Electrophysiological Modulation of Cardiomyocytic Tissue by Transfected Fibroblasts Expressing Potassium Channels
A Novel Strategy to Manipulate Excitability

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Background—Traditional pharmacological therapies aiming to modify the abnormal electrophysiological substrate underlying cardiac arrhythmias may be limited by their relatively low efficacy, global cardiac activity, and significant proarrhythmic effects. We suggest a new approach, in which transfected cellular grafts expressing various ionic channels may be used to manipulate the local electrophysiological properties of cardiac tissue. To examine the feasibility of this concept, we tested the hypothesis that transfected fibroblasts expressing the voltage-sensitive potassium channel Kv1.3 can modify the electrophysiological properties of cardiomyocytic cultures.

Methods and Results—A high-resolution multielectrode mapping technique was used to assess the electrophysiological and structural properties of primary cultures of neonatal rat ventricular myocytes. The transfected fibroblasts, added to the cardiomyocytic cultures, caused a significant effect on the conduction properties of the hybrid cultures. These changes were manifested by significant reduction in extracellular signal amplitude and by the appearance of multiple local conduction blocks. The location of all conduction blocks correlated with the spatial distribution of the transfected fibroblasts assessed by vital staining. All electrophysiological changes were reversed after the application of Charybdotoxin, a specific Kv1.3 blocker. In contrast, conduction remained uniform in the control hybrid cultures when nontransfected fibroblasts were used.

Conclusions—Transfected fibroblasts are able to electrically couple with cardiac myocytes, causing a significant local and reversible modification of the tissue’s electrophysiological properties. More broadly, this study suggests that transfected cellular grafts expressing various ionic channels may be used to modify cardiac excitability, providing a possible future novel cell therapy strategy. (Circulation. 2002;105:522-529.)

Key Words: electrophysiology ■ gene therapy ■ arrhythmia ■ ion channels ■ mapping
Animal Study Committee of the Technion Faculty of Medicine. Briefly, after excision, the ventricles were minced in Dulbecco’s phosphate-buffered saline (Biological Industries) and later treated with RDB (IIBR). After centrifugation, the dispersed cells were suspended in culture medium (Ham’s F10), 5% FCS, 5% horse serum, 100 U/mL penicillin, 100 mg/mL streptomycin (all from Biological industries), 1 mmol/L CaCl₂, and 50 mg/100 mL bromodeoxyuridine (BrdU, Sigma). BrdU was used during the preparation of the cultures to reduce the number of nonmyocytic cells. Cells were then cultured on a microelectrode array culture plate at a density of 1.5×10⁶ cells/mL.

Multielectrode Mapping Technique

Extracellular recording from the cultured myocytes was performed using a microelectrode array (MEA) data acquisition system (MultiChannel Systems). The MEA consists of a matrix of 60 electrodes (30-µm diameter) with an interelectrode distance of 100 or 200 µm (Figures 1A and 1B), allowing simultaneous recording of extracellular potentials from all electrodes at a sampling rate of 10 to 25 kHz (Figure 1C). The MEA mapping area, however, covers only a relatively limited zone (1.4×1.4 mm²) of the cardiomyocytic cultures.

During recordings, cultures were perfused with a gas mixture consisting of 5% CO₂ plus 95% air. Temperature was kept at 37.0±0.1°C. Local activation time (LAT) at each electrode was determined by the timing of the maximal negative intrinsic deflection (dV/dtₘᵟₐₓ) of the unipolar recordings (Figure 1D). The measured LATs at all electrodes were then used for the generation of high-resolution color-coded activation maps (Figure 1E) using Matlab’s standard two-dimensional plotting function.

Expression System and Electrophysiological Recording

The plasmid pRC/CMV/Kv1.3 was kindly provided by Dr I. Levitan. Stable transfection was achieved in NIH 3T3 fibroblasts by electroporation with a single pulse of 200 V, 960 µF delivered from a gene pulser transfection apparatus (Bio-Rad). After 48 hours, 400 µg/mL of G-418 (Gibco) was added to select for cells expressing neomycin resistance. Two weeks later, colonies were picked and tested for channel expression. Whole-cell recordings were conducted at room temperature using Axopatch 200 (Axon Instruments). Data were collected using a Quadra 800 (Apple computers) with PULSE software (HEKA Electronic), low-pass filtered at 5 to 10 kHz and sampled at 20 kHz. Electrodes for voltage-clamp experiments were made from fire-polished aluminum silicate glass, with a resistance of 5 to 6 MΩ. The pipette solution contained (in mmol/L) KCl 140, Na₂ATP 10, EGTA 10, HEPES 5, CaCl₂ 1, and MgCl₂ 1 (pH 7.3). The bath solution contained (in mmol/L) NaCl 140, KCl 1, KCl 3, HEPES 10, glucose 10, MgCl₂ 2, and CaCl₂ 2.

Preparation of Cocultures

Once a well-synchronous spontaneous activity was established in the cardiomyocyte cultures, the fibroblasts were seeded in clusters into the cardiac cultures without any specific predetermined pattern.
Labeling of Fibroblasts

To track the transplanted cells within the cocultures, fibroblasts were labeled with a fluorescent lipophilic tracer, Fast DiO (3,3'-dilinoleylxocarbocyanine perchlorate [FAST DiO, molecular probes]), and identified throughout the experiment using an inverted Zeiss fluorescent microscope (Axiovert 135).

Immunohistochemistry

Cocultures were grown on top of the MEA or on cover slips for 5 days and then fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 (Sigma). Cells were blocked with 10% goat serum (Biological Industries) for 1 hour at 37°C. Immunostaining was performed using monoclonal rabbit anti-mouse Kv1.3 (Alomone Labs, Jerusalem, Israel) and mouse anti–myosin heavy chain (anti-MHC, Chemicon) antibodies in the blocking buffer for 24 hours at 4°C. The preparations were then incubated with FITC-conjugated anti-mouse IgG and Rhodamine-conjugated anti-rabbit IgG secondary antibodies (both from Chemicon) for 1 hour. The preparations were analyzed using the inverted fluorescent microscope (Zeiss, Axiovert 135) or by confocal microscopy (Nikon Eclipse E600 microscope and Bio-Rad Radiance 2000 scanning system at a magnification ×60 and a z resolution of 0.5 μm).

Experimental Protocol

Two groups were studied, a control group consisting of cardiomyocytes and transfect Fibroblasts expressing the Kv1.3 channels (n=5). The cultures were studied daily, and the conduction properties were assessed at baseline and after application of the specific Kv1.3 blocker, Charybdotoxin (CTx), at increasing dosages (1, 10, and 100 nmol/L).

The changes induced were quantified by measuring the average peak-to-peak amplitude of the local extracellular potentials recorded from all 60 electrodes and the presence of local conduction delays. A conduction block factor (CBF) was defined as the number of electrodes in which the time difference between the measured LAT and at least 1 of their 4 neighboring electrodes was ≥25% of total activation time of the culture, indicating the presence of significant local conduction delay.

Statistical Analysis

All results are expressed as mean±SEM. Possible differences between the control and study groups in the conduction properties (CBF and signal amplitude) at each day were evaluated using Mann-Whitney rank-sum test. Friedman repeated measures ANOVA on ranks, and post hoc multiple comparisons using Dunnett’s method, when applicable, were used to assess differences in the electrophysiological properties before and on consecutive days after fibroblast seeding and also to assess for possible effects of CTx. Results were considered statistically significant if P<0.05.
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Results

Voltage-Clamp Analysis of the Transfected Fibroblasts

The voltage-clamp studies performed (Figure 2A) and the corresponding reconstructed current-voltage ($I$–$V$) curve (Figure 2B) demonstrated the presence of a high-magnitude outward potassium current with rapid activation kinetics in the transfected fibroblasts. The time to peak current at a voltage where the channel is fully activated was in the order of 4 to 5 ms. In contrast, no significant transmembrane currents were observed in the fibroblasts without transfection (Figure 2C). The unique properties of the Kv1.3 channel can also be appreciated in Figure 2D, in which a computer simulation compares the rapid opening of the Kv1.3 channel to that of typical cardiac sodium and total potassium currents.

Structural Analysis of the Hybrid Cultures

The presence of the fibroblasts within the hybrid cultures was assessed during the experiments using vital Fast-DiO staining. Figure 3A displays a typical fibroblast distribution in a control culture, whereas Figure 3B presents a similar distribution using the transfected fibroblasts. The ability to accurately locate the fibroblasts with relevance to the microelectrode array enabled the accurate assessment of their possible local electrophysiological effects. Additional containing experiments with anti-MHC and anti-Kv1.3 antibodies (Figures 3C and 3D) confirmed the presence of fibroblasts expressing Kv1.3 channels in the study group and absence of these channels in the cardiomyocytes and in the nontransfected fibroblasts.

Electrophysiological Assessment of the Hybrid Cultures

MEA recordings obtained during culture development demonstrated, in both groups, frequent changes in the rate and position of the firing focus. In most cases, the site of spontaneous firing was outside the mapping area, and changes in pacemaker position were identified by shifts in the earliest activation sites within the MEA.

Figure 4A presents a typical clustered fibroblast-seeding pattern in the control group lacking transfection. Figures 4B and 4C show activation maps obtained before and 5 days after fibroblast seeding, respectively. Note in both cases the absence of a uniform and relatively fast conduction without regional conduction delays. After application of the specific Kv1.3 blocker, CTx, no significant changes were noted in the conduction properties (Figure 4D).

The hybrid cultures containing transfected fibroblasts expressing Kv1.3 channels, in contrast, developed several areas of significant conduction delays and conduction blocks. Figure 5A demonstrates a typical fibroblast-seeding pattern in the study group. Figure 5B depicts the activation map before cell grafting with a uniform conduction. Figure 5C shows an activation map of the same culture 5 days after grafting of the transfected fibroblasts. Note the appearance of a conduction block (black arrows) identified by the significant time delay on electrodes on opposite sides of the block and by the change in wave front orientation circumventing the fibroblast clusters (white arrow). The presence of conduction block was also evident by the significant increase in activation time from a baseline value of 40 to 95 ms. These conduction changes were fully reversible after administration of CTx (Figure 5D) with resumption of uniform conduction and a return of total activation time to 45 ms.

The localized electrophysiological effects of the transfected fibroblasts can also be appreciated by the changes in the morphology of the recorded electrograms. Electrogram amplitudes did not change significantly from baseline in electrodes located away from the fibroblasts clusters (Figure 5E) but were reduced significantly in electrodes located near transfected fibroblast clusters (Figure 5F) and were associated with double potentials in areas of conduction blocks (Figure 5G). These changes were also reversible after CTx application (Figures 5F and 5G, right).

Summary of the Electrophysiological Changes

Signal Amplitude

Seeding of fibroblasts without transfection did not reduce, and even increased, the amplitude of the recorded electrograms during culture maturation (Figure 6A). In contrast, seeding of the transfected fibroblasts generated a significant reduction in the peak-to-peak electrogram amplitude by 47%, 55%, 66%, and 79% from its baseline value at 2, 3, 4, and 5 days after fibroblast grafting ($P<0.05$, Figure 6B). Consequently, mean electrogram amplitude was significantly lower in the study group as early as day 2 after fibroblast seeding. CTx application did not change electrogram amplitude in the control group (Figure 6C) but had a dose-related effect in the study group (Figure 6D) with a 23% increase in
the normalized electrogram amplitude at a CTx concentration of 100 nmol/L ($P<0.05$).

**Conduction Blocks**

A conduction block factor (CBF) was defined to evaluate the number of electrodes associated with significant conduction delays (>25% of the total MEA activation time). Figures 6E and 6F depict the changes in the CBF at baseline (day 1) and on consecutive days after fibroblast seeding. CBF did not change and was minimal during the recording period in the control group (Figure 6E). In contrast, in the study group, CBF increased gradually from a baseline value of 0.8±0.8 up to 6.7±1.8 at 6 days after fibroblast seeding ($P<0.05$). The average time delay in these electrodes was 25.8±0.1 ms.

CTx application did not change the CBF in the control group but had a dose-related effect in the study group with a reduction of CBF from an initial value of 6.0±1.1 to 2.0±0.8 at a CTx dose of 100 nmol/L (Figure 6G). In addition, significant differences were noted in CBF values between the control and the study group before CTx application and at 1 and 10 nmol/L ($P<0.05$). Application of 100 nmol/L of CTx restored CBF in the study group to a value not significantly different from that of the control group.

**Discussion**

The work presented in this study represents the first evidence of the ability to modify the electrophysiological substrate of cardiac tissue by transfected cell grafts expressing ionic channels. Our results demonstrate the following: (1) Fibroblasts transfected with the voltage-sensitive potassium channel Kv1.3 are able to integrate structurally and functionally with preexisting cardiac cultures; (2) transplanted cells are able to significantly alter the conduction properties of the hybrid cultures; and (3) electrophysiological effect is localized to the site of the transplanted cells and is reversible after application of a specific Kv1.3 channel blocker.

**Generation of Transfected Cellular Grafts**

A fibroblast cell line was transfected with a construct, allowing robust expression of the voltage-dependent potassium channel Kv1.3. The detailed voltage-clamp studies performed demonstrated some of the possible advantages of this channel for our study. First, the relatively rapid activation and relatively slow kinetics from open to close states9–11 may allow maximal modulating effects on the neighboring cells during various phases of the action potential. Second, the robust expression of the potassium channel within the fibroblasts generated a very high-magnitude outward potassium current after membrane depolarization. Third, the high affinity of the Kv1.3 channels to the inhibitor CTx allowed the usage of low dosages (not affecting the cardiomyocytic tissue) in the coculture studies.

**Structural and Functional Integration Between Transplanted and Host Cells**

Several methods were used to demonstrate fibroblast survival and structural integration within the cocultures. These included vital staining with Fast-DiO throughout the experiments and immunostaining with anti Kv1.3 at the end of the experiments. Co-staining with anti-MHC and anti Kv1.3
antibodies also ruled out the possibility of endogenous expression or transfection of the cardiomyocytes by showing the absence of Kv1.3 staining in these cells.

Electrophysiological coupling between fibroblasts and cardiomyocytes was demonstrated previously. Rook et al. reported on electrotonic interactions in pairs of fibroblasts and myocytes with a transient increase in the fibroblast’s membrane potential during the appearance of action potential in the neighboring myocyte. These investigators also demonstrated by electron microscopy and immunostaining for connexin43 the presence of gap-junction in some of these fibroblast-cardiomyocyte pairs and absence of such structures in fibroblast pairs. Similarly, Fast et al. using high-resolution optical mapping of cardiomyocytic monolayers, noted that fibroblasts within the cultures were electrically connected to myocytes and served as sinks for electrotonic currents.

The results reported here also support electrical coupling between fibroblasts and myocytes. The transfected fibroblasts caused significant changes in the electrophysiological properties of the hybrid cultures manifested by the generation of multiple conduction blocks. To study whether these marked changes were attributable to the presence of the Kv1.3 channels within the fibroblasts or merely attributable to the physical presence of the fibroblasts, we conducted two sets of experiments. First, a control group was studied in which fibroblasts lacking transfection were added to the cardiomyocytic cultures. Interestingly, these grafts did not cause any significant electrophysiological changes during culture development. Second, a specific Kv1.3 blocker, CTx, caused a dose-related effect with partial or complete reversibility of the electrophysiological changes on specific blockade of the Kv1.3 channel.

The surprising results of the present study, in which the fibroblasts lacking Kv1.3 activity (either without transfection or functionally blocked with CTx) did not cause a major effect on the conduction properties of the cardiomyocytic
Figure 6. Electrophysiological changes induced by fibroblast seeding. A and B, Changes in electrogram amplitude at baseline (day 1) and after fibroblast seeding (days 2 through 6) in the control (A) and study (B) groups. *P<0.05 compared with baseline values. C and D, Changes in the normalized electrogram amplitude (normalized to the maximal amplitude value at each experiment) after CTx application in the control (C) and study (D) groups. *P<0.05 when compared with baseline values. E and F, Changes in the conduction block factor (CBF) at baseline (day 1) and after fibroblast seeding in the control (E) and study (F) groups. Note the significant (†P<0.05) gradual increase in CBF induced by the transfected fibroblasts (F). G, Changes in CBF after CTx application in the control (●) and study (●) groups. *P<0.05 when the CBF values in the study group were compared with the control group for each CTx concentration. †P<0.05 when the CBF values following CTx application were compared in the same group with baseline values.
cultures, differ from the above-mentioned study of Fast et al. This difference may stem from the lower spatial resolution of the MEA technique relative to the optical mapping technique used by these investigators, precluding the ability to identify very small and localized effects. Alternatively, the difference may lie in the structural properties of the cultures in the two studies. Hence, in the study of Fast et al., the effect of well-coupled fibroblasts already present during culture preparation and maturation was assessed, whereas in the present study, the effects of externally grafted cells to an already well-coupled culture were evaluated.

The mechanism underlying the electrophysiological changes induced by the transfected fibroblast seeding may be multifactorial and can be divided into passive and active properties. The passive effects of the fibroblasts are determined by the membrane’s capacitance, the electrophysiological coupling with the myocytes, the density, and the location of the fibroblasts within the coculture. The active effects of the transfected fibroblasts depend on the transfected channel density and the kinetics and type of the channel. The Kv1.3 channel used in the present study differs significantly from cardiac potassium channels by its very rapid opening kinetics, which approaches that of the fast inward sodium channels (see computer simulation in Figure 2D).9–12 The combination of the rapid opening kinetics of the Kv1.3 channels, the high density of the Kv1.3 channels within the fibroblasts, and the relatively ill-developed sodium current in neonatal rat cardiomyocytes14 may explain the significant effects that were observed on conduction in the hybrid cultures.

Limitations

There are several important limitations to the present study. First, the concept tested in the present study was evaluated in an in vitro system. Long-term applications of this concept, however, rely more on the prospect of in vivo use, which may present several obstacles, which may be common to all forms of cell-based therapies. Initially, there is the problem of immune rejection, which may require the use of autologous cell sources. In addition, the ability of the cells to survive and integrate with host tissue in the intact heart must be evaluated as well as the ability to prevent abnormal cell growth.

Another limitation of the present study is the relatively short time (up to 1 week) during which the fibroblasts effects could be monitored because of the inherent limitations of the in vitro model. An additional limitation is the heterogeneity in fibroblast seeding in the different cultures, resulting in relatively large variabilities in some of the measured parameters. This limitation was partially addressed by using each culture as its own control.

Despite the aforementioned limitations, we demonstrated for the first time that cellular grafts transfected with specific ionic channels could be used to alter the electrophysiological substrate of cardiac tissue. In contrast with other suggested cell-based therapies, which seek to replace dysfunctional or diseased tissue, the approach suggested here aims to alter the tissue’s excitable properties. In that respect, this approach is more analogous to pharmacological therapy but may surpass the latter by having a localized effect. Creative manipulation of ion channel composition within the transfected cellular grafts may ultimately be used to develop different strategies for modification (either increasing or decreasing) of the cardiac excitable properties for the treatment of a variety of cardiac arrhythmias.

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