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Carbon nanotube-composite hydrogels promote intercalated disc assembly in engineered cardiac tissues through β1-integrin mediated FAK and RhoA pathway

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ABSTRACT

Carbon nanotube (CNT)-based hydrogels have been shown to support cardiomyocyte growth and function. However, their role in cellular integrity among cardiomyocytes has not been studied in detail and the mechanisms underlying this process remain unclear. Here, single walled CNTs incorporated into gelatin with methacrylate anhydride (CNT/GelMA) hydrogels were utilized to construct cardiac tissues, which enhanced cardiomyocyte adhesion and maturation. Furthermore, through the use of immunohistochemical staining, transmission electron microscopy and intracellular calcium transient measurement, the incorporation of CNTs into the scaffolds was observed to markedly enhance the assembly and formation in the cardiac constructs. Importantly, we further explored the underlying mechanism behind these effects through the use of immunohistochemical staining and western blotting. The β1-integrin-mediated FAK and RhoA signaling pathways were found to be responsible for CNT-induced upregulation of electrical and mechanical junction proteins respectively. Together, our study provides new insights into the facilitative effects of CNTs on ID formation, which has important significance for improving the quality of engineered cardiac tissue and applying them to cardiac regenerative therapies.

Statement of Significance

Currently, the bottleneck to engineering cardiac tissues (ECTs) for cardiac regeneration is the lack of efficient cellular integrity among adjacent cells, especially the insufficient remodeling of intercalated discs (IDs) in ECTs. Recently, carbon nanotube (CNT) hydrogels provide an advantageous supporting microenvironment and therefore benefit greatly the functional performance of ECTs. Although their beneficial effect in modulating ECT performance is evident, the influence of CNTs on structural integrity of ECTs has not been studied in detail, and the mechanisms underlying the process remain to be determined. Here, we utilized carbon nanotube incorporated into gelatin with methacrylate anhydride (CNT/GelMA) hydrogels to construct cardiac tissues, determined the influence of CNTs on intercalated discs (IDs) assembly and formation and explored the underlying mechanisms.

1. Introduction

Cardiac tissue engineering aims to fabricate functional cardiac tissues *in vitro*, that later will be transplanted into to the damaged myocardium for replacement therapy [1–5]. Although significant improvement has been made in this respect, there are still many challenges that remain to be addressed. A major roadblock/challenge in the development of functional engineered cardiac tissues (ECTs) is the lack of efficient cellular integrity among adjacent cells [6–8], which ensures the structural integrity and functionality of the native myocardium.

The structural integrity of the heart is maintained by the end-to-end connections between cardiomyocytes known as the

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intercalated disc (ID). The ID, a highly specialized cell-cell contact structure, is comprised of different junctions, including adherens-, desmosomal-, and gap junctions, which electromechanically couple cardiomyocytes (CMs) into a syncytium and are essential for the morphogenesis, differentiation, and maintenance of tissues [9,10]. Furthermore, studies have demonstrated that the mutation of even one ID protein can cause inherited heart diseases, such as arrhythmogenic right ventricular cardiomyopathy (ARVC) [11,12]. Therefore, in order to induce proper cardiac tissue assembly into functional ECTs, it is essential to investigate the development mode of IDs in the microenvironment provided by the scaffold biomaterial.

Among various biomaterials, nanocomposite hydrogels have received significant and growing interest for biomedical applications, such as drug delivery, tissue engineering, and regenerative medicine [13,14]. In this regard, carbon nanotubes (CNTs) based hydrogels present important advantages for cardiac tissue engineering since they mimic the native extracellular matrix (ECM) [15–22]. These CNT hydrogels provide topographical, electrical and mechanical cues for proper organization and tissue formation. To date, CNTs have been incorporated to various synthetic and natural polymers to fabricate various conductive nanocomposite hydrogels, which exhibit excellent electrical and mechanical properties. In a series of studies, we and other groups have previously shown that CNTs embedded within natural hydrogels, such as gelatin, alginate and chitosan, support or direct the attachment, electrical coupling and function of CMs [18–20]. Most notably, Shin et al. cultured cardiomyocytes on CNTs incorporated into gelatin methacrylate (GelMA) hydrogels and found the enhanced electrophysiological activities of CMs and ultimately developed functional ECTs [21,22]. These works highlight that CNT hydrogels provide an advantageous supportive microenvironment and improving the functional performance of constructed cardiac tissue. Although their beneficial effect in modulating ECT performance is evident, the influence of CNT hydrogels on structural integrity of ECTs has not been studied in detail, and the mechanisms underlying the process remain to be determined.

In our recent work, we have shown that CNT-collagen substrates enhanced ID assembly in cultured cardiomyocytes [23]. Here, we utilized CNT/GelMA hydrogels to construct cardiac tissues and determined the influence of CNTs on ID assembly and formation on the tissue level. Furthermore, we explored the underlying mechanisms by which signaling pathway CNTs regulate this process.

2. Materials and methods

2.1. Preparation of CNT/GelMA hydrogels

All chemicals were purchased from Sigma-Aldrich. Single wall carbon nanotubes (SWCNTs; 0.7–1.3 nm in diameter and 5–20 μm in length) were purchased from US Nanomaterials Research Inc. CNT/GelMA hydrogels were fabricated and characterized as previously described [21]. In brief, GelMA precursors were first obtained by adding methacrylate anhydride (MA) into 10% (w/v) of gelatin solution at 50 °C. CNTs with various concentrations of 0, 0.5, 1 and 2 mg/mL were prepared by dispersing in 2% Pluronic copolymer solution. Subsequently, the CNT solution was added to GelMA solution containing photoinitiator (2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone, 0.5% w/v). The mixtures were ultra-sonicated for 1 min × 5 times in an ice bath, resulting in homogenous solutions. Finally, the mixtures were cast into 24 well dishes, and then left under UV (wavelength 360–480 nm, 6.9 m W/cm^2) for 30 min to allow the cross-linking reaction to occur.

2.2. Characterization of CNT/GelMA hydrogels

Scanning electron microscopy (SEM) was utilized to observe the surface topography of CNT/GelMA and GelMA hydrogels. The samples were dried through a freeze-drying procedure and sputter-coated with gold (Autoconductavac IV, See-Vac). The microscope was operated under a working voltage of 10 kV. For the porosity analysis, six SEM images were obtained and the data was analyzed using ImageJ software.

For atomic force microscope (AFM) analysis, the microscope (Nanoscope IIIa, Digital Instruments Inc., USA) was registered in tapping mode with a scanning rate of 1 Hz and scanning line of 512. A scan field of 1 × 1 μm was used for measurements and the data was analyzed using NanoScope imaging software.

For mechanical testing, the compressive modulus of the hydrogels was obtained from a TA.XT2i Texture Analyzer (Texture Technologies Corp.). The samples were measured from the slope in the linear region corresponding to 5–15% strain at 0.1 mm/s.

For degradation analysis *in vitro*, the hydrogels were immersed in PBS (pH = 7.4) at 37 °C for 3 and 7 days respectively. The detailed method was characterized and determined as previously described [24].

2.3. Construction and evaluation of ECTs

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-day-old Sprague-Dawley (SD) rats as previously described [23]. All SD rats were purchased from Chengdu Da Shuo Biotech Co., Ltd. (Chengdu, China). All experiments in the study were in compliance with the Committee on the Ethics of Animal Experiments of Chengdu Military General Hospital (Chengdu, China). Following isolation and enrichment through 2 h preplating, NRVMs (>90% purity) were immediately seeded into either CNT/GelMA or GelMA hydrogels to construct ECTs (7×10^7 cells/cm^3). In some experiments, the gelatin-coated dish was used as control. All samples were cultivated in DMEM (Invitrogen, Carlsbad, CA, USA) culture medium containing 15% FBS (Invitrogen, Carlsbad, CA, USA) at 37 °C and 5% CO2. The culture medium was changed daily.

2.4. Cell viability assays

A Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen, Grand Island, NY) was utilized to assess the viability of cardiac constructs based on CNT/GelMA and GelMA hydrogels. At day 1, cardiac constructs were incubated with 2 μM calcein AM and 4 μM EthD-1 in phosphate-buffered saline (PBS) for 30 min. Ten randomly selected fields of each group were visualized under a fluorescence microscope ((Nikon AZ-100 multipurpose microscope) using a 10× objective lens. The obtained images were processed by ImageJ software. Additionally, cytotoxicity evaluation was performed by AlamarBlue based colorimetric assays. Experiments were conducted in triplicate per each group.

2.5. Immunofluorescence staining and confocal microscopy

Immunofluorescence was performed as previously described [27]. Cardiac constructs based on CNT/GelMA and GelMA hydrogels were fixed in 4% formaldehyde for 10 min and permeabilized in 0.3% Triton X-100 for 10 min, and then blocked in 2% BSA in PBS for 1 h at room temperature. The samples were then incubated with primary antibodies overnight at 4 °C, including mouse monoclonal anti-alpha-actinin (sarcomeric) (EA-53, α-SA) (1:100, AbCam, Cambridge, MA), rabbit monoclonal anti-Troponin I (TnI) (1:100, AbCam, Cambridge, MA), and rabbit polyclonal anti-connexin-43 (1:1000, AbCam, Cambridge, MA), anti-N-cadherin (1:200, Abcam Cambridge, MA), rabbit monoclonal anti-plakophilin2