomyocytes.

Detailed information regarding telocytes may be found at www.telocytes.com.

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