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SHORT COMMUNICATION

Growth factors mediated differentiation of mesenchymal stem cells to cardiac polymicrotissue using hanging drop and bioreactor

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Abstract

Heart disease is the major leading cause of death worldwide and the use of stem cells promises new ways for its treatment. The relatively easy and quick acquisition of human umbilical cord matrix mesenchymal stem cells (HUMSCs) and their properties make them useful for the treatment of cardiac diseases. Therefore, the main aim of this investigation was to create cardiac polymicrotissue from HUMSCs using a combination of growth factors [sphingosine-1-phosphate (S1P) and suramin] and techniques (hanging drop and bioreactor). Using designated culture conditions of the growth factors (100 nM S1P and 500 μM suramin), cardiomyocyte differentiation medium (CDM), hanging drop, bioreactor and differentiation for 7 days, a potential specific cardiac polymicrotissue was derived from HUMSCs. The effectiveness of growth factors alone or in combination in differentiation of HUMSCs to cardiac polymicrotissue was analysed by assessing the presence of cardiac markers by immunocytochemistry. This analysis demonstrated the importance of those growth factors for the differentiation. This study for the first time demonstrated the formation of a cardiac polymicrotissue under specific culture conditions. The polymicrotissue thus obtained may be used in future as a 'patch' to cover the injured cardiac region and would thereby be useful for the treatment of heart diseases.

Keywords: cell differentiation

Introduction

Cardiac disease, a disorder that affects heart, is a major cause of death worldwide (Mensah and Brown, 2007). It has been proved that replacement of injured heart cells by healthy cardiac cells through mitotic division has limited activity (Karabekian et al., 2011). To overcome this obstacle, three types of stem cells [Pluripotent stem cells (PSCs), MSCs and induced PSCs (iPSCs)] have been used (Williams and Hare, 2011; Mazhari and Hare, 2012; Yamanaka, 2012). However, iPSCs may not have clinical applications in the near future because their differentiation is achieved through re-programming. This obstacle confirmed the necessity of using MSCs, which is one step forward of differentiation from PSCs use, for replacing injured heart cells. Nevertheless, their ability to differentiate into cardiac cells is a complex biologic process and poorly understood, where two major signaling pathways are involved. They are the Wnt canonical pathway and transforming growth factor-β (TGF-β) superfamily pathway (Augello and De Bari, 2010).

Sphingosine-1-phosphate (S1P) and suramin are potential growth factors for the differentiation of human umbilical cord MSCs (HUMSCs) and mouse embryonic stem cells into cardiomyocytes, respectively (Wiese et al., 2011; Zhao et al., 2011). S1P sphingolipid has potent effects on the embryonic and neural stem cell differentiation, proliferation and maintenance (Avery et al., 2008; Price et al., 2009). Suramin, a derivative of urea, exerts many effects on various cell functions including proliferation, migration and differentiation (Wiese et al., 2011).

The primary aim of this study was the in vitro differentiation of HUMSCs towards cardiomyocytes rendering that as a new way for the treatment of heart diseases. To achieve that, different concentrations of S1P and suramin have been used separately on monolayer cells (2D) and in

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combination for creating polymicrotissues (3D) with cardiomyocyte characteristics. At the same time, the proper differentiation and maturation stage of the cardiac single polymicrotissue has been characterized by assessing the expression of a specific cardiac marker. The main advantage of 3D over 2D cell culture is that it maintains the in-vivo tissue characteristics. In this study the 'hanging drop' microtissue procedure was used that may lead to an alternative and more effective result than the artificial tissue used in the past (Kelm et al., 2005). Providing the proper microenviromental culture conditions and refining the procedure, the creation of polymicrotissue with potential specific cardiomyogenic characteristics, from HUMSCs, was succeeded. Polymicrotissue thus obtained could be used to replace the injured part of the heart. In addition to the growth factors, the other major factors contributing to the formation of polymicrotissue, which consists of 20,000 cells or more, were the 'hanging drop' technique, where a single monomicrotissue consisting of approximately 1000 cells was formed, and a bioreactor, where monomicrotissue converted into polymicrotissue.

Materials and methods

Cell culture conditions

HUMSCs derived from the human umbilical cords that were obtained from health donors at Singleton Hospital, Swansea with informed consent and approval by South West Wales Research Ethics Committee. HUMSCs were cultured in DMEM-F12 (Invitrogen) basal medium or Cardiomyocyte Differentiation Medium (CDM, Millipore) containing 10% fetal bovine serum (FBS, Biosera), 100 units/mL penicillin and 0.1 mg/mL streptomycin. The cells were maintained in a humidified incubator at 37°C and 5% CO_2 and passaged when the cellular confluence was over 80%.

Differentiation of HUMSCs to cardiac cells in a monolayer form

To differentiate HUMSCs to cardiac cells, HUMSCs were subcultured in 96-well plates and treated individually with DMEM-F12 medium containing three different concentrations of S1P (0.1 μM, 0.5 μM and 1 μM) or suramin (0.1 mM, 0.2 mM and 0.5 mM) for 7 days. Cardiac differentiation was assessed morphologically using phase-contrast microscope and immunocytochemically by staining the cells with specific cardiac marker antibodies.

Preparation and live/dead cell staining of individual microtissues

HUMSCs were seeded at the density of 1000 cells in 20 μL DMEM-F12 medium per well into 60-well Terasaki plates (Greiner BioOne) and the plates were incubated upside down in an incubator at 37°C/5% CO_2 for 24 h to form microtissue in hanging drops. Then the medium with microtissues was transferred into a 96-well-plate containing 80 μL of medium in each well and subjected to live/dead cell staining. The microtissues were washed twice with PBS and stained for 30 min at 37°C with Live/Dead cell viability kit [Invitrogen, ethidium homodimer-1 (2 μL/mL in PBS) and calcein-AM (0.5 μL/mL in PBS)]. The tissue was then washed twice with PBS and imaged by Carl-Zeiss LSM710 confocal microscope.

Differentiation of HUMSCs to cardiac polymicrotissue

One millilitre microtissue suspension (CDM plus 20 single monomicrotissues) in a 1.5 mL microfuge was rotated end to end, using a bioreactor, at 37°C and 5% CO_2 to form a polymicrotissue. Rotating forces application to microtissues led them to form a polymicrotissue. The position of the microfuge was horizontal on the bioreactor resulting in the single monomicrotissues meeting at the same point forming one single polymicrotissue. The treatment conditions were CDM plus 100 nM S1P for 5 days and then CDM plus 100 nM S1P and 500 μM suramin for 2–days. After the 7th day, the polymicrotissue was maintained in CDM by changing the medium every 3 days.

Immunocytochemistry

The monolayer of cells in 96-well plate were washed with PBS and fixed for 10 min with 10% formalin. Next, the cells were washed thrice with PBS and permeabilised with PBS plus 0.1% Triton X-100. Afterwards, the cells were incubated with the primary antibody [rabbit anti-hyperpolarization-activated cyclic nucleotide-gated potassium channel (HCN)4, Alomone, or mouse anti-cardiac troponin (cTn)T, Abcam] diluted 1:50 in PBS at 4°C overnight. The cellswere then washed three times with PBS and incubated with the secondary antibody (FITC-conjugated anti-rabbit IgG or FITC-conjugated anti-mouse IgG or FITC-conjugated anti-rabbit IgG; Cy5-conjugated anti-mouse IgG, and TRITC-conjugated phalloidin, which is to stain the cytoskeleton) for 45 min in the dark at room temperature. The dilutions for the secondary antibodies and phalloidin were 1:200 and 1:5000 in PBS, respectively. The cells were then washed three times with PBS, stained with DAPI (stains the nucleus) at a dilution of 1:1000 in PBS and stored at 4°C until the time for confocal microscope use. All images presented are single optical sections and processed using LSM710 Image Bowser (Zeiss Ltd).

The polymicrotissue was washed five times with PBS, fixed for 10 min with 10% formalin and immunostained as described above using anti-HCN4 and anti-rabbit Cy3-IgG antibodies.

Results

Immunocytochemical confirmation of 2D cardiomyogenic differentiation

Firstly, the 2D cardiomyogenic differentiation of HUMSCs in the presence of growth factors suramin or S1P was assessed. For this purpose, HUMSCs were treated with different concentrations of the growth factors, and the differentiation was assessed by immunostaining for the presence of cardiac markers HCN4 and cTnT. HUMSCs treated with 0.2 mM and 0.5 mM suramin, and 0.1 μM, 0.5 μM and 1 μM S1P showed immunostaining with an anti-HCN4 antibody (Figure 1). Similarly, HUMSCs incubated with 0.5 mM suramin and 0.5 μM S1P showed immunostaining with an anti-cTnT antibody. However, untreated HUMSCs grown for 7 days in the absence of growth factors not only maintained the MSCs morphology but also showed no immunostaining with either the anti-HCN4 or an anti-cTnT antibodies (data not shown).

Immunohistochemical confirmation of 3D cardiomyogenic differentiation

We next determined the therapeutic potentiality of cardiomyogenic differentiation of HUMSCs by generating

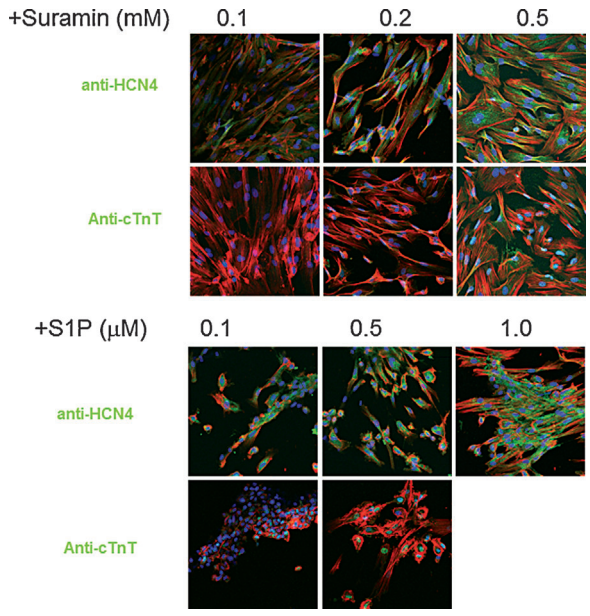

Figure 1 Immunostaining of HCN4 or cTnT in HUMSCs treated with suramin or S1P. HUMSCs treated with indicating concentrations of either suramin for 2 days (from 5th day plating) or S1P for 7 days, fixed and immunostained using an anti-HCN4 or anti-cTnT. Blue colour corresponds to DAPI staining (which stains the nucleus), red colour corresponds to phalloidin staining (which stains the cytoskeleton) and green colour corresponds to HCN4 or cTnT antibody staining.

3D spheroid microtissues of cardiac cell types from HUMSCs. The viability of the single monomicrotissue was assessed using live/dead cell staining (Figure 2A) where the percentage of dead cells corresponds to physiological experimental level of cell mortality.

Next, we differentiated HUMSCs into poly-microcardiomyotissues using a bioreactor (for culturing cells) and a combination of S1P (100 nM) and suramin (500 μM). The differentiation at different time points of incubation with the growth factors was assessed immunocytochemically using HCN4 antibody (Figure 2). According to Figure 1, 100 nM S1P and 500 μM suramin are optimal to trigger the differentiation of HUMSCs towards cardiomyocytes. We, therefore, used 100 nM S1P for 7 days and 500 μM suramin for 48 h from the 5th day to generate 3D spheroid microtissues. CDM was chosen as a feeding factor for 3D culture to provide the proper microenvironmental conditions. HUMSCs differentiation to potential cardiac polymicrotissues was assessed by immunostaining (Figure 2). Polymicrotissue was also immunostained without HCN4 antibody (Figure 2B) to assess the background staining. Figures 2C and D correspond to immunostaining with HCN4 antibody of polymicrotissue under untreated and treated conditions respectively, which both showed the same level of HCN4 expression.

Furthermore, fibrogenesis has already taken place consisted of a syncytium of asymmetrically elongated mononuclear fibres. The latter fused and formed an elongated multinucleated fibre visible in Figures 2B–E. Figures 2D and E correspond to the improper developmental maturation time points due to the improper location of the fibres. Figure 2F shows that the differentiation and proper maturation time point was 15 days (7 days with the growth factors and 8 days without them) whereby the mononuclear cardiac fibres have already moved to reside in their final position in a vertical manner to cardiomyocytes. Supporting Information movies S1 and S2 show the rotated movement in 3D of the polymicrotissue and the potential cardiac polymicrotissue, respectively, from Figures 2E and F.

Discussion

The easy and quick acquisition of HUMSCs from placenta and the fact that iPSCs do not have clinical applications, so far, and also the embryonic stem cells is one step of differentiation behind of HUMSCs, render them a major tool for the treatment of many diseases (Anzalone et al., 2010). However, HUMSCs differentiation into cardiomyocytes is less studied so far. In this study, we report the transformation of HUMSCs into cardiac cell types. In particular, we demonstrated, by immunostaining with specific antibodies, the expression of HCN4 and cTnT cardiac myocyte markers in HUMSCs differentiated using growth factors suramin and S1P. We

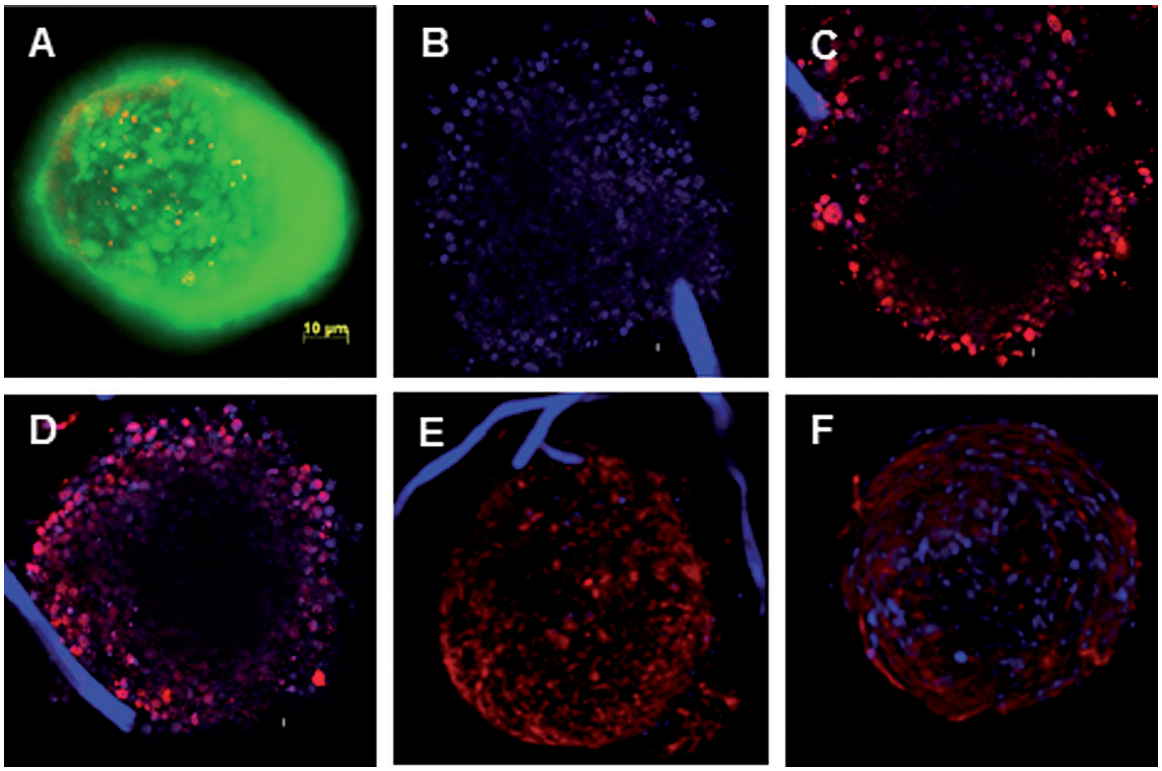

Figure 2 Live/dead cells staining of a single microtissue and immunostaining of HCN4 in HUMSCs polymicrotissues. A single monomicrotissue stained with EThD1 (orange) and calcein-AM (green). Green corresponds to live cells and orange corresponds to dead cells (A). HUMSCs grown in the absence of S1P and suramin for 7 days were fixed and immunostained without (B) or with (C) an anti-HCN4 antibody. A single polymicrotissue derived by growing HUMSCs for 7 (D) or 11 (E) or 15 (F) days in the presence of S1P (100nM) and suramin (500μM) for first 7 days. This was fixed and immunostained using an anti-HCN4 antibody. Blue corresponds to DAPI staining (which stains the nucleus) and red corresponds to HCN4 antibody staining.

further demonstrated the successful differentiation and maturation of HUMSCs into a single cardiac polymicrotissue by using hanging drop/bioreactor, for providing the suitable microenvironmental space, and a combination of the growth factors S1P and suramin. The expression of HCN4 protein and the residence of the mononuclear fibres in the differentiated tissue (Figure 2F), suggest, together, the proper differentiation and maturation stage. Two differentiation time points, over 7 days, were presented and the most proper, according to this study, was 7 (polymicrotissue treated with the growth factors) plus 8 (untreated conditions) days (Movies S1 and S2).

The molecular mechanism of the combined growth factors action is poorly understood; nevertheless, an assumption could be possible. The two major signaling pathways involved in this differentiation process are the Wnt and TGF-β superfamily pathway (Augello and De Bari, 2010). The growth factors, S1P and suramin, act through these two pathways. S1P binds to G-protein coupled receptors (GPCRs), mainly the sphingosine-1-phosphate receptor 1 (S1PR1), that constitute the main receptors of Wnt pathway. The ligand binding of GPCRs is connected with G-protein activity, which is an important signal transduction pathway in cells (Overington et al., 2006; Rao and Kuhl, 2010). Our unpublished results suggest that S1P regulates cell division at higher concentration and promotes cell adhesion at lower concentration. For this reason, the lowest concentration (100 nM) was preferred so that S1P's role was limited only for triggering the differentiation of HUMSCs to cardiomyocytes (Zhao et al., 2011).

Suramin causes the contraction of the myocyte and the rapid release of Ca^{+2} from the sarcoplasmic reticulum via the ryanodine receptor/calcium release channel (Fabiato, 1983; Wolner et al., 2005). In addition, suramin inhibits the TGF-β signaling and thereby prevents HUMSCs differentiaing into other cell types (Kloen et al., 1994). Suramin has also been shown to reduce the aggregation of Chinese hamster ovary cells (Zanghi et al., 2000). Although suramin may affect monomicrotissues aggregation, S1P eliminates that. This specifies the significance of the rotating forces action, generated by the bioreactor, on the monomicrotissues leading to the creation of a single polymicrotissue.

The roles of S1P on Wnt pathway and suramin on TGF-β pathway, together with the use of CDM and hanging drop/

bioreactor, lead to differentiation and maturation of HUMSCs into cardiomyocyte microtissues. Importantly, these conditions also bypass proarrythmic and tumorigenic effects of in vitro differentiation of HUMSCs, which are two important factors that could affect the safe use of MSCs (Fabiato, 1983). An argument could take place here that HUMSCs differentiated towards neuron cells, because this cell type also expresses HCN4 protein (Cho et al., 2009). However, the use of the designated conditions, such as the suitable growth factors and CDM, confers that the HUMSCs differentiation and maturation were limited not only into cardiomyocytes and cardiac fibres but also to a potential specific cell type of them which was the cardiac pacemaker cells. In addition, another argument is that the cardiac polymicrotissue could be the result of overgrowing cells; however the use of the growth factors, inducing the differentiation of HUMSCs, and the proper culture conditions weakens that. This study is useful for generating differentiated cardiomyocytes, which could be used for functional assessment of electrophysiological activities. Our immediate future work will concentrate on further optimising the polymicrotissue techniques and electrophysiology of these polymicrotissues to confirm their cardiomyocyte phenotypes.

Conclusion

The limited clinical applications of iPSC and the low number of studies on the differentiation of HUMSCs towards cardiomyocytes renders the current investigation an important tool for the future. In this study, we managed to create, on a small scale, a microcardiac tissue that, in a large scale form, could be used as a 'patch' to the injured cardiac region, contributing, at the same time, to the regenerative medicine field as well.

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Disclosure

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Movie S1. 3D rotating form of a prospective cardiac polymicrotissue. The cardiac fibers have not moved to reside in their final position resulting in formation of elongated multinucleated fibers. Blue colour corresponds to DAPI staining (which stains the nucleus) and red colour corresponds to HCN4 antibody staining.

Movie S2. 3D rotating form of the potential cardiac polymicrotissue. The mononuclear fibers, almost individually, have resided in a vertical manner to cardiomyocytes. Blue colour corresponds to DAPI staining (which stains the nucleus) and red colour corresponds to HCN4 antibody staining.