Human and Murine Embryonic Stem Cell-Derived Cardiomyocytes Serve Together as a Valuable Model for Drug Safety Screening

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Key Words

Human embryonic stem cells • Cardiomyocytes • Antiarrhythmic drugs • QT-screening • Repolarization • Drug screening

Abstract

Aims: Screening of drug safety is typically performed in diverse non-human healthy species with an intact repolarization reserve. Nevertheless, these drugs are later applied in diseased humans with a reduced repolarization reserve. It would be optimal to set up a preclinical screening tool to estimate the proarrhythmic potential of drugs in human cardiac tissue with a reduced repolarization reserve *in vitro*. **Methods and Results:** In our study spontaneously beating human embryonic stem cell-derived cardiomyocytes clusters (hESCM) and murine ES cell-derived cardiomyocytes (mESCMs) were plated onto micro-electrode arrays (MEAs) to record the extracellular field potentials (FPs) as well as effects of several antiarrhythmic drugs. In line with clinical observations the class III antiarrhythmic drugs ($\pm$)-sotalol, E4031 and class I antiarrhythmic drug quinidine led to a prolongation of the cardiac repolarization phase (FP duration, FPdur) and a decrease of the FP frequency. Verapamil (a class IV

antiarrhythmic drug) decreased the FP frequency and shortened FPdur. Both, quinidine and verapamil, but not ($\pm$)-sotalol or E4031 decreased conduction velocities in hESCM clusters. Moreover, ($\pm$)-sotalol exerted stronger effects on FPdur in early developmental stages of hESCMs, as proof for a reduced repolarization reserve. The EC₅₀ of the ($\pm$)-sotalol-induced prolongation of the FPdur was higher in mESCMs than in hESCMs implying species-dependent differences in cardiac repolarization. Likewise, the incidence of drug-induced early recurrent depolarization (ERDs) was higher in mESCMs than hESCMs. **Conclusion:** The combined measurement of drug effects on FP parameters in hESCMs and mESCMs serves as a reliable *in vitro* model for preclinical studies of drug safety.

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Introduction

Safety pharmacology focuses on drug-induced prolongation of the QT-interval in the electrocardiogram since the use of drugs that prolong the QT interval as readout for the cardiac repolarization reserve is considered to increase the risk of torsade de pointes (TdP), a life-threatening polymorphic ventricular tachycardia [1-4].

To ensure that only the most effective and safest drugs enter clinical trials, preclinical safety-pharmacology studies assessing the effects on cardiac repolarization *in vitro* may help to enhance drug safety. Beside others, animal models have been used as a valuable model to study drug safety [5-7]. However, due to the well-known species-dependent differences of cardiac electrophysiology, data derived from animal models cannot directly be transferred to humans.

Recently, systems allowing high temporal and spatial recordings of extracellular field potentials (FPs) such as the microelectrode array (MEA) system [8], have been proposed as a pharmacological screening tool for cardioactive drugs in human cardiac tissue [9]. We could recently show that with such experimental models drug effects on the cardiac repolarization phase and the proarrhythmic as well as the cardiac conduction prolonging potential can easily be determined.

Human embryonic stem cells (hESC), which have already been shown to propagate *in vitro* towards cardiomyocytes [10, 11], appear as an attractive model for drug safety screening as they provide an infinite source of cardiac tissue. In fact, we have recently shown that ($\pm$)-sotalol leads to a strong prolongation of the hESCMs repolarization phase strongly suggesting a reduced repolarization reserve [12] that is also a key feature of the diseased human heart.

In the present study we therefore focused on the effects of antiarrhythmic drugs on FP parameters and their proarrhythmic potential in cardiomyocyte clusters derived from the hES line H1. In line with clinical observations, class I, III and IV antiarrhythmic drugs led to typical effects on FP frequency, cardiac repolarization and conduction. Most importantly, as the diseased human adult heart, especially early developmental stages of hESCMs showed a significantly reduced repolarization reserve making them to an attractive model candidate of the diseased heart. Moreover, in accordance to previous studies important species-dependent differences between mESCMs and hESCMs were found that emphasize the need for an implementation of novel preclinical research techniques into daily drug safety screening routines.

Materials and Methods

Cardiac Differentiation of murine ES cells (mESC) and human ES Cells (hESC)

The mESC line R1 was cultivated and differentiated to cardiac spontaneously beating clusters as described [13]. The hESC line H1 (passage numbers between 30 and 70; WiCell

Research Institute, Madison, WI,) was imported according to German law and after approval by the Robert Koch Institute (Berlin) (No. AZ 1710-79-1-4-2; AZ 1710-79-1-4-2-E2). H1 cells were differentiated to spontaneously beating cardiac clusters as described previously [14]. Briefly, H1 colonies were cultivated on CF1 feeder cell layers for approximately seven days before they were transferred as clusters of approximately 500-800 cells onto cell layers of a visceral endoderm-like cell line (END-2) pretreated with mitomycin C (Serva GmbH, Heidelberg, Germany). hESCs were cultured in knockout Dulbecco's modified Eagle's medium supplemented with 20% serum replacer (Invitrogen, Carlsbad, CA), 1 mmol/l L-glutamine, 1% nonessential amino acids, 1 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 g/ml streptomycin sulfate, and 4 ng/ml basic fibroblast growth factor. Then, hESCs and END-2 cells were cocultured for up to 2 weeks without serum or serum replacer and without basic fibroblast growth factor and scored for the presence of beating areas from five days onward. For electrophysiological extracellular FP measurements, beating areas were cut out of the differentiating EB outgrowths by the use of a micro-scalpel and were directly plated onto MEAs.

MEA measurements

After plating and attachment of hESCMs or mESCMs on MEAs, extracellular recording was performed using a MEA data acquisition system (Multi Channel Systems, Reutlingen, Germany) [15]. The standard substrate-integrated MEA culture dish contains 60 Titanium Nitride coated gold electrodes (30 μ m diameter) arranged in an 8x8 electrode grid with an inter-electrode distance of 200 μ m, allowing simultaneous recording of extracellular FPs from all electrodes at a sampling rate of 1 to 25 kHz by the use of the MEA amplifier system. Standard measurements were performed at 1-2 kHz (bandwidth 1-5 kHz) in standard Tyrode solution. The extracellular solution contained (in mmol/L): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, Na-Pyruvic acid 2, HEPES 10, Glucose 10, pH=7.4 adjusted with NaOH. During recordings, temperature was kept constant at 37.0°C. Data were analyzed off-line with a customized toolbox programmed with MATLAB (The Mathworks, Natick, MA, USA) to detect and characterize FPs as described [16].

Substances

E4031 dihydrochloride was obtained from Tocris bioscience (Eching, Germany), ($\pm$)-sotalol hydrochloride, quinidine, and verapamil were purchased from Sigma-Aldrich GmbH (Deisenhofen, Germany) and were dissolved in extracellular solution and stored according to the manufacturer's guidelines. If not otherwise stated we applied drug concentrations that have been reported to be expected as serum concentration *in vivo* or as suggested by preclinic research.

Data analysis

The beating frequency was calculated from the distance between respective FP minima (FPmin) and denoted inter-spike interval (ISI). The FP duration (FPdur) was calculated as the distance from FPmin to FPmax. To exclude influence of the beating frequency on FP parameters we further corrected our data according to the Bazzett's formula (FPDc= FPdur/(60/

Fig. 1. Effects of ($\pm$)-sotalol on FPdur and incidence of ERDs. The EC₅₀ of ($\pm$)-sotalol in mESCMs and hESCMs were 74.0 μ M and 144.6 μ M, respectively. (A) ($\pm$)-sotalol concentration-dependently induced ERDs in mESCMs but not in hESCMs (B). Note the significant prolongation of FPdc in hESCMs. (C) Concentration-response curve of ($\pm$)-sotalol in hESCMs and mESCMs.

Fig. 2. Development-dependent prolongation of FPdur in hESCMs. (A) At early development stages (=15 days), FPdc was significantly prolonged by ($\pm$)-sotalol. Statistical analysis showed prolongation by 66.9 $\pm$ 9.9% (n=5, *p<0.05) (B). However, at later developmental stages (=30 days, C), FPdc was increased by 19.1 $\pm$ 6.1% (n=5, *p<0.05) (D). 1, control; 2, ($\pm$)-sotalol.

frequency (1/min))^{1/2}). Conduction velocities were calculated by dividing the distance between individual electrodes by the delay of the respective electrode-related FP. Results are presented as mean $\pm$ standard error of the mean. Statistical analysis was done using paired or unpaired student's *t*-test where applicable. P values of <0.05 were considered significant.

Results

Higher Incidence of Early Recurrent Depolarizations (ERDs) in mESCMs

Several established cardioactive pharmacological agents were tested for the occurrence of early recurrent depolarizations (ERDs) as a read out for their proarrhythmic potential. Taken together all tested agents induced ERDs in mESCMs but rarely in hESCMs. In detail, 300 μ M ($\pm$)-sotalol induced ERDs in all tested mESCMs clusters (Fig. 1A, n=5). In six out of seven beating hESCMs no arrhythmia was induced by ($\pm$)-

sotalol even at a concentration of 1 mM (Fig. 1B). Also E4031 (5 μ M) did not lead to ERDs in hESCMs (n=7) but in all mESCMs (n=4). Likewise, quinidine (20 μ M) induced ERDs in three out of eight mESCMs samples but in none of the tested hESCMs (n=12). The dose dependence of ($\pm$)-sotalol to prolong FPdc with an EC₅₀ of 74.0 μ M in mESCMs and of 144.6 μ M in hESCMs (Fig. 1C) further underlined the species-dependent differences. Note the prolongation of cardiac repolarization due to sotalol application (Fig. 1A, B)

Fig. 3. Effects of quinidine on FP frequency and FP morphology in hESCMs. Quinidine (20 μ M) decreased FP frequency, and prolonged FPDc (original tracings, A, control; B, quinidine; C, washout). (D) Overlay of single FPs showing the prolongation of FPDc. (E) Time course of the effect of quinidine on FPDc. (F) Time course of the effect of quinidine on FP frequency. (G) Effect of quinidine on FPDc and FP frequency. Effects were reversible after washout.

Development-dependent differences of the cardiac repolarization reserve

Since especially early stages of hESCMs are believed to serve as a good model for the diseased human heart *in vitro* due to their proposed reduced repolarization reserve we aimed to study the repolarization phase at different developmental stages. ($\pm$)-sotalol as a standard QT prolonging drug was applied and the FPDc studied. At early developmental stages (≤ 15 days), ($\pm$)-sotalol prolonged FPDc by $66.9 \pm 9.9\%$ ($n=5$, $p<0.05$) (Fig. 2A-B), whereas at later developmental stages (≥ 30 days) FPDc was only increased by $19.1 \pm 6.1\%$ ($n=5$, $p=0.29$) (Fig. 2C-D). Thus, the repolarization reserve increases during development.

Fig. 4. Effect of ($\pm$)-sotalol on FP frequency and FP morphology in hESCMs. ($\pm$)-sotalol (300 μ M) decreased FP frequency and prolonged FPDc (original tracings, A, control; B, ($\pm$)-sotalol; C, washout). (D) Overlay of single FPs showing the prolongation of FPDc. (E) Time course of the effect of ($\pm$)-sotalol on FPDc. (F) Time course of the effect of ($\pm$)-sotalol on FP frequency. (G) Effect of ($\pm$)-sotalol on FPDc and FP frequency. Effects were reversible after washout.

Drug-induced changes of FP parameters in hESCMs

By calculating several FP parameters, i.e. FPDc, FP frequency, FPmin and conduction velocities, effects of the above mentioned drugs on early stage hESCMs were evaluated. 20 μ M quinidine, the maximum therapeutic plasma concentration in clinical settings [17], were administered first. As expected, quinidine reversibly prolonged the FPDc by $25.2 \pm 5.2\%$ ($n=6$, $p<0.05$). Furthermore it decreased the FP frequency by $32.8 \pm 3.4\%$ ($n=6$, $p<0.01$, Fig. 3A-E, G, Fig. 7A-B) and decreased FPmin by $35.3 \pm 8.0\%$ ($n=6$, $p<0.05$). Likewise, conduction velocities were reduced by $20.6 \pm 4.1\%$ ($n=6$, $p<0.05$, Fig. 7C).

Fig. 5. Effect of E4031 on FP frequency and FP morphology in hESCMs. E4031 (5 μ M) decreased FP frequency, and prolonged FPdur (original tracings, A, control; B, E4031; C, washout). (D) Overlay of single FPs showing the prolongation of FPdur. (E) Time course of the effect of E4031 on FPDc. (F) Time course of the effect of E4031 on FP frequency. (G) Effect of E4031 on FPDc and FP frequency.

($\pm$)-sotalol (300 μ M) and E4031 (5 μ M) both evoked reversible changes of the FPDur and the FP frequency. In detail, the beating frequency decreased after application of ($\pm$)-sotalol by $22.2 \pm 4.0\%$ ($n=6$, $p<0.01$) and after application of E4031 by $29.5.4 \pm 10.8\%$, ($n=7$, $p<0.05$, Fig. 4 and 5A-C, E-G, Fig. 7B). FPDc increased significantly by $69.2 \pm 10.1\%$, ($n=6$, $p<0.05$) and by $85.1 \pm 12.3\%$ ($n=7$, $p<0.01$, Fig. 4 and 5D-E, G, Fig. 7A), respectively. However, neither ($\pm$)-sotalol (300 μ M) nor E4031 affected FPmin or the conduction velocities ($p>0.05$) (Fig. 7C).

The class IV antiarrhythmic drug verapamil (0.2 μ M) leads to a block of transsarcolemmal Ca^{2+} influx through L-type Ca^{2+} channels and a slowing of cardiac conduction. Verapamil significantly decreased the FP frequency by $26.6 \pm 8.3\%$ ($n=6$, $p<0.05$) (Fig. 6A-C, E-G,

Fig. 6. Effect of verapamil on FP frequency and FP morphology in hESCMs. Verapamil (0.2 μ M) decreased FP frequency and shortened FPDur (original traces, A, control; B, verapamil; C, washout). (D) Overlay of single FPs showing the shortening of FPDur. (E) Time course of the effect of verapamil on FPDc. (F) Time course of the effect of verapamil on FP frequency. (G) Effect of verapamil on FPDc and FP frequency.

Fig. 7B) and conduction velocities by $36.2 \pm 7.5\%$ ($p<0.01$, Fig. 7C). Unlike quinidine, ($\pm$)-sotalol or E4031, verapamil significantly shortened FPDc by $38.9 \pm 7.2\%$ ($p<0.05$, Fig. 6D-E, G, Fig. 7A).

Discussion

The present study aimed at evaluating hESC and mESC-derived cardiac tissue for their potential to uncover proarrhythmic side effects of cardioactive drugs. Our study shows that the measurement of drug effects on FP parameters in hESCMs and mESCMs serves as a good *in vitro* model for preclinical studies of drug safety. In line with clinical observations the tested antiarrhythmic drugs led to typical effects on FP frequency, FPDur and

Fig. 7. Differences among different drugs. Quinidine, ($\pm$)-sotalol, and E4031 significantly prolonged the FPdur. Verapamil, however, shortened FPdur (A). All substances decreased the beating frequency (B) without significant difference among these drugs. Both, quinidine and verapamil decreased the conduction velocity whereas ($\pm$)-sotalol and E4031 did not exert significant effects (C).

cardiac conduction. As the diseased heart, early developmental stages of hESCMs showed a significantly reduced repolarization reserve. Moreover, significant species-dependent differences were found.

In line with clinical reports and *in vitro* research, quinidine, ($\pm$)-sotalol, and E4031 prolonged repolarization indicated by a prolongation of FPdur in hESCMs, a finding that is being explained by the inhibition of hERG channels [18, 19]. In contrast, verapamil shortened FPdur, indicating blockage of L-type Ca^{2+} channels [20, 21]. Both, quinidine and verapamil decreased the conduction velocity as expected [22, 23], whereas neither ($\pm$)-sotalol nor E4031 showed significant effects at the concentrations used. This observation is in accordance with the ($\pm$)-sotalol and E4031-induced inhibition of I_{Kr} that does not lead to alterations of conduction velocities [24, 25]. However, quinidine and verapamil inhibit Na^{+} and Ca^{2+} channels, respectively, thereby leading to a decrease of the conduction velocity [22, 23]. The differences among the effects of these drugs are in good agreement with previous clinical observations [26].

As also reported from clinical studies, all tested antiarrhythmics decreased the beating frequency regardless of their different pharmacological mechanisms [27]. There was no significant difference among their effects on hESCMs. Interestingly, quinidine, ($\pm$)-sotalol and E4031-induced ERDs were only rarely found in

hESCMs which is in line with the incidence of the drugs' associated TdP in patients [28]. However, ERDs were found in most of the mESCMs tested implying significant species-dependent differences. Likewise, hESCMs were less sensitive to ($\pm$)-sotalol than mESCMs as indicated by a higher EC_{50} . The EC_{50} of ($\pm$)-sotalol in mESCMs was similar to the one reported for ($\pm$)-sotalol effects on I_{Kr} in animal models [29–31], whereas an EC_{50} of more than 500 μM ($\pm$)-sotalol is reported for the human $\text{K}_{\text{v}}1.4$ and human I_{to} in different models [32]. One might speculate that a contribution of I_{to} and I_{Kr} to the repolarization phase may account for that finding [33, 34].

In addition, since ($\pm$)-sotalol evoked stronger effects on FPdur at early developmental stages of hESCMs, a decreased repolarization reserve must be present as reported for the diseased human heart [35]. Therefore, hESCMs, especially at the early developmental stage, serve as a more promising system for preclinical pharmacological testing. Findings that contribute to that statement show that the biology and electrophysiology of the diseased heart resemble to some extent the one of the immature heart and *vice versa* [36, 37].

Nevertheless, the high proarrhythmic potential of these drugs in mESCMs and/or the higher sensitivity of mESCMs to these antiarrhythmics with respect to the incidence of ERDs, makes additionally mESCMs to an important and interesting *in vitro* model for drug safety

screening.

Up to date, this is the first paper comparing in mESCMs and hESCMs electrophysiological parameters derived from high temporal and spatial resolution MEA recordings and antiarrhythmic drug effects hereon. Our results propose that both tissue models are together a reliable model for drug safety screening.

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