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Original article

Skeletal muscle stem cells propagated as myospheres display electrophysiological properties modulated by culture conditions

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ABSTRACT

In cardiac regenerative therapy, transplantation of stem cells to form new myocardium is limited by their inability to integrate into host myocardium and conduct cardiac electrical activity. It is now hypothesized that refining cell sorting could upgrade the therapeutic result. Here we characterized a subpopulation of skeletal muscle stem cells with respect to their electrophysiological properties. The aim of our study was to determine whether electrophysiological parameters are compatible with cardiac function and can be influenced by culture conditions. Low-adherent skeletal muscle stem cells were isolated from the hind legs of 12–20 week old mice. After 6 days of culture the cells were analysed using patch-clamp techniques and RT-PCR, and replated in different media for skeletal muscle or cardiac differentiation. The cells generated action potentials (APs) longer than skeletal muscle APs, expressed functional cardiac Na^+ channels (~46% of the total channel fraction), displayed fast activating and inactivating L-type Ca^{2+} currents, possibly conducted through cardiac channels and did not show significant Cl^- conductance. Moreover, a fraction of cells expressed muscarinic acetylcholine receptors. Conditioning the cells for skeletal muscle differentiation resulted in upregulation of skeletal muscle-specific Na^+ and Ca^{2+} channel expression, shortening of AP duration and loss of functional cardiac Na^+ channels. Cardiomyogenic conditions however, promoted the participation of cardiac Na^+ channels (57% of the total channel fraction). Nevertheless the cells retained properties of myoblasts such as the expression of nicotinic acetylcholine receptors. We conclude that skeletal muscle stem cells display several electrophysiological properties similar to those of cardiomyocytes. Culture conditions modulated these properties but only partially succeeded in further driving the cells towards a cardiac phenotype. This article is part of a special issue entitled, "Cardiovascular Stem Cells Revisited".

1. Introduction

Over the past decade, much work has focused on the identification of a stem cell population that could be implanted in infarcted myocardium and promote cardiac repair. Because of appealing characteristics (easy accessibility and expansion, no risk of tumorigenicity, resistance to ischemia, and intrinsic ability to develop a contractile apparatus) and promising results from pre-clinical studies, autologous skeletal myoblasts were the first cells used in clinical trials (for review see [1]). The ability of skeletal myoblasts to engraft in post-infarct scars, survive and improve left ventricular function has been repeatedly demonstrated. Long-term improvements were however limited and a recent randomized controlled trial failed to show any substantial benefit [2]. These unsatisfactory results are mostly due to the fact that instead of integrating with host tissue, skeletal myoblasts formed isolated islets of differentiated myotubes [3]. Improvement of cardiac function observed earlier is now believed to result from the release of growth factors and cytokines [4].

In order to upgrade the therapeutic outcome, many groups have attempted to isolate sub-populations of myogenic progenitors that could have a greater potential for cardiac repair. Of particular interest is the repeated description of skeletal muscle stem cells featuring a cardiomyogenic protential [5–9]. Evidence for cardiac differentiation included mRNA and protein expression of cardiac transcription factors and contractile, junctional and secretory proteins, as well as spontaneous beating. In coculture experiments, the cells expressed connexin 43 at junction sites with cardiac cells [5], and after injection into murine models of myocardial infarction, they showed a greater and more persistent engraftment, induced more angiogenesis and elicited greater improvements in left ventricular function than unsorted myoblasts [10,11].

Surprisingly, despite the primordial role of electrophysiology in cardiac function, little interest has been shown in the electrical properties of these cells. Electrical coupling between engrafted cells and host cardiomyocytes is a prerequisite for formation of new functional myocardium. Propagation of action potentials (APs)

Abbreviations: MDCs, myosphere-derived cells; APs, action potentials; TTX, tetrodotoxin; ACh, acetylcholine; nAChRs, nicotinic acetylcholine receptors; mAChRs, muscarinic acetylcholine receptors.

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requires effective cell communication through gap junctions as well as Na^+ channels that allow rapid regenerative APs.

The objective of this study was to characterize the electrophysiological properties of skeletal muscle stem cells and to investigate whether culture conditions can influence these properties. We concentrated on characteristics that could allow the distinction between cardiac and skeletal muscle phenotypes, including the presence of Na^+, Ca^{2+}, K^+ and Cl^- channels, and acetylcholine receptors. Among other properties, Na^+ channels can be distinguished by their sensitivity to tetrodotoxin (TTX). The major cardiac Na^+ channel isoform, Nav1.5, is TTX-resistant (IC_{50} ~2 μM) whereas the skeletal muscle isoform, Nav1.4, is TTX-sensitive (IC_{50} ~5–15 nM) [12]. Divalent cations such as Cd^{2+} or Zn^{2+}, can block Na^+ channels with an affinity opposite to that of TTX. Thus the TTX-sensitive channel Nav1.4 is blocked by millimolar concentrations of Cd^{2+} whereas micromolar concentrations are enough to block the TTX-resistant channel Nav1.5 [12]. TTX and Cd^{2+} therefore allow distinction between cardiac and skeletal muscle Na^+ channels. The skeletal muscle and cardiac L-type Ca^{2+} channels, Cav1.1 and Cav1.2, show similar sensitivities to drugs and hence are difficult to distinguish pharmacologically. They nevertheless exhibit differences in their activation and inactivation kinetics, i.e. Cav1.1 activates and inactivates more slowly than Cav1.2 (for review see [13]). In cardiomyocytes, stabilization of the membrane potential and repolarization of action potentials are achieved by K^+ channels. In contrast, Cl^- channels largely participate in these tasks in skeletal muscle (for review see [14]). Finally, acetylcholine (ACh) receptors are present in both cardiac and skeletal muscle tissues but have opposite functions. In cardiac tissue, muscarinic ACh receptors (mAChRs) activate inward-rectifier K^+ channels resulting in hyperpolarization of the membrane. In contrast, skeletal muscle expresses nicotinic ACh receptors (nACHRs), unselective cation channels that allow Na^+ influx upon activation leading to membrane depolarization.

Using these electrophysiological differences as tools to distinguish between skeletal muscle and cardiac phenotypes, we demonstrated that (i) skeletal muscle stem cells show some electrophysiological characteristics similar to those of cardiomyocytes, and (ii) culture conditions induce changes in these electrophysiological properties.

2. Material and methods

Adult male C57Bl/6 mice (12–20 weeks old) were used for this study. Animal care and experimental protocol were conducted in accordance with the German federal animal protection law and approved by the institutional committee at the Dresden University of Technology. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Isolation of myosphere-derived cells (MDCs)

Cells were isolated as described before with slight modifications [6]. Briefly, skeletal muscle tissue from hind legs, was minced after carefully removing adipose tissue and large vasculature, and digested with 5 mg/ml collagenase II (Biochrom, Berlin, Germany) for 70 min at 37 °C. The suspension was cleared of undigested fragments by filtration through 100 μm and 40 μm meshes. The cells were incubated in DMEM (Sigma-Aldrich, Steinheim, Germany), 5% FCS (Biochrom) for 2 h at 37 °C to discard fibroblasts. The cell suspension was then plated in 3 ml of M1 medium: DMEM, 5% FCS, 10 ng/ml EGF and bFGF (Sigma-Aldrich), 1% ITS-X supplement (Insulin, Transferrin, Selenium, Invitrogen, Karlsruhe, Germany) and antibiotics (Biochrom). Within 3 days, triangular or spindle-shaped cells attached onto the plate. Another population of cells, however, remained floating, growing as clusters (myospheres). After 6 days of culture myosphere-derived cells (MDCs 6d) were either used for RNA isolation and patch-clamp, or replated and further cultivated for 1 week in 3 different media: 1) M2: identical to M1 except that no growth factor was added; 2) RA: M2 supplemented with 10^{-7} M of retinoic acid (9-cis-retinoic acid, Sigma-Aldrich); 3) HS: M2 with 2% horse serum (Biochrom) instead of 5% FCS. After 1 week of culture, the cells were used for RNA isolation and patch-clamp.

2.2. Isolation of ventricular cardiomyocytes

For ventricular myocytes isolation, mice received heparin (5000 U/kg) by intraperitoneal injection 60 min before cervical dislocation. Hearts were quickly excised, mounted on a Langendorff apparatus and perfused with Ca^{2+}-free solution (in mM: NaCl 150, KCl 5.4, $MgCl_2$ 2, glucose 11, and HEPES 10, pH = 7.4, adjusted with NaOH) to wash out blood. After 5 min, the perfusion was switched to 125 U/ml collagenase solution (Worthington, Lakewood, USA) supplemented with 0.08% BSA (Sigma-Aldrich). After 5–10 min of tissue digestion, the hearts were taken off the apparatus, atrial tissue was removed and ventricles were cut into small pieces that were gently agitated in recovery solution (in mM: KOH 70, KCl 40, EGTA 0.5, taurine 20, glutamic acid 50, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, and HEPES 10, pH = 7.4, adjusted with KOH). Tissue debris were removed by filtration through a 250 μm mesh. The myocytes were then permitted to settle under gravity for 15 min, resuspended in recovery solution supplemented with 0.5 mM Ca^{2+} and stored at room temperature until use.

2.3. RNA isolation

Total RNA from tissues (skeletal muscle, heart and brain) was isolated using the peqGOLD RNAPure™ reagent (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. Total RNA from cells was isolated using the peqGOLD Total RNA kit (PeqLab) according to the manufacturer's instructions. RNA was cleared off genomic DNA by DNase digestion (DNase I digest kit, PeqLab).

2.4. Semi-quantitative RT-PCR

cDNA was synthesized from 500 ng of total RNA with the Moloney murine leukaemia virus reverse transcriptase system (Promega, Mannheim, Germany). PCR experiments were performed using the Taq polymerase system (Roche, Mannheim, Germany) following the manufacturer's recommendations. Primers were designed to span 1 intron and ordered from Eurofins MWG Operon. The products were analysed by agarose gel electrophoresis and confirmed by sequence analysis.

2.5. Quantitative RT-PCR

Comparative quantitative PCR (qPCR) was performed using the QuantiTect SYBR® Green RT-PCR kit (Qiagen, Hilden, Germany) and a Corbett Rotor-Gene 2000 cycler (Qiagen). The primers were ordered from Qiagen. Data were normalized to the expression of polymerase II polypeptide A (Polr2a).

2.6. Electrophysiological recordings

Action potentials and membrane currents were measured with standard whole cell current- and voltage-clamp techniques. ISO-2 software (MFK, Nierdernhausen, Germany) was used for data acquisition and analysis. Borosilicate glass microelectrodes had tip resistances of 2–5 MΩ when filled with pipette solution. Series resistance and cell capacitance were compensated up to 70%. All chemicals and drugs were purchased from Sigma-Aldrich.

Action potentials were recorded at 37 °C with the following bath solution (mM): NaCl 150, KCl 5.4, $CaCl_2$ 2, $MgCl_2$ 2, and glucose 11, Hepes 10 (pH 7.4, adjusted with NaOH). The pipette solution