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A novel modified physiologically relevant model for cardiac angiogenesis

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A B S T R A C T

Angiogenesis assays are important tools for studying both the mechanisms of cardiac angiogenesis and the potential development of therapeutic strategies to ischemic heart diseases. Currently, various assays have been used to quantitate cardiac tubule formation, yet no consensus has been reached regarding a suitable assay for evaluating the efficacy of angiogenic stimulants or inhibitors. Most in vivo angiogenesis assays are complex and difficult to interpret, whereas traditional in vitro angiogenesis models measure only one aspect of this process. To bridge the gap between in vivo and in vitro angiogenesis assays, here, we have developed a novel modified cardiac explants matrigel assay. We observed the morphology of vascular sprouts formed in three forms of cardiac angiogenesis assays then used quantitative image analyses to further compare the morphological features of vascular sprouts formed in two cardiac explants angiogenesis assays. Vascular sprouts formed in the fibronectin group were less and short, whereas those formed in the matrigel group were significantly longer, consisting of more area and branch points. Moreover, we found the benefits of this matrigel model by observing the ability of cardiac explants to form vascular sprouts under normoxia or hypoxia condition in the presence of angiogenic stimulant and inhibitor, VEGF and PEDF. In summary, the above analyses revealed that the morphology of vascular sprouts formed in this model appears more representative of myocardial capillary formation in vivo, and this accessible, reliable angiogenic assay is a more physiologically relevant assay which allows further assessment of pharmacologic compounds on cardiac angiogenesis.

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1. Introduction

Cardiac angiogenesis is of major interest because of its involvement in numerous cardiac pathologies. Studies have demonstrated that stimulation of angiogenesis is beneficial to ischemic and infarcted heart (Zhao et al., 2010), while impaired angiogenesis may delay cardiac repair and cause cardiac rupture or immature scar tissue formation (Barandon et al., 2004). Examining the physical process of cardiac angiogenesis requires experimental systems in which the formation of new capillary vessels can be easily observed and manipulated (Nguyen et al., 2013). Currently, numerous in vivo and in vitro angiogenesis assays are in general use, each with their own advantages and limitations (Jain et al., 1997; Auerbach et al., 2003).

The most commonly used in vivo angiogenesis assays include the chick embryo chorioallantoic membrane (CAM) assay (Tay et al., 2012; Ribatti, 2008), the matrigel plug assay (Passaniti et al., 1992), the zebrafish embryo system (Hlushchuk et al., 2016), the retina angiogenesis assay (Pitulescu et al., 2010; Hu et al., 2014), and the rat/mouse hind limb ischemia model (Silvestre et al., 2000). In vivo assays simulate the natural, sequential process of angiogenesis and have the benefit of potentially involving all of the relevant cell types and growth factors, thus providing deep insights into the angiogenic process (Norrby, 2006). However, despite their relevance, in vivo assays are extremely complex and time-consuming, labor-intensive, expensive, and require significant skill in surgery, and thus are unsuitable as routine assays for widespread adoption or for high-throughput testing.

By contrast, in vitro angiogenesis assays tend to be more rapid, less expensive, easier to interpret, and closely mimic the in vivo situation. Several in vitro angiogenesis assays are used widely to investigate and quantify angiogenesis, such as the endothelial tube formation assay (Bootle-Wilbraham et al., 2000; Roberts et al., 2010), the endothelial co-culture assays (Chen et al., 2009; Nehls and Drenckhahn, 1995) or the endothelial spheroid sprouting assay (Klose et al., 2015). However, these cell-based assays are limited by the fact that they are largely focused on observing direct effects on endothelial cells and measuring certain steps in angiogenic process under controlled conditions (Goodwin, 2007), whereas cardiac angiogenesis implies different cell type communication and interactions.

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Therefore, an urgent need exists for the development of improved cardiac angiogenesis assays that can offer high-throughput capacity and simple convenient operation while significantly enhancing the physiological relevance of the in vitro tissue microenvironment. Due to the limitations of the in vivo and in vitro angiogenesis models, organ culture models remain the more reliable choices for cardiac angiogenesis studies. Organ culture models, such as the aortic ring assay (Baker et al., 2012; Stiffey-Wilusz et al., 2001), unlike the in vitro ones, are more complex and incorporate multiple tissue cell types, but simpler to prepare than in vivo methods.

In this study, we developed a novel modified cardiac explants matrigel assay which is more representative of physiologically relevant myocardial angiogenesis, maintaining important elements of the complex tissue microenvironment. As the name suggests, this model involves the embedding of cardiac explants in an extracellular matrigel matrix and culture in a defined medium. Matrigel contains a mixture of basement membrane components, which stimulate endothelial cells to form capillary-like hexagonal structures (Hughes et al., 2010), and is often preferred over in vitro assays because of its ease of use, rapidity and the ability to measure key steps in angiogenesis (Lawley and Kubota, 1989). In this study, we observed the morphology of vascular sprouts formed in three forms of cardiac angiogenesis assays then used quantitative image analyses to further compared the vascular sprout formation activity in two cardiac explants angiogenesis assays and proved that this model has many advantages in researching cardiac angiogenic process under normoxia or hypoxia condition in the presence of angiogenic stimulant or inhibitor, vascular endothelial growth factor (VEGF) (Makiewicz et al., 2016) or pigment epithelium-derived factor (PEDF) (Zhang et al., 2015a). As knowledge of the molecular signals that modulate cardiac angiogenesis continues to emerge, this reliable and cost-effective model will provide an opportunity to investigate cardiac angiogenesis in greater detail and improve therapeutic strategies for ischemic and infarcted heart diseases.

2. Materials and methods

2.1. Reagents

Recombinant rat PEDF (GenBankTM accession number: NM_177927) was synthesized by Cusabio Biotech, Co., Ltd. (Wuhan, China) (Zhang et al., 2015b; Zhang et al., 2015c; Zhang et al., 2016a). Recombinant rat VEGF was purchased from R&D Systems (Minneapolis, MN) (Zhang et al., 2016b). Matrigel Basement Membrane Matrix was purchased from BD Bioscience (catalog #356234, USA). Fibronectin was purchased from Sigma (catalog # F0895, USA). FITC-Lectin was purchased from Sigma (catalog #103M4035V, USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) purchased from Sciencell (CA, USA, Cat.No.8000) were used for studying angiogenesis. All cells were cultured in endothelial cell medium (ECM) (Sciencell, CA, USA, catalog #1001) supplemented with 5% fetal bovine serum (FBS, ScienCell, catalog #0025), 1% endothelial cell growth supplement (ECGS, ScienCell, catalog #1052) and 1% penicillin/streptomycin solution, in a humidified 37 °C incubator with 5% CO_2 and 21% O_2. The medium was changed after 24 h and every other day. HUVECs used in this study were obtained between passages 3 and 6.

2.3. In vitro tubule formation assay

The formation of vascular-like structures by HUVECs on matrigel was performed as previously described with minor modifications (Chen et al., 2016). The 24-well culture plates (Corning, USA) were coated with 250 μL of Matrigel Basement Membrane Matrix per well and then allowed to polymerize for 30 min in a humidified CO_2 incubator at 37 °C. Cultured HUVECs were seeded on coated plates at a density of 6×10^4 cells per well in ECM (500 μL) supplemented with 5% fetal bovine serum, 1% endothelial cell growth supplement and 1% penicillin/streptomycin solution and then incubated for 18 h at 37 °C before observation. The micrographs of tube formation were taken and processed by an inverted microscopy (Olympus IX73, Tokyo, Japan).

2.4. Animals

Neonatal Sprague–Dawley (SD) rats (1–3 d old, weighing 5–7 g) were obtained from the Experimental Animal Centre of Xuzhou Medical University and housed in a controlled environment. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). The animal care and experimental protocols were approved by the Xuzhou Medical University Committee on Animal Care.

2.5. Cardiac explants angiogenesis model

The cardiac explants angiogenesis was performed as followed. Briefly, isolated myocardial tissue from neonatal SD rats (1–3 d old, weighing 5–7 g, means 6.2 ± 0.5 g) was minced into 1- to 2-mm^3 pieces, washed with Ca^{2+}–Mg^{2+}–free phosphate-buffered solution (PBS) (Invitrogen). After 7-minutes digestion with 0.2% trypsin, the cardiac explants were seeded on 24-well tissue culture plates (Corning, USA) coated with 250 μL fibronectin (15 μg/ml) or 250 μL Matrigel Basement Membrane Matrix. Then, the cardiac explants were cultured in ECM at 37 °C and treated with VEGF (10 ng/ml) or PEDF (10 nmol/L). Hypoxia was achieved by culturing the explants in an tri-gas incubator (Heal Force, Shanghai, China) saturated with 5% CO_2/1% O_2 at 37 °C for the indicated time periods. Angiogenesis was evaluated at 2 days. Tubular structures were photographed with an inverted microscopy (Olympus IX73, Tokyo, Japan). Endothelial network formation was quantified in randomly captured microscopic fields by measuring the number and length of formed vascular sprouts.

2.6. Immunofluorescence staining

Vascular sprouts were also visualized by immunostaining of lectin. The cardiac explants were treated with 4% paraformaldehyde for 10 min. After blocking (1× PBS, 5% fetal bovine serum, and 0.1% Triton X-100) for 1 h, the explants were incubated for 1 h at room temperature with FITC-Lectin (Sigma, catalog #103M4035V). Then after washing, the explants were observed under a fluorescence microscope (Olympus IX73, Tokyo, Japan). Several micrographs were obtained randomly from each explants for vascular sprout analyses.

2.7. Image analysis

The micrographs were quantitative analyzed using the Wimasis Image Analysis, where users can load their images and have their images analyzed by the web platform (https://mywim.wimasis.com). Wimasis creates image analysis algorithms specific to the needs of the researcher, to help automate and standardize image analysis. In this study, the TIFF images of vascular sprout formation were uploaded on to the Wimasis platform. Vascular sprouts length was assessed by drawing a line along each vascular sprout and measuring the length of the line in pixels. Vascular sprout area was calculated as the total number of pixels in thresholded images. Vascular sprouts were automatically counted. Branch points, tip cells and filopodias were manually counted. In each assay, at least five randomly selected fields of view were photographed in each of triplicate wells per condition.

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