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Functional improvement and maturation of rat and human engineered heart tissue by chronic electrical stimulation

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ABSTRACT

Spontaneously beating engineered heart tissue (EHT) represents an advanced in vitro model for drug testing and disease modeling, but cardiomyocytes in EHTs are less mature and generate lower forces than in the adult heart. We devised a novel pacing system integrated in a setup for videooptical recording of EHT contractile function over time and investigated whether sustained electrical field stimulation improved EHT properties. EHTs were generated from neonatal rat heart cells (rEHT, n = 96) or human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes (hEHT, n = 19). Pacing with biphasic pulses was initiated on day 4 of culture. REHT continuously paced for 16–18 days at 0.5 Hz developed 2.2× higher forces than nonstimulated rEHT. This was reflected by higher cardiomyocyte density in the center of EHTs, increased connexin-43 abundance as investigated by two-photon microscopy and remarkably improved sarcomere ultrastructure including regular M-bands. Further signs of tissue maturation include a rightward shift (to more physiological values) of the Ca^{2+}-response curve, increased force response to isoprenaline and decreased spontaneous beating activity. Human EHTs stimulated at 2 Hz in the first week and 1.5 Hz thereafter developed 1.5× higher forces than nonstimulated hEHT on day 14, an ameliorated muscular network of longitudinally oriented cardiomyocytes and a higher cytoplasm-to-nucleus ratio. Taken together, continuous pacing improved structural and functional properties of rEHTs and hEHTs to an unprecedented level. Electrical stimulation appears to be an important step toward the generation of fully mature EHT.

1. Introduction

Cardiac tissue engineering aims at providing advanced in vitro models for drug testing and disease modeling as well as heart muscle tissue for cardiac regeneration [1]. With the recent progress in human pluripotent stem cell (hPSC) technologies and the principally unlimited availability of hPSC-derived cardiomyocytes, 3D human engineered heart tissues (hEHTs) will soon be widely available. Even so rodent EHTs are easier to produce, generally develop higher contractile force and are considerably cheaper than human EHT. They therefore still represent the workhorse in most laboratories. The 3D culture form of EHTs combined with directed mechanical load allows cardiomyocytes in EHTs to reach a higher degree of differentiation and maturation than standard 2D cultures [2], probably reaching the highest level possible

Abbreviations: cp-p, continuously paced group-paced state; cp-s, continuously paced group-spontaneous state; EC_{50}, half maximal effective concentration; ECM, extracellular Matrix; EHT, engineered heart tissue; FC, fold change; FS, fractional shortening; hEHT, human engineered heart tissue; hiPSC, human induced pluripotent stem cell; hPSC, human pluripotent stem cell; MLC2v, myosin regulatory light chain 2, ventricular isoform; rEHT, rat engineered heart tissue.

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to date. However, both rodent [3] and human EHTs [4] and related 3D engineered cardiac constructs [5,6] show signs of cardiomyocyte immaturity. Differences compared to adult cardiomyocytes include the following. (i) Adult ventricular cardiomyocytes are quiescent and need a stimulus to beat [7]. Hence, spontaneous beating of EHTs from neonatal rodent cardiomyocytes or differentiated human pluripotent stem cells argues for immaturity. (ii) Twitch forces of freshly isolated human cardiomyocytes reach >50 mN/mm^2 [8], while regularly sized rodent EHTs rarely develop more than 1 mN/mm^2 (very thin ones reach >5 mN/mm^2) and human EHTs are frequently even weaker [9] although the group of Bursac recently reported considerably higher forces [10]. Part of this difference is due to a much lower volume fraction occupied by cardiomyocytes (compared to cell-free matrix) [11]. (iii) EHTs display hypersensitivity toward external calcium with an EC_{50} of 0.15 mM and 1.05 mM in rat and human EHTs, respectively [12]. This compares with 2.1 mM [13] and 3.0 mM [14] in adult rat and human muscle strips, respectively. (iv) The positive inotropic response to the beta-adrenergic agonist isoprenaline is less pronounced in EHTs than in adult hearts [9]. (v) Cardiomyocytes in native heart tissue are rod-shaped and couple end-to-end in intercalated disks which are very rich in gap junctions. This degree of spatial organization is not reached in EHT in which cardiomyocytes rather couple side to side [3] and, even under optimized conditions, display an abnormal length to width ratio (>11 compared to 7) [15].

Thus, a major goal in the field is to advance maturation of cultured cardiomyocytes in general and within EHT in particular. The simplest idea is waiting, and indeed culture times of several months are feasible for both rodent and human EHTs and led to improved maturation [9,15]. This is very likely facilitated by the beneficial 3D growth environment which enables interaction with fibroblasts and extracellular matrix [16], and optimal biomechanical loading [6,11]. However, most cardiac diseases occur in early or even late adult life, i.e. in cells decades older than their hPSC-derived counterparts in vitro, and long culture times are expensive and prone to infections, making this approach practically unfeasible. Growth factors and hormones, e.g. triiodothyronine (T3) [17], neuregulin-1β [18] or insulin-like growth factor-1 [19], and high ambient oxygen concentrations [20,21] promote cardiac maturation and 3D tissue development. Another method for tissue maturation is electrical field stimulation, which may play a natural role in tissues with electrical field strengths in the range of 0.01–2.0 V/cm [22]. Beneficial effects of pacing have been shown in cardiomyocyte-seeded collagen sponges [5] and EHTs [23], but the final tissue quality did not exceed that of standard fibrin EHTs cultured under high oxygen and in the presence of insulin and horse serum (with endogenous T3) [3].

In this study we established a protocol to electrically stimulate rat and human (induced pluripotent stem cell derived)-EHTs for several weeks. Stimulated rat EHTs were considerably stronger than controls, showed reduced spontaneous beating activity, a lower Ca^{2+}-sensitivity, a stronger inotropic response to isoprenaline and remarkably improved structure and ultrastructure. Likewise, force of human EHTs increased and their structural organization ameliorated considerably.

2. Material and methods

2.1. Generation of rat and human EHT

Rat engineered heart tissue (rEHT) was produced according to previously published protocols in a 24-well cell culture format [3,11]. All procedures were approved by the Hamburg Ethics Commission. In brief, ventricles from neonatal Wistar and Lewis rats (postnatal day 0 to 3) were digested repeatedly by DNAse and Trypsin. For one tissue strip of 100 μL 500,000 ventricular heart cells, fibrinogen, thrombin and DMEM were mixed and immediately cast into agarose casting molds around silicone posts protruding from a silicone rack. Within a period of two hours the fibrin polymerized and built an extracellular matrix for the ventricular heart cells. Afterwards the silicone racks each carrying 4 EHTs were transferred to a cell culture dish filled with rEHT medium. This consisted of DMEM (Biochrom F0415), 10% horse serum inactivated (Gibco 26050), 2% chick embryo extract, 1% penicillin/streptomycin (Gibco 15140), insulin (10 μg/mL, Sigma-Aldrich 857653), and aprotinin (33 μg/mL, Sigma-Aldrich A1153) and was changed three times per week.

Human engineered heart tissue (hEHT) was produced from human induced pluripotent stem cell (hiPSC) clone C25 which was a generous gift from the group of Laugwitz [24]. HiPSCs were differentiated into cardiomyocytes according to our previously published protocol for human embryonic stem cells (hESCs) [4]. Further enrichment was performed as recently described [25]. After the differentiation process 500,000 cells (cardiomyocyte content: 80–90%) were used for the generation of human EHTs (initial volume 100 μL). The procedure was carried out analogous to the production of rat EHTs with the exception that the fibrin matrix was supplemented with 10% Matrigel (BD Bioscience 356235) in the reconstitution mix and that EHTs from this lactate-enriched cell population were maintained in lactate-based EHT medium. This human EHT medium was changed daily and consisted of DMEM without glucose (Gibco 11966-025), 10% fetal calf serum inactivated (Biochrom S0615), 1% penicillin/streptomycin (Gibco 15140), insulin (10 μg/mL, Sigma-Aldrich 857653), aprotinin (33 μg/mL, Sigma-Aldrich A1153), and sodium L-lactate (4 mM, Sigma-Aldrich 71718). Both rat and human EHTs were maintained in 37 °C, 7% CO_2, and 40% O_2 humidified cell culture incubators throughout experiments and video-optical recordings. Aprotinin in the culture medium was required to prevent rapid dissolution of the fibrin matrix in the presence of serum.

2.2. Continuous electrical stimulation of EHT

EHTs were paced by mounting the silicone racks onto custom-made electrical pacing units. During development of these units their scaffold material was changed from acrylic glass over brass to stainless steel and electrode material from stainless steel to carbon. In the final version (Fig. 1A), which was exclusively used in this study, two stainless steel square bars (austenitic grade EN 1.4301, UNS S30400; Koch + Krupitzer, Germany) served both as conducting material and as scaffold for four pairs of carbon electrodes (CG 1290 Carbon Graphite Consulting Klein, Siegen, Germany). They were isolated from each other by polyamide plastic screws (GHW, Niederkrüchten, Germany). Only stainless steel connecting screws and nuts, rust-proof tools and unleaded tin-solder were used in the assembly process of the pacing units. A readily assembled pacing unit was narrow enough to fit into the wells of a standard 24-well dish (Fig. 1B). By connecting 6 pacing units in parallel 24 EHTs could be stimulated simultaneously. Yet, a significant disadvantage of electrical stimulation in buffer solutions is the formation of hypochlorous acid (HOCL), a strong oxidizer [26], and oxygen-derived free radicals [27]. Not to interfere with redox-reactions in cardiomyocytes we have refrained from supplementing our medium with an antioxidant such as sodium ascorbate [28], but employed known strategies to reduce the production of toxic metabolites and to optimize the stimulation setting. We used comparatively large carbon [29] electrodes in relation to the size of a single well which stood in a distance of 1 cm parallel to one another yielding a homogeneous electrical field. Symmetric biphasic pulses [23] of short duration [29] (4 ms overall, 2 ms in both polarities) were applied and both culture medium and pacing units were changed frequently (three times a week for rEHT and daily for human EHT). They were reconditioned by washing them at least 4 times 12 h in distilled water before autoclaving.

At day 4 of culture continuous electrical stimulation was initiated. The signals were generated by a Grass S88X Dual Output Square Stimulator (Natus Neurology Incorporated, Warwick, USA). An output voltage of 2 V (yielding an electrical field strength of 2 V/cm) in biphasic pulses of 4 ms was applied both to rEHT and hEHT.