Proarrhythmic Potential of Mesenchymal Stem Cell Transplantation Revealed in an In Vitro Coculture Model

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Background—Mesenchymal stem cells (MSCs) are bone marrow stromal cells that are in phase 1 clinical studies of cellular cardiomyoplasty. However, the electrophysiological effects of MSC transplantation have not been studied. Although improvement of ventricular function would represent a positive outcome of MSC transplantation, focal application of stem cells has the potential downside of creating inhomogeneities that may predispose the heart to reentrant arrhythmias. In the present study we use an MSC and neonatal rat ventricular myocyte (NRVM) coculture system to investigate potential proarrhythmic consequences of MSC transplantation into the heart.

Methods and Results—Human MSCs were cocultured with NRVMs in ratios of 1:99, 1:9, and 1:4 and optically mapped. We found that conduction velocity was decreased in cocultures compared with controls, but action potential duration (APD80) was not affected. Reentrant arrhythmias were induced in 86% of cocultures containing 10% and 20% MSCs (n/H1100536) but not in controls (n/H110057) or cocultures containing only 1% MSCs (n/H110054). Immunostaining, Western blot, and dye transfer revealed the presence of functional gap junctions involving MSCs.

Conclusions—Our results suggest that mixtures of MSCs and NRVMs can produce an arrhythmogenic substrate. The mechanism of reentry is probably increased tissue heterogeneity resulting from electric coupling of inexcitable MSCs with myocytes. (Circulation. 2006;113:1832-1841.)

Key Words: arrhythmia ■ conduction ■ electrophysiology ■ tachyarrhythmias

Heart failure affects an estimated 4.9 million people in the United States, with 550 000 new cases reported annually.1 Despite major improvements in medical therapy, a significant proportion of patients remain symptomatic. Recent encouraging results from cellular therapy in animal models2,3 have greatly increased the enthusiasm for clinical trials in patients suffering from heart failure. Several cell types, including bone marrow mononuclear cells,4–6 have been used for transplantation in preclinical and clinical studies,7 the most recent involving mesenchymal stem cells (MSCs). MSCs are bone marrow stromal cells that have the potential of differentiating toward a number of cellular phenotypes.8

Advantages of cellular therapy based on MSCs include their rapid expansion in culture without discernible chromosomal abnormalities or loss of telomerase activity; in addition, MSCs lack B7 costimulatory molecules CD80 and CD86 and do not stimulate an allogeneic immune cell proliferation.9 Therefore, MSC therapy offers the tantalizing opportunity for “off-the-shelf” allogeneic stem cell therapy soon after a patient presents with myocardial infarction or heart failure.

CLINICAL PERSPECTIVE

Like most undifferentiated cells, MSCs have a resting potential of −30 to −40 mV,10 MSCs possess delayed rectifier channels, and a small fraction expresses the L-type calcium channel. However, MSCs do not express sodium or inwardly rectifying potassium channels.10 Furthermore, despite expression of some cardiac muscle–specific proteins, complete cardiac transdifferentiation of MSCs to cardiomyocytes has not been confirmed.11 Significantly, the gap junction protein Cx43 is expressed in MSCs and forms functional gap junctions between MSCs as well as between MSCs and myocytes12; this attribute has been used to generate pacemaker-like areas of focal ventricular automaticity in animal models.13

On the basis of evidence that MSCs migrate to sites of tissue injury, allogeneic MSCs are in phase 1 clinical trials in the United States, where postmyocardial infarction patients are being enrolled for intravenous MSC therapy. Numerous animal studies have demonstrated the beneficial effects of MSC transplantation on cardiac hemodynamics11,14–19; the arrhythmic and electrophysiological consequences have not

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been systematically studied, however. The only study addressing possible proarrhythmic consequences of MSC therapy was performed by Pak et al., who found an increase in heterogeneously distributed sympathetic nerve sprouting in pigs after MSC transplantation. Amado and coworkers recently reported no significant increase in sudden death in pigs receiving intramyocardial injections of MSCs compared with placebo. However, they did not perform ECG monitoring or electrophysiological studies and were unable to accurately quantify MSC engraftment in these animals. Phase 1 studies of human skeletal myoblast transplantation have highlighted the importance of thorough assessment of arrhythmia risk before human trials.

In the present study we used a reductionist in vitro coculture approach that allows a controlled study of the arrhythmogenic potential of MSCs. We hypothesized that inexcitable MSCs would act as microscopic current sinks, constitute abnormal electric loads, and predispose to conduction block and reentrant arrhythmias when electrically coupled with myocytes. The main advantage of an in vitro model of MSC transplantation is that it permits the evaluation of pure MSC–myocyte interactions without the confounding proarrhythmic effects of heart failure or sympathetic hyperinnervation seen in animal models. It also lends itself to manipulation of stem cell number and location and may provide important insights into the electrophysiological effects of MSC transplantation.

We found that cocultures containing ≥10% MSCs exhibited decreased conduction velocity and were predisposed to reentrant arrhythmias, which are the most common cause of post–myocardial infarction arrhythmias.

Methods

Cell Culture

Human MSCs were obtained from Cambrex (Walkersville, Md) and grown in MSC basal growth medium (MSCBM, Clonetics Corp, Bio Whittaker, Inc, San Diego, Calif). MSCs were positive for CD105, CD166, CD29, and CD44 and negative for CD14, CD34, and CD45. Human MSCs were obtained from Cambrex (Walkersville, Md). MSCs were positive for CD105, CD166, CD29, and CD44 and negative for CD14, CD34, and CD45. The cells were plated at 5000 to 6000 cells/cm² and subcultured when they attained a density of 90%. Cells were frozen in liquid nitrogen at their third passage and were amplified up to 10 population doublings.

Immunostaining and Western Blot
MSCs were grown to confluence; immunostaining and Western blot were performed as previously described.

Dye Transfer

Cell-to-cell coupling via gap junctions was assessed by dye transfer of calcein between human MSCs and neonatal rat ventricular myocytes (NRVMs). Details are included in the online-only Data Supplement.

Cardiac Myocytes

NRVMs were dissociated from ventricles of 2-day-old neonatal Sprague-Dawley rats (Harlan, Indianapolis, Ind) as previously described.

Cocultures

MSCs and NRVMs were mixed and plated in ratios of 1:4 (20% MSCs), 1:9 (10% MSCs), and 1:99 (1% MSCs). The cell mixtures were cocultured for 9 to 11 days as isotropic monolayers on 21-mm plastic coverslips coated with human fibronectin (25 μg/mL, BD Biosciences, Becton, Dickinson and Co, San Jose, Calif) and then used for optical mapping (NRVMs mature to form rod-shaped, striated cells after 6 days in culture). On day 2 after cell plating, serum was reduced to 2%. For imaging of cocultures, MSCs were labeled with CMFDA green (10 μmol/L, Molecular Probes, Carlsbad, Calif) to enable cell tracking (n = 12).

Recent reports by Amado et al. of improved functional effects after focal intramyocardial injection of MSCs prompted us to perform a second set of experiments to simulate the in vivo situation in which MSCs would exist as islands mixed with myocytes (n = 7). For this purpose, we preplated MSCs using poly-dimethyl-siloxane stencils and then added NRVMs 4 hours later, generating islands (6-mm diameter) of 20% MSC-NRVM mixtures surrounded by NRVMs. Optical mapping was performed after 9 days in culture.

Optical Mapping

Electric activity was monitored by optical mapping as previously described. Unipolar point stimulation via a platinum electrode was applied just above the monolayer with platinum field electrodes at the sides of the chamber acting as the return line.

Experimental Protocol

A 1-second recording was initially made to check for spontaneous activity. Thirty-five–beat drive trains of 10-ms monophasic pulses (1.5× diastolic threshold) were subsequently used for stimulation throughout the experiment. Stimulation was begun at 1 Hz and increased progressively by 1 Hz until 1:1 capture was no longer observed or reentry was initiated. Reentry was considered initiated if it lasted for >5 rotations and was considered sustained if it lasted for >5 minutes.

Data Analysis

Data was analyzed in MATLAB (The MathWorks Inc, Natick, Mass) with the use of custom-written scripts. Details are included in the online-only Data Supplement.

Statistics

Data are expressed as mean ± SD. Differences between means were tested with the paired t test with the use of the statistical software GraphPad Prism (Graphpad Software, Inc, San Diego, Calif). Unpaired t tests were used to compare action potential duration (APD₉₀) and conduction velocity between cocultures and controls, and paired t tests were used in all other cases. A probability value of <0.05 was considered statistically significant.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

MSCs Express Cx43 and Are Electrically Coupled With Myocytes

Structural characterization was performed before the electrophysiological phenotype of the cocultures was studied. Transmitted light (Figure 1A) and fluorescence microscopy (Figure 1B) revealed that MSCs are randomly distributed in culture and that large numbers of MSCs existed in a plane below the NRVMs with some interspersed in between the myocytes. This is perhaps due to the fact that MSCs adhere to the coverslips within 3 to 4 hours, whereas NRVMs take up to 24 hours to fully adhere.

An important characteristic of MSCs is their ability to form gap junctions between themselves and with neighboring cardiomyocytes. Figure 2A and 2B confirm Cx43 expression in MSC cultures by immunostaining and Western blot, respectively. We found high levels of Cx43 expression in
MSCs, but, in contrast to adult myocytes in which Cx43 exists mainly as plaques, Cx43 was predominantly expressed in a punctate fashion throughout the plasma membrane. We confirmed the presence of functional gap junctions between MSCs and NRVMs by demonstrating calcein but not Di-I (membrane dye; Invitrogen) transfer from MSCs to NRVMs (Figure 2C and 2D).

**Electrophysiological Characterization Using Optical Mapping**

In the first set of experiments, we characterized cocultures containing MSCs and NRVMs that were coplated in several ratios. We found that the conduction velocity (Figure 3C) was decreased ($P=0.003$, t-test) by an average of 33% in 20% MSC/NRVM cocultures ($14.7\pm2.8$ cm/s; $n=7$) compared with controls ($21.8\pm3.0$ cm/s; $n=7$). The voltage maps for both the NRVM controls (Figure 3A) and 20% MSC/NRVM cocultures (Figure 3B) illustrate the differences in conduction velocity observed. Additionally, we found that, with the exception of 1-Hz pacing ($P=0.007$), APD$_{80}$ as a function of the pacing interval (APD restitution) was not significantly changed in 20% MSC/NRVM cocultures ($P=0.244$) compared with controls (Figure 3D and 3E).

**Reentrant Arrhythmias Are Easily Inducible in Mixtures of MSCs and Cardiomyocytes**

Reentrant arrhythmias (spiral waves) were induced by rapid pacing between 5 to 7 Hz and were preceded by wavebreaks. This is seen in Figure 4A, which shows 6-Hz pacing in which wave propagation is fairly uniform in the second frame; with continued pacing, wavebreaks appear in the third frame and precede reentry initiation. Reentrant arrhythmias were in-
duced in 86% of the 10% and 20% MSC cocultures that were mapped (n/H11005 36) but in none of the 1% MSC cocultures or NRVM-only controls. Reentries that sustained for 30 seconds and 5 minutes were observed in 83% and 64%, respectively, of the cocultures that were mapped. Single spiral waves similar to those seen in Figure 4B occurred most frequently; however, a variety of other reentry morphologies were also observed. Of the reentries that were sustained (n/H11005 23), 61% were initially single spirals (Figure 4B), 17% figure-of-eight spirals (Figure 4C), 13% dual-arm reentries (Figure 4D), and 9% complex reentries (Figure 4E). Single spiral wave reentry consists of a single spiral and usually manifests as monomorphic ventricular tachycardia in the clinical setting. Figure-of-eight reentry consists of 2 counterrotating spirals, and dual-arm reentry consists of 2 individual spirals that are coupled. Finally, complex reentry exhibits many spirals that may collide with one another, forming significant wavebreaks, and this morphology most resembles ventricular fibrillation. In all experiments in which reentries started out as dual-arm, complex, or figure-of-eight, the morphology eventually simplified to single spiral waves when reentry was sustained.

**Cycle Length Adaptation in Sustained Reentry**

Sustained reentry cycle length (CL) varied from 106 to 196 ms (n=20), with the average being 150±28 ms. Interestingly, the reentries accelerated (Figure 5A) by 17% from a mean of 185±2.9 versus 12.9±2.8 cm/s; P<0.005) whereas APD_{80} (103±16 versus 90±15 ms; P<0.001), curvature (0.33±0.02 versus 0.28±0.04; P=0.08), and pathlength (6.7±2.4 versus 5.1±2.5 mm; P<0.1) decreased without a significant change in reentry wavelength (7.87±0.37 versus 7.93±0.27 mm; P>0.4). Interestingly, the majority of the tip trajectories revealed initial hypocycloidal phenotypes that evolved into irregular phenotypes (Figure 5G) during sustained reentry.

**Optical Mapping of MSC-NRVM Islands Surrounded by NRVMs**

Because uniform mixtures of MSCs and NRVMs were arrhythmogenic, we investigated whether MSC-NRVM islands surrounded by myocytes could support reentry. Optical mapping of these cocultures (n=7) revealed delayed activation and repolarization of the MSC-NRVM region even at low pacing rates (Figure 6A), reentry initiation at high pacing rates, and pinning of the spirals to the MSC-NRVM islands (Figure 6B) in 6 of 7 cultures that were mapped. This suggests that MSCs have the potential of introducing microscopic and macroscopic heterogeneities that are proarrhythmic.

**Triggered Activity and Increased Automaticity Are Not Observed in MSC-NRVM Cocultures**

Because abnormal automaticity and triggered activity can also induce arrhythmias, we examined the susceptibility of these cocl-
tured to triggered activity. Early afterdepolarizations were not observed spontaneously or at low pacing rates; delayed afterdepolarizations were only seen with rapid pacing in the presence of 2.5 μmol/L BayK 8644, an L-type calcium channel agonist.

Discussion

Our study characterizes for the first time the electrophysiological consequences of mixing MSCs and myocytes at a syncytial level. The main findings of this study are (1) that mixtures of MSCs and NRVMs containing 10% MSCs resulted in an arrhythmogenic substrate that is characterized by decreased conduction velocity and easily inducible sustained reentrant arrhythmias and (2) that the mechanism underlying arrhythmogenicity of the cocultures is probably coupling of cardiomyocytes with inexcitable MSCs.

This study points to possible undesirable consequences of MSC therapy and emphasizes the importance of quantifying MSC engraftment and performing ECG monitoring and detailed electrophysiological studies in large-animal models of intramyocardial MSC therapy. On the basis of our results, we would not expect a proarrhythmic effect of intravenous MSC therapy, which is currently in phase 1 trials. This is due to the fact that in mouse models of intravenous MSC therapy, the injected cells were monodispersed and tended to resemble our 1% MSC/NRVM cocultures.

Possible Mechanisms Underlying Decreased Conduction Velocity Observed in MSC-NRVM Cocultures

Conduction velocity was significantly depressed in MSC cocultures containing >10% MSCs despite the ability of MSCs to couple with their cardiomyocyte and MSC neighbors. These observations can be explained by the dependence of conduction velocity on both gap junction coupling and excitability. The observed decrease in conduction velocity in these MSC cocultures may be attributable to the inexcitable nature of the MSCs and their ability to act as current sinks. The coupled MSCs, which have a resting membrane potential of ~−40 mV, could partially depolarize neighboring myocytes, inactivate Na⁺ channels, and decrease conduction velocity.

Surprisingly, APD₈₀ was not significantly changed in our cocultures compared with controls. MSCs secrete an important array of growth factors and cytokines including macrophage, granulocyte, and granulocyte-macrophage colony stimulating factors, stem cell factor 1, leukemia inhibitory factor, stromal cell–derived factor-1, Flt-3 ligand, and interleukin (IL)-1, -6, -7, -8, -11, -14, and -15. On the basis of our results with human skeletal myoblast and NRVM cocultures, we speculate that these paracrine factors secreted by MSCs may induce myocyte hypertrophy and consequently increase APD, which is balanced by increased electric load imposed by coupled MSCs, resulting in a net insignificant change in APD.

Factors Underlying Initiation and Maintenance of Reentrant Arrhythmias

Factors predisposing to reentrant arrhythmias include a heterogeneous distribution of MSCs and electric coupling of inexcitable MSCs with myocytes. Consequently, MSCs
may induce Na\(^+\) channel inactivation in myocytes in a heterogeneous fashion. These effects would be exacerbated at high pacing rates because of incomplete recovery of Na\(^+\) channels and result in conduction block, wavebreak, and reentrant arrhythmias. We also observed lower conduction velocities at all pacing rates in MSC cocultures compared with NRVM-only (Figure 7A) controls, which could predispose to reentrant arrhythmias in the in vivo setting after intramyocardial injection and engraftment of large numbers of MSCs. Delayed activation of areas containing a mixture of MSCs and myocytes (as seen in Figure 4) would also delay the time of recovery of that region. For this reason, high heart rates or premature impulses may encounter refractory tissue in the MSC area, leading to localized block of impulse propagation and susceptibility to reentry.

Because dynamic instabilities in the setting of steep APD restitution slopes (slopes >0.5, when CL is plotted against APD) can also result in wavebreak and reentry initiation,\(^33\) we measured APD restitution (CL dependence of APD) slopes at different reentry CLs (Figure 3E). We found that APD\(_{80}\) slopes were <0.5, ruling out dynamic instability as cause of wavebreaks (Figure 7B).

**Functional, Not Anatomic, Reentry Is the Cause of Arrhythmias Observed in MSC-NRVM Cocultures**

Anatomic reentry occurs because of rotation of the cardiac impulse around a fixed obstacle like scar, whereas functional reentry can occur in homogeneous tissue and takes the form of an Archimedean spiral that rotates around an unexcited but excitable core.\(^34,35\) Several observations suggest that reentries observed in our cocultures are functional. (1) Tip trajectories of the reentries reveal hypocycloidal or irregular patterns of tip meandering.
(Figure 8A) that have been described experimentally in ventricular and atrial tissue as well as in numerical models of functional reentry. (2) Spiral drift, in which the reentry would move from one distinct location to another, was frequently observed (Figure 8B). (3) The reentry wavefront could be closely fit to an Archimedean spiral (Figure 8C). (4) In addition, we did not find evidence of action potential activation in the core (at 1-mm resolution); the core region was partially depolarized during reentry with a “double-humped” morphology characteristic of such regions (Figure 8D) and excited when the reentry wave drifted.

Mechanism of Reentry CL Adaptation
Previous work has revealed the importance of reentry wavelength and pathlength on the CL and stability of anatomic reentry. However, to date, there has been very little quantitative experimental analysis of CL adaptation in functional reentry. In our experiments, acceleration of reentry was associated with a decrease in reentry APD, tip pathlength, and wavefront curvature and increase in reentry conduction velocity without any change in reentry wavelength.

Reentry rate is dependent on APD (tissue refractoriness), tissue excitability, wavefront curvature, source-load effects, and reentry core size (pathlength). Decreased APD may occur because of an increase in intracellular calcium resulting from high-frequency stimulation that occurs during reentry, enhancing calcium-dependent I_{CaL} inactivation. Decreased APD would also be expected to shorten refractoriness that would decrease wavefront–waveback interactions, resulting in increased wavefront conduction velocity. Because calcium is a major contributor to transmembrane potential at sites of steep wavefront curvature, as seen in the core, the increased intracellular calcium, by increasing excitability in the region of the spiral tip, may also decrease core size.

Limitations
One potential limitation of this study is the use of a 2-dimensional experimental model to predict effects of cell therapy in the 3-dimensional heart. Because of differences in electric loading of cells in the 2-dimensional monolayer versus the 3-dimensional heart, the impact of a noncardiac cell is clearer and would be expected to be magnified in 2-dimensional compared with 3-dimensional tissue because there are fewer shunt pathways available for propagation to circumvent the noncardiac cell. Thus, on this basis, one might expect to find a higher incidence of arrhythmia in 2-dimensional than in 3-dimensional tissue. However, the difference in arrhythmia incidence would be a quantitative and not a qualitative difference and would not preclude our results showing an increased incidence of arrhythmias with increasing numbers of MSCs. Indeed, our results are to be expected on the basis of fundamental biophysical principles.

Bearing in mind this limitation, in a separate study we were able to reproduce features of clinical arrhythmias seen after skeletal myoblast transplantation that were not seen in small-animal models of myoblast transplantation. This underscores the importance of using both in vitro and in vivo approaches to arrhythmic risk assessment before clinical application of intramyocardial stem cell therapy.

Another limitation of our investigation involves the use of NRVMs for our cultures. NRVMs are electrophysiologically different from adult human cardiomyocytes. However, it is not possible at the present time to culture cells of other species that more closely resemble the human ventricular myocyte. Nevertheless, the NRVM has proven to be an effective model for the study of cardiac conduction and reentry.

Conclusions
Our results indicate that in vitro, mixtures of MSCs and cardiomyocytes result in an arrhythmogenic substrate when the MSC concentration is ≥10%. Reentry, not
triggered activity or increased automaticity, was observed to underlie arrhythmias seen in these cocultures. Despite the difficulty in extrapolating in vitro results to clinical cardiology, these findings of easily inducible sustained reentrant arrhythmias motivate a careful assessment of arrhythmogenic potential by performing electrophysiological studies in large-animal models of MSC transplantation before proceeding to intramyocardial MSC therapy in patients. Our results also suggest that intramyocardial MSC injections restricted to areas of scar and intravenous MSC therapy (which results in few MSCs surrounded by myocytes) would probably be safe from an arrhythmia standpoint. Finally, these findings indicate that if current engraftment rates are increased by genetic modification or other means to improve therapeutic outcome, the resulting tissue substrate may then be prone to arrhythmias.

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**Disclosures**

None.

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CLINICAL PERSPECTIVE

Is mesenchymal stem cell (MSC) transplantation potentially arrhythmogenic? In the present study, this question is addressed with optical mapping of an in vitro coculture model of MSC transplantation. Sustained reentrant arrhythmias, the in vitro equivalent of sustained monomorphic ventricular tachycardia, were easily induced by rapid pacing in cocultures containing at least 10% MSCs but not in cocultures containing 1% MSCs. These results indicate that MSCs may be proarrhythmic if large numbers of cells were to engraft at injection sites in the heart and suggest a need for arrhythmia risk assessment in large-animal models of MSC transplantation before clinical trials of intramyocardial MSC therapy.
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