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Behavior of CMPCs in unidirectional constrained and stress-free 3D hydrogels

Mieke H. van Marion [a,*], Noortje A.M. Bax [a,b], Mark.C. van Turnhout [a], Arianna Mauretti [a], Daisy W.J. van der Schaft [a], Marie José. T.H. Goumans [c], Carlijn V.C. Bouten [a,b]

[a] Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands
[b] Institute for Complex Molecular Systems, Eindhoven University of Technology, Eindhoven, The Netherlands
[c] Department of Molecular Cell Biology and Center for Biomedical Genetics, Leiden University Medical Center, The Netherlands

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ABSTRACT

Cardiomyocyte progenitor cells (CMPCs) are a candidate cell source for cardiac regenerative therapy. However, like other stem cells, after transplantation in the heart, cell retention and differentiation capacity of the CMPCs are low. Combining cells with biomaterials might overcome this problem. By serving as a (temporal) environment, the biomaterial can retain the cells and provide signals that enhance survival, proliferation and differentiation of the cells. To gain more insight into the effect that the encapsulation of CMPCs in a biomaterial has on their behavior, we cultured CMPCs in unidirectional constrained and stress-free collagen/Matrigel hydrogels. CMPCs cultured in 3D hydrogels stay viable and keep their cardiomyogenic profile independent of the application of strain. Moreover, the increased expression of Nkx2.5, myocardin and cTnT in 3D hydrogels compared to 2D cultures, suggests enhanced cardiomyogenic differentiation capacity of cells in 3D. Furthermore, increased expression of collagen I, collagen III, elastin and fibronectin and of the matrix remodeling enzymes MMP-1, MMP-2, MMP-9, and TIMP-1 and TIMP-2 in the 3D hydrogels is indicative of an enhanced matrix remodeling capacity of CMPCs in a 3D environment, independent of the application of strain. Interestingly, the additional application of static strain to the 3D hydrogels, as imposed by hydrogel constrainment, stabilized CMPC viability and proliferation, resulted in enhanced cardiac marker protein expression and appeared crucial for cellular organization and morphology. More specifically, CMPCs cultured in 3D collagen/Matrigel constrained hydrogels became readily mechanosensitive, had a rod-shaped morphology, and responded to the applied strain by orienting in the direction of the constraint. Overall, our data demonstrate the applicability of CMPCs in a 3D environment since encapsulation of CMPCs may stabilize survival and proliferation, can enhance the differentiation and remodeling capacity of the cells, and could induce cellular re-organization, which all may contribute to an improved efficiency of cardiac stem cell therapy.

1. Introduction

Stem cell therapy is a new approach for heart regeneration, which aims at creating de novo cardiac tissue and preventing fibrosis. Cardiac stem cell therapy efficiency has been studied extensively in both animal studies and clinical trials [1–5]. Overall, a small increase in cardiac function is reported [1–3,6], mainly ascribed to paracrine signaling since cell retention is very low and stem cell differentiation is often not observed [3,6,7]. One possibility to improve the efficiency and effectiveness of stem cell therapy is to combine cells with biomaterials [8]. The biomaterial can be applied as tissue patch or serve as injectable cell carrier. Injection of biomaterials with cells or biomaterials alone has shown to prevent negative remodeling after MI and passively contributes to the regeneration process by changing the local mechanical properties of the heart tissue [9–14]. By encapsulating stem cells in a biomaterial, cell survival and retention are increased and paracrine signaling is induced [9–13]. Furthermore, biomaterials may serve as a proper microenvironment for stem cells to integrate and differentiate.

Traditional culture methods rely on two-dimensional (2D) adhesive substrates. However, upon encapsulation in a biomaterial, cells experience a three-dimensional (3D) rather than a 2D environment. The interactions of cells with the biomaterial, cellular organization and spatial distribution in a 3D environment resemble the in vivo situation much more [15]. The transition towards a 3D environment

* Corresponding author.
E-mail addresses: m.h.v.marion@tue.nl (M.H. van Marion), n.a.m.bax@tue.nl (N.A.M. Bax), m.c.v.turnhout@tue.nl (M.C. van Turnhout), a.mauretti@tue.nl (A. Mauretti), d.w.j.v.d.schaft@tue.nl (D.W.J. van der Schaft), M.J.T.H.Goumans@lumc.nl (M.J.T.H. Goumans), c.v.c.bouten@tue.nl (C.V.C. Bouten).

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can result in different cellular behaviors, such as changes in proliferation and differentiation [16]. For example, Liu et al. [17] demonstrated that embryonic stem cells cultured in 3D show an increased expression of genes that regulate cell growth, proliferation, differentiation and extracellular matrix (ECM) production and remodeling. More specifically, Duan et al. [18] demonstrated that ESCs cultured in hydrogels composed of collagen and decellularized ECM, promoted cardiomyogenic differentiation and maturation. Therefore, 3D culture systems can give important insight on how stem cells respond after transplantation into the heart. Upon transplantation into the heart, stem cells also become exposed to mechanical loads. The contractile and pulsatile forces of the beating heart can be considered as cyclic strains and stresses at the cellular level [19]. Strain is known to affect the proliferation, cellular organization and differentiation of stem cells [20]. For example, Huang et al. [21] showed that cyclic mechanical straining of bone marrow derived mesenchymal stem cells results in increased expression of cardiomyocyte genes and an increase in the gap-junction protein connexin43. Furthermore, Fink et al. [22] and Hansen et al. [23] demonstrated that neonatal rat cardiomyocytes cultured in 3D longitudinally constrained hydrogels show alignment, increased cardiomyocyte maturation, and gap junctional coupling. This indicates that the application of constraint or dynamic strain to stem cells may stimulate differentiation towards a more mature phenotype tissue integration. However, to be able to sense and respond to mechanical forces, cells need to be able to interact with the ECM or biomaterial via specific anchoring proteins [24].

There are multiple stem cell sources for cardiac regenerative therapy. Cardiomyocyte progenitor cells (CMPCs) form an interesting candidate cell source for regenerative cell therapy since these cells reside in the heart itself. CMPCs have the ability to differentiate into mature cardiomyocytes and are able to improve cardiac function after MI via paracrine effects. Additionally, these cells have matrix production and remodeling capacity [25–30]. Although the properties of CMPCs are promising for application in cardiac regenerative therapy, the effects observed in vivo are only temporarily beneficial [25,26,29]. Therefore, this present study aims to elucidate whether 3D culture of CMPCs, by encapsulation in a biomaterial, can increase the regenerative capacity of CMCPs. By comparing unidirectional constrained hydrogels with stress-free (unconstrained) 3D culture conditions, the importance of contact with the host tissue can be elucidated. The constrained culture also provides application of static strain which gives insight in the ability of CMPCs to respond to mechanical forces. Therefore, viability and proliferation of CMCPs in a 3D collagen/Matrigel based hydrogel were studied in both constrained and stress-free culture conditions. Additionally, cardiac differentiation capacity and the ability of CMPCs to produce ECM and matrix remodeling proteins in 3D were investigated.

This study shows that CMPCs can be cultured in 3D without losing their early cardiomyogenic profile. Differentiation as well as the ECM remodeling capacity of CMCPs is increased in 3D hydrogels compared to 2D cultures, independent of the application of strain. Moreover, CMPCs cultured in 3D collagen/Matrigel hydrogels respond to static mechanical strain as induced by constrainment by cell alignment and constrained culture stabilizes cell proliferation and organization. These results demonstrate the applicability of CMPCs in a 3D environment, and that pretreatment of CMPCs in a 3D culture system may enhance their in vivo regenerative capacity for cardiac regenerative therapy compared to 2D cultured CMPCs.

2. Materials and Methods

2.1. Culture of cardiomyocyte progenitor cells

Human fetal CMPCs were isolated from fetal hearts that were collected after elective abortion based on individual informed consent and after approval by the Medical Ethics committee of the Leiden University Medical Centre. The isolated CMPCs were cultured and subsequently immortalized by lentiviral transduction of hTert and BMI-1 as described previously [29,30]. Immortalized L9TB CMPCs were cultured in growth medium (GM) consisting of M199 (Gibco)/EGM2 (3:1) supplemented with 10% fetal bovine serum (FBS) (Greiner bio-one), 1% non-essential amino acids (Gibco), and 1% penicillin/streptomycin (Lonza) on 0.1% gelatin (Sigma)/PBS (Sigma) coated flasks.

2.2. Culture of 3D cell-hydrogel constructs

For 3D culture, CMPCs were encapsulated in collagen/Matrigel hydrogels, composed of 50% rat tail collagen type I (3.2 mg/ml, BD Biosciences), 8.3% growth factor reduced Matrigel™ (BD Bioscience), 2.7% NaOH (0.5 M, Sigma-Aldrich) and 39% SP^{++} growth medium. To induce static strain, cell-hydrogel constructs were casted in and between two house-shaped Velcro attachment points (5 × 5mm, 12 mm interspace) in non-adhesive 6-Well tissue culture plates (Fig. 1A) as previously described [31–33]. These constructs are further referred to as unidirectional constrained constructs. To create stress-free constructs, cell–hydrogel mixture was casted as droplets in non-adhesive 6-well plates (Fig. 1B).

Both types of constructs consisted of 400 μl collagen/Matrigel hydrogel solution mixed with 800,000 CMPCs. After polymerization of the constructs for 1 h at 37 °C, growth medium was carefully added to the plates. It was ensured that the constructs did not attach to the culture plates. Constructs were cultured for 9 days and growth medium was replaced twice a week. To induce cardiomyogenic differentiation, half of the constructs were subjected to the differentiation protocol as previously described for 2D monolayer culture [29]. Briefly, after 1 day of culture the constructs were treated with 5 μM 5-azacytidine (Sigma) for 72 h in differentiation medium (IMDM (Iscove's Modified Dulbecco's Medium)/Ham-F12 (1:1) (Gibco), 2% horse serum, 10^{-4} M ascorbic acid (Sigma), 1% non-essential amino acids, 1% insulin–transferrin–selenium (Lonza), and 1% penicillin/streptomycin) to inhibit proliferation of the cells [29]. After 72 h, the medium was changed followed by transforming growth factor (TGF-β1) stimulation on day 7 (1 ng/ml; Sigma) (IFDIFF media) [27,29]. Constructs were sacrificed at day 1 and day 9, and analyzed for viability, proliferation, cardiac differentiation, and mechanical responsiveness. Furthermore, expression of ECM genes and proteins was investigated. Cell culture and differentiation of both types of 3D constructs were performed in three independent experiments.

2.3. Cell viability

Viability of CMPCs in constrained and stress-free constructs was determined using a cell tracker green (CTG) (Molecular Probes) and propidium iodide (PI) staining (Molecular Probes). Constructs were washed twice with PBS and incubated with 10 μM OregonGreen 488 labeled CTG in growth or differentiation medium for 15 min at 37 °C. After vigorous washing with PBS, constructs were incubated with 10 μM PI in growth or differentiation media for 30 min at 37 °C. After incubation, constructs were washed with PBS, submerged in media and visualized using a Zeiss confocal microscope (N = 6 per test group). Images were analyzed for viability using a custom-made MATLAB script (based on [34]). Briefly, based on visual inspection a threshold was defined for each channel (red (PI)/green (CTG)), separately. These channel specific thresholds were used to convert each image into a binary image. In these binary images, the number of connected regions (i.e., groups of connected pixels) per channel was counted using built-in MATLAB functions. A connected region was considered to represent a cell when it contained at least 6 pixels (pixel size 900 nm × 900 nm). Dead cells were counted in the PI channel, and living cells were counted in the CTG channel.