Biomaterials 33 (2012) 2032–2040

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

The effect of vitronectin on the differentiation of embryonic stem cells in a 3D culture system

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ARTICLE INFO

Article history:
Received 7 November 2011
Accepted 24 November 2011
Available online 12 December 2011

Keywords:
Extracellular matrix
Niche
Cardiovascular tissue engineering
Stem cell
Scaffold

ABSTRACT

While stem cell niches *in vivo* are complex three-dimensional (3D) microenvironments, the relationship between the dimensionality of the niche to its function is unknown. We have created a 3D microenvironment through electrospinning to study the impact of geometry and different extracellular proteins on the development of cardiac progenitor cells (Flk-1$^+$) from resident stem cells and their differentiation into functional cardiovascular cells. We have investigated the effect of collagen IV, fibronectin, laminin and vitronectin on the adhesion and proliferation of murine ES cells as well as the effects of these proteins on the number of Flk-1$^+$ cells cultured in 2D conditions compared to 3D system in a feeder free condition. We found that the number of Flk-1$^+$ cells was significantly higher in 3D scaffolds coated with laminin or vitronectin compared to colIV-coated scaffolds. Our results show the importance of defined culture systems *in vitro* for studying the guided differentiation of pluripotent embryonic stem cells in the field of cardiovascular tissue engineering and regenerative medicine.

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1. Introduction

Stem cells are defined by their ability to self-renew and differentiate into different cell types. They are the most promising cell source for transplantation therapy, tissue regeneration and drug development. However, despite the remarkable potential clinical applications of different stem-cell populations, their use is currently hindered by different hurdles that must be addressed [1]. Thus, a major goal is to develop new culture-based approaches, using advanced biomaterials that more closely mimic what the body already does so well and promotes differentiation of pluripotent cells or propagation of specialized adult stem cells without loss of 'stemness.'

The relative importance of specific substrate components for stem cell adhesion, survival, and undifferentiated growth is still insufficiently characterized. However, an increasing emphasis is on designing biomaterials, based on basic mechanisms of cell–matrix interactions and cell signaling for applications in stem cell biology. This application has the potential to revolutionize our understanding of extrinsic regulators of cell fate, as matrices can be made using technologies that recapitulate the features of stem-cell microenvironments, or niches, down to the molecular level [2].

During embryonic development, the extracellular matrix (ECM) plays a critical role in regulating stem cell differentiation into different lineages, as well as in cell migration and proliferation [3–7]. *In vivo*, stem cells reside within instructive, tissue-specific niches that physically localize them and maintain their stem-cell fate [8–10]. Within the niche, stem cells are exposed to complex, spatially and temporally controlled biochemical mixtures of soluble chemokines, cytokines and growth factors, as well as insoluble transmembrane receptor ligands and ECM molecules. While an important function of the ECM is to provide the structural framework to support cellular functions, this scaffold of proteins, proteoglycans, and glycosaminoglycans also provides cell adhesion sites and important signaling cues [10–12]. The ECM interacts with cells via cell surface receptors such as integrins; serves as a reservoir for growth factors; and provides a substrate for cell attachment and spreading, contact guidance for cell migration, and a scaffold for building tissues. The morphology of cells determined by their contact with ECM or with nonbiological surfaces may be associated with particular patterns of cell differentiation and proliferation [13–15].

The geometry of the matrix (i.e., 2D versus 3D) also plays an important role in determining how a cell will respond to biochemical and mechanical cues, since in many native tissues cells

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0142-9612/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.biomaterials.2011.11.065

are completely surrounded by ECM [16,17]. Conventional 2D cell culture has provided important insight into how cells interact with their environment. The use of 3D culture systems is gaining popularity due to their promise as improved models of tissue physiology and because such systems can potentially be developed into engineered tissues for the treatment of the disease. The field of tissue engineering therefore is in need of a better understanding of how cells interact with 3D matrices and how cell function can be controlled via cell-matrix interactions.

In an effort to elucidate the mechanism through which the complex 3D ECM microenvironment enhances cardiovascular differentiation of ES cells, we have investigated the effect of collagen IV, fibronectin, laminin and vitronectin on the adhesion and proliferation of mES cells in 2D and 3D feeder free condition. Further, we have isolated Flk-1$^+$ cells from partially differentiating mES cultured on vitronectin-coated substrates and investigated their ability to differentiate into cardiovascular lineage i.e. cardiac myocytes (CMs), smooth muscle cells (SMCs) and endothelial cells (ECs).

2. Materials and methods

2.1. Human procurement and processing

First-trimester (7–12 week) human hearts were purchased from Novogenix laboratories (Los Angeles, CA). All heart tissues were fixed in 10% buffered formalin for 12 h and transferred to 70% ethanol prior to receiving. The fixed specimens were embedded in paraffin and cut into 5 μm sections by the UCLA Translational Pathology Core Laboratory (TPCL).

2.2. Mouse ES cell cultures, In vitro differentiation assays and magnetic cell sorting in 2D condition

Unless otherwise noted all reagents were purchased from Sigma Aldrich (St. Louis, MO). Murine Flk-1 GFP-labeled embryonic stem cells (mES) were a kind gift from Dr. MacLellan's laboratory at the Department of Medicine/Cardiology at the University of California Los Angeles. mES cells were maintained in an undifferentiated state on mitomycin-C-treated primary mouse embryonic stem fibroblasts (MEF) in leukemia inhibitory factor (LIF) supplemented medium (Knockout Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% ES-FCS (Invitrogen), 0.1 mM β-mercaptoethanol, 2 mM glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen) and 1000 U/ml recombinant LIF (Chemicon, Temecula, CA) and HEPES (2 mM, Invitrogen). For adaptation of the cells to a feeder free condition, the cells were detached from the culture dish using accutase (Chemicon) and cultured in 90% LIF-medium as described before and 10% ESGRO Complete™ medium (Chemicon) on gelatin coated (0.1% gelatin in PBS, coated for 2 h at 37 °C) T-75 flasks at 37 °C, 5% CO2, in a humidified incubator. All cells were passaged every other day and cultured in a reducing LIF-medium and increasing ESGRO combination. After several passagings all mES cells were cultured in 100% ESGRO medium (Chemicon). The feeder free mES cells were then expanded for two additional passages before being used in experiments.

For differentiation assays, the mES cells were either introduced into a dynamic suspension culture system for generating embryoid bodies (EBs) or cultured on coated plates with collagen type IV (ColIV, 5 μg/cm^2, BD Biosciences, San Jose, CA), vitronectin (50 ng/cm^2, Chemicon), fibronectin (5 μg/cm^2), laminin (5 μg/cm^2, BD Biosciences) or Matrigel (BD, 4/μg/cm^2, used as a positive control for cell attachement). Briefly, for EB formation, the cells were dissociated, resuspended in α-minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% ES-FCS (Invitrogen), 0.1 mM β-mercaptoethanol, 2 mM glutamine (Invitrogen), and 0.1 mM nonessential amino acids (Invitrogen) HEPES (2 mM, Invitrogen) and transferred into 60-mm ultralow-attachment dishes (4×10^5 cells per dish; Corning Life Sciences, Acton, MA), placed onto an orbital rotary shaker (Stovall Belly Button; ATR, Laurel, MD), and cultured under continuous shaking at approximately 50 rpm for up to two weeks. For morphometric analysis, phase-contrast images of mES-derived EBs were acquired every day during the course of culture.

Further for differentiation assay, mES cells were detached from the culture dishes and transferred to colIV- vitronectin-, fibronectin-, and laminin-coated plates for 2D culture. After 4 days, the cells were either harvested for FACS analysis, or they were detached and the Flk-1-positive cells were isolated by indirect magnetic cell sorting using a purified rat anti-mouse Flk-1 antibody (BD Pharmingen, San Diego) and magnetic microbeads (Miltenyi Biotec, Auburn, CA). The Flk-1-positive progenitor (Flk-1$^+$) cells were then plated on fibronectin-coated culture slides (BD Biosciences) in either α-MEM for cardiac differentiation, smooth muscle growth medium (SMGM-2; Lonza, Walkersville, MD) supplemented with 10 ng/ml platelet-derived growth factor-BB (PDGF-BB, R&D Systems Inc., Minneapolis) for SMC differentiation, or endothelial growth medium (EGM-2; Lonza) supplemented with 50 ng/ml vascular endothelial growth factor (VEGF, R&D Systems, Minneapolis, MN) for EC differentiation for up to 12 days at 37 °C and 5% CO2. To expand the mES cell-derived SMCs or ECs, cells were grown to >80% confluence in either SMGM-2 or EGM-2 and passaged into gelatin-coated plates with a 1:3 ratio every 3–4 days. Bright-field images and movies of undifferentiated and differentiated mES cells, as well as EBs, were acquired using the Olympus microscope (Center Valley, PA).

2.3. Alkaline phosphatase activity

The alkaline phosphatase (AP) activity of mES cells cultured in the ESGRO medium for 4 days on gelatin coated plates was detected with a Fast Red substrate kit (Chemicon) according to manufacturer's protocol. Briefly, the cells were fixed in 4% glutaraldehyde for 1 min before incubating with the staining mix (Fast Red Violet:Naphthol:water (2:1:1)) for 15 min in the dark. The cells were then rinsed with PBS and the red stem cell colonies were detected using Olympus microscope.

2.4. Scaffold fabrication, cell culture and in vitro differentiation assays in 3D condition

Electrospinning has been used to produce a scaffold with nano- to micro-diameter fibers with similar structural properties to the ECM as described before [18]. Briefly, gelatin type B (bovine skin, 10% w/v) and PCL (10% w/v) were mixed together and dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). The solution was then loaded into a 10 ml-syringe, to which to an 18-gauge blunt ended needle (spinning nuzzle) was attached. A core solution of 5% w/v PU dissolved in HFIP was loaded into a 3 ml-syringe, to which a 25-gauge needle was attached. This syringe and needle was then loaded into the 10 ml-syringe containing the sheath solution. The entire syringe system was then loaded into a modified syringe pump. The positive output lead of a high voltage supply (25 kV; Glassman High Voltage Inc., NJ, USA) was attached to the needle on the 10 ml-syringe, spinning nuzzle. In the created electric field, a thin jet was ejected from the solution in the syringe at a speed of 70 μL/min. The grounded copper target (5 cm × 5 cm) was placed ~15 cm under the needle tip and upon introduction of the electric field Taylor cone formation at the base of the spinning nuzzle was observed. A dry fibrous scaffold was collected in the form of a flat 3D mat (100–200 μm thick). The electrospun scaffolds were (1 cm × 1 cm) then sterilized by soaking scaffolds in 70% EtOH for 30 min, and then washed with sterile PBS three times and coated with colIV, fibronectin, laminin, vitronectin, Matrigel and gelatin for 3D *in vitro* studies.

2.5. Scanning electron microscopy

For ultrastructural analysis, unseeded, seeded scaffolds as well as EBs were processed for characterization by scanning electron microscopy (SEM) as described previously [19]. Briefly, cell-seeded samples and EBs were rinsed with SEM buffer (0.1 M sodium cacodylate buffer, pH 7.2, supplemented with 5% sucrose) for 10 min. The samples were then fixed for 30 min in 2% paraformaldehyde/2% glutaraldehyde in SEM buffer, followed by dehydration through grades of ethanol, 30, 50, 70, 80 and 95% for 10 min each, followed by 3 incubations in 100% ethanol for 10 min and a final incubation in 100% ethanol for 40 min. The samples were dried by incubating in one-half volume 100% ethanol and one-half volume hexamethyldisilazane for 20 min followed by 100% hexamethyldisilazane for 20 min. Finally, the 100% hexamethyldisilazane solution was evaporated during 20 min air-drying. Once dry, the samples were mounted onto stubs and sputter coated by gold/palladium (Au/Pd, thickness of ~10 nm) using JEOL JSM-6490 (JEOL USA, Inc. Peabody, MA) scanning electron microscope.

2.6. Immunofluorescent staining

The Flk-1$^+$ cells, plated on fibronectin-coated culture slides, as well as undifferentiated, mES cells were washed and fixed with 4% paraformaldehyde in PBS, for 20 min and rinsed with PBS. The EBs were also fixed and mixed with 50 μL Histogel (Fisherscientific, Pittsburgh, PA) prior paraffin embedding and sectioning at TPCL. All the sections were deparaffinized using xylene for 10 min, rehydrated in an ethanol gradient by incubating for 5 min in 100%, 90%, 85%, and 70% ethanol solutions and washed in deionized water for 3 min. All the samples were then blocked with 1% bovine serum albumin (BSA) and 2% goat serum in PBS for 30 min at room temperatue, followed by incubation with primary antibodies (ES markers: Nanog, Oct-4, Sox-2, SSEA-1 (Abcam, Cambrige, MA)); smooth muscle specific markers: SM-α-actin (Dako, Carpinteria, CA), h-caldesmon (Dako), basic calponin (Dako), SM-myosin (Dako); endothelial specific markers: CD31 (Dako), VE-cadherin (CD144, Santa Cruz Biotechnology, Santa Cruz, CA) and von Willebrand Factor (vWF, Dako); cardiomyocyte specific markers: MF20 (Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA), connexin-43 (Santa Cruz Biotechnology, Inc), Troponin-C (Santa Cruz), Nkx2.5 (Santa Cruz) overnight at 4 °C followed by several washes with PBS. The feeder free mES cells were also stained for early markers for ectodermal (nestin (Abcam)), endodermal (β-catenin (Abcam), α-fetoprotein (Santa Cruz Biotechnology)), and mesodermal (brachyury (Santa Cruz Biotechnology), SM-α-actin (Dako)) markers. Alexa Fluor 488- or 546-conjugated secondary antibodies