Arrhythmia Surgery

Alteration of Parasympathetic/Sympathetic Ratio in the Infarcted Myocardium After Schwann Cell Transplantation
Modified Electrophysiological Function of Heart
A Novel Antiarrhythmic Therapy

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Background—Neural remodeling after myocardial infarction (MI) may cause fatal ventricular arrhythmia. Schwann cells (SCs), which are important for neurogenesis, are dramatically reduced after MI. We investigated the feasibility of modifying nervous system regeneration after MI and the efficacy by which it may prevent ventricular arrhythmia following SC transplantation.

Methods and Results—Immediately after creation of MI, syngenic Lewis rats were randomized into cell transplantation (n=80) and control groups (n=72). SCs were isolated from sciatic nerves, and 5×10^6 cells were intramyocardially injected into the infarct region. Expression levels of myocardial nerve growth factor, vascular endothelial growth factor, growth-associated protein 43, connexin 43, and laminin in the SC group were significantly higher than control at 7 and 14 days after cell transplantation. Immunohistochemical staining illustrated increases in sympathetic and parasympathetic nerves in both groups. However, SC transplantation significantly increased the parasympathetic/sympathetic ratio at 14 days after cell injection. Dynamic electrocardiography and programmed electric stimulation were also performed. The SCs significantly decreased the low-/high-frequency ratio and arrhythmia score of programmed electric stimulation-induced ventricular arrhythmia at 2 weeks after cell injection. However, SCs did not restore heart function.

Conclusion—Transplanted SCs in the infarcted myocardium secrete multiple biological molecules, which alter the ratio of parasympathetic/sympathetic nerve density to normalize irritable myocardium. SC transplantation might be a novel cell-based antiarrhythmic therapy following MI. (Circulation. 2010;122[suppl 1]:S193–S200.)

Key Words: myocardial infarction • ventricular arrhythmia • cell transplantation • neural remodeling

Fatal ventricular arrhythmia (VA) is a common complication after acute myocardial infarction (MI) and accounts for nearly 50% of sudden cardiac deaths among patients surviving an MI.1

Cardiac autonomic nerves include sympathetic and parasympathetic nerve fibers and compose a complicated nerve network to control heart rhythm. After MI, the nerve fibers in necrotic myocardium are destroyed and then have the potential for regeneration. However, Cao et al2 demonstrated that increased sympathetic nerve density, termed hyperinnervation, was significantly higher in patients with a history of cardiac arrhythmia than those without such histories. Their work brought to light the strong correlation between abnormally increased nerve sprouting and the occurrence of VA and sudden cardiac deaths.

Schwann cells (SCs), the myelinating cells of the nervous system, play a critical role in neural remodeling. SCs exert repair functions by providing physical supports to growing axons, ensheathing and myelinating these axons, and producing a diversity of cell adhesion molecules, extracellular matrix molecules, and neurotrophic factors.3,4 Unfortunately, at the early stages of MI, acute myocardial lesions are also associated with a dramatic loss of SCs, which could result in denervation and unbalanced neural remodeling.

To suppress a potentially fatal VA, various efforts have been used to normalize the irritable myocardium following infarction. Biological therapy, such as local targeted gene injection, has become an attractive option for future antiarrhythmic therapies. In the current study, we injected SCs into the injured myocardium and hypothesized a novel cell-based therapy that could modify cardiac autonomic neural remodeling and thus exert an antiarrhythmic effect. Therefore, in this study, we wanted to address: (1) the survival and

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Presented at the 2009 American Heart Association meeting in Orlando, Fla, November 14–18, 2009.

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Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.109.922740
paracrine function of transplanted SCs in infarcted myocardium; (2) whether SC transplantation reduces VA and improves heart function; and (3) the underlying mechanisms behind SCs transplantation.

Methods

Study Protocol and Animal Model
All animal experimental procedures conformed to the guidelines mandated by the Regulation to the Care and Use of Experimental Animal (1996) of the Beijing Council on Animal Care and were approved by the Ethics Committee for Animal Study of Fuwai Hospital. The study protocol is shown in Figure 1.

Preparation of SCs
Ten syngenic male Lewis rats were used to prepare SCs. The bilateral sciatic nerves were obtained under aseptic conditions and were placed in L-15 medium. After removal of epineurium and connective tissue, the nerves were minced into 2-mm³ segments and digested with 0.25% trypsin and 0.125% collagenase. The precipitate was resuspended in Dulbecco’s minimum essential medium supplemented with 10% FBS, 2 μmol/L forskolin (Sigma-Aldrich), and 20 mg/L bovine pituitary extract (Sigma-Aldrich).

Before transplantation, SCs were labeled with 4’,6-diamidino-2’-phenylindole (DAPI) (Sigma) as previously described. In brief, sterile DAPI stock solution was added to culture medium at a final concentration of 50 μg/mL for 30 minutes. After labeling, cells were washed 6 times in PBS to remove excess unbound DAPI. The DAPI stained 100% of the SCs nuclei.

Transplantation of SCs
MI was induced as described previously. Thirty minutes after induction of MI, Lewis rats with confirmed MI were randomized into 2 groups: SC transplantation (cell transplantation group, n=80) and serum-free media injection group (control group, n=72); 5×10⁶ of cells were transplanted intramyocardially in and around the infarcted region. The infarcted region was identified by color changing from bright red to dark blue.

Heart Function
2D echocardiography was performed at before and 2 weeks after MI and cell injection. Left ventricular end-diastolic diameters (LVEDds) and LVE-systolic diameters were measured at the middle papillary muscle level and left ventricular fractional shortening were calculated as follows: left ventricular fractional shortening (%)=(LVEDd-LVE-systolic diameter)/LVEDd×100. All measurements were averaged on 3 consecutive cardiac cycles and were analyzed by 2 independent observers.

Dynamic Electrocardiography and Ventricular Programmed Electric Stimulation

Dynamic Electrocardiography
Before coronary ligation, a telemetric transmitter (TA10EA-F20; Data Sciences International) was inserted into the abdominal cavity as pervious described. The positive and the negative leads were tunneled to the left hind limb and to the right shoulder, respectively. A 24-hour period dynamic electrocardiography was obtained at the time of 2 weeks after the cell transplantation, and data were record by a blinded operator, using ECG Auto 1.5.7 software (EMKA Technologies). A random of 5-minute ECG recordings exclusion of nonsinus beats was selected for the analyze of heart rate variability as described by others. The peak of the R spike served as a reference point for the temporal location of the R wave. In the time domain, the mean interval between beat-to-beat intervals (NN-interval, ms), SD of NN-interval, and the coefficient of variance (100 SD of NN-interval/mean NN-interval) were calculated. In the frequency domain, 2 regions of interest were defined: low-frequency (LF) (0.5 Hz<LF<0.8 Hz) and high-frequency (HF) bands (0.8 Hz<HF<4.5 Hz), and the LF/HF ratio was calculated.

Ventricular Programmed Electric Stimulation (PES)
An epicardial electrode (Medtronic) was tied to the viable left ventricular myocardium during open-chest surgery for coronary ligation and tunneled under the skin, allowing easy access to the heart.

Table 1. Heart Function and Infarction Area Before and After MI

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Cell Transplantation</th>
<th>Control</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDd (mm)</td>
<td>6.81±0.43</td>
<td>7.80±0.62</td>
<td>8.00±0.31</td>
<td>0.07</td>
</tr>
<tr>
<td>LVEsd (mm)</td>
<td>4.41±0.41</td>
<td>5.78±0.38</td>
<td>5.99±0.23</td>
<td>0.15</td>
</tr>
<tr>
<td>FS (%)</td>
<td>35.14±3.61</td>
<td>24.35±2.19</td>
<td>23.83±1.99</td>
<td>0.09</td>
</tr>
<tr>
<td>Infarction area (%)</td>
<td>None</td>
<td>36.19±2.7</td>
<td>35.32±3.05</td>
<td>0.51</td>
</tr>
</tbody>
</table>

LVEDd, LVE-end systolic diameter; FS, fractional shortening.
*Cell transplantation vs control group.
stimulator. A blinded operator performed dynamic electrocardiography and PES at 2 weeks after cell transplantation. Another 5 healthy rats were served as the sham group.

Programmed electrical stimulation was performed through electrodes sewn on the epicardial surface of the right ventricular outflow tract. Induced arrhythmias were effected using an electric Bloom stimulator (Chengdu Electronic Machine Company). The induction of VAs was then attempted by ventricular stimulation at a basic cycle length of 150 ms (S0) with single (S1), double (S2), and triple (S3) extrastimuli delivered after 8 paced beats. Pacing protocols were interrupted if sustained ventricular tachycardia was induced. Ventricular tachyarrhythmias, including ventricular tachycardia and ventricular fibrillation, were considered nonsustained when it lasted $\leq 15$ beats and sustained when it lasted $>15$ beats. An arrhythmia scoring system was modified as previously described. When multiple forms of arrhythmias occurred in one heart, the highest score was used. The experimental protocols were typically completed within 10 minutes.

Western Blot Analysis
Western blot analysis was performed to identify the in situ expression of nerve growth factor (NGF), vascular endothelial growth factor (VEGF), growth-associated protein 43 (GAP43), connexin 43 (Cx43), and laminin (LN) (Sigma-Aldrich). Tissues were obtained from the treated area of the LV wall at 24 hours, 1 week, and 2 weeks after MI. Another 15 rats, which received chest-opening without coronary ligation, served as a sham group at various time points. Tissue lysates were subjected to SDS-PAGE, followed by standard Western blot analysis procedures with $\beta$-actin as internal standard. Horseradish peroxidase-linked secondary antibodies (Zhongshan Golden Bridge) were used, and proteins were visualized by enhanced chemiluminescence. The bands were then exposed to radiography film, followed with densitometric quantification by TotalLab image analysis software (Total Laboratory Systems Ltd).

Immunohistochemistry
At 24 hours, 1 week, and 2 weeks after MI, 5 rats were euthanized for CD68 (a marker for macrophages) staining. Two weeks after MI, slides dried at room temperature for 2 hours were placed in sodium citrate buffer and heated for 10 minutes. The primary antibody for CD68 (Sigma-Aldrich) was added to the sections and incubated overnight at 4°C. Sections were rinsed with PBS buffer and exposed to secondary antibody.

Two weeks after MI, 5 rat hearts from each group were harvested after dynamic ECG study, and then immunofluorescence was used.
for S-100, tyrosine hydroxylase (TH), acetylcholinesterase enzyme (AChE), and GAP43 staining. Samples taken from liquid nitrogen were embedded immediately into optimal cutting temperature compound (Miles-Bayer) and cut into 5-μm sections. Slices were rinsed and then incubated with the mouse polyclonal antibody targeted against rat S-100 (Sigma-Aldrich), AChE, TH (both from Abcam, Inc), and GAP43 (AbD Serotec). The slices were then rinsed 5 times and incubated prior to the addition of secondary antibody. Another 5 healthy rats were served as sham group, and the free wall of left ventricle was harvested to perform the AChE, TH, and GAP43 staining.

Two weeks after MI, 10 rat hearts from each group were harvested after echocardiography study and then processed in standard formalin-fixed, paraffin-embedded tissues for regular immunohistochemistry staining. The slices from 5 rats from each group were prepared as pervious described. Ten to 12 sections were obtained and stained by Masson’s trichrome, the lengths of infarct and total left ventricle on both epicardial and endocardial surfaces of each section were measured using calibrated digitizer software (Image-Pro Plus version 5.0; Media Cybernetics, Inc). The infarct size was determined as previously described. The slices from another 5 rats from each group were prepared for S-100 staining, which was following the same protocol as CD68 staining. Another 5 healthy rats were served as sham group to perform the S-100 staining.

Cell counting was performed on snapshot images obtained under a 40× field of a light microscope equipped with a computerized image system (Image-Pro Plus version 6.0; Media Cybernetics, Inc). Five fields of each section were randomly selected, and the percentage of positively staining cells was calculated per field by 2 double-blinded investigators. The density of TH-positive, AChE-positive, and GAP43-positive nerve fibers, as well as CD68-positive cells, was determined in a 40× field (0.13 mm²). Five fields were randomly selected, and the density was expressed as their average (μm²/mm²).

**Statistical Methods**

Data are expressed as means±SEM and frequencies (expressed as percentages). Statistical analyses were performed using SPSS software (SPSS, Inc). Continuous variables were compared using the Student’s t test. Electrophysiological data (scoring of PES-induced

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**Figure 3.** Nerve fibers were stained with TH (indicated by white arrows), AChE (indicated by yellow arrows), and GAP43 (indicated by pink arrows) 2 weeks after MI. The nerve sprouting was observed in both groups. However, the ratio of parasympathetic to sympathetic nerves was higher in the cell transplantation group 2 weeks after MI.
arrhythmias) were compared by a Kruskal–Wallis test followed by a Mann–Whitney test. A value of $P < 0.05$ was considered significant.

**Results**

**Identification of SCs**

SCs were shuttle shaped with oval nuclei and 2 thread-like processes at each cell pole. More than 95% of cells were S-100-positive SCs during the entire culture process.

**Mortality**

Mortality at the various time points is depicted in Figure 1. There was no difference between the 2 groups.

**Heart Function**

As shown in Table 1, although the induced MI resulted in the deteriorated heart function, there was no difference in LVEDd, LVSDd, and left ventricular fractional shortening between the cell transplantation and control group, and furthermore, the SCs injection didn’t reduce the infarction area.

**Immunohistochemistry**

Increasing numbers of S-100-positive cells were seen in both groups at 2 weeks after MI (Figure 2B and 2C). Compared with control group, DAPI-positive transplanted cells induced local sprouting of neo-S-100-positive cells in the cell transplantation group at 2 weeks after transplantation (Figure 2F).

As revealed by immunofluorescence, the density of sympathetic and parasympathetic nerves increased in both groups after MI, but the cell transplantation group increased more rapidly (Figure 3 and Table 2). A similar trend was noted in the density of GAP43-positive new nerve fibers. Additionally, the parasympathetic/sympathetic ratio (calculated by dividing the density of parasympathetic nerves by the density of sympathetic nerves) in the cell transplantation group outgrew the control group at 2 weeks.

More CD68-positive macrophages were observed in the cell transplantation group after 24 hours, whereas this difference was less evident at 1 and 2 weeks after MI (Figure 4).

**Western Blot Analysis**

Western blot results are presented in Figure 5. Twenty-four hours after cell transplantation, no difference in targeted protein expression was observed. However, the expression of NGF, VEGF, Cx43, GAP43, and LN, 1 and 2 weeks after MI, were significantly higher in cell the cell transplantation group.

**Dynamic Electrocardiography and Ventricular Programmed Electric Stimulation**

As showed in Table 3, 2 weeks after MI, we observed no major changes in coefficient of variance and SD of NN-interval in the time domain analysis. However, frequency domain, assessed by the LF/HF ratio, was significantly decreased compared with control in the cell transplantation group ($P < 0.01$).

A typical ECG after PES is shown (Figure 6A). The inducibility quotient of ventricular tachycardia was significantly lower in the cell transplantation group versus control 2 weeks after MI (Figure 6B).

**Discussion**

Sympathetic remodeling, and the potential for accompanying arrhythmic events, is a main factor to affecting patient

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Table 2. Density of Nerve Fiber in the Infarction Area at 2 Weeks After Cell Transplantation

<table>
<thead>
<tr>
<th>Nerve Density</th>
<th>Sham</th>
<th>Cell Transplantation</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sympathetic (um²/mm²)</td>
<td>996±226</td>
<td>6988±111*</td>
<td>2529±117</td>
</tr>
<tr>
<td>Parasympathetic (um²/mm²)</td>
<td>178±89</td>
<td>4039±534*</td>
<td>1088±93</td>
</tr>
<tr>
<td>Par-/sympathetic</td>
<td>0.18±0.04</td>
<td>0.58±0.03*</td>
<td>0.43±0.03</td>
</tr>
<tr>
<td>GAP43 (um²/mm²)</td>
<td>1396±530</td>
<td>8397±416*</td>
<td>4775±50</td>
</tr>
</tbody>
</table>

*vs control group, $P<0.01$.
survival after MI. SCs that are vitally important for neurogenesis during the postinfarction period are dramatically reduced early after MI. In the present study, we injected SCs in the early phase of MI in attempt to supply fresh SCs and modify nerve regeneration. The main findings are as follows: (1) injected SCs survived for 2 weeks in the infarcted region, and the release of NGF and VEGF were noted; (2) injected SCs improved nerve regeneration and enhanced the parasympathetic/sympathetic ratio but did not restore heart function; (3) higher levels of Cx43, GAP43, and LN were observed in the cell transplantation group, and more macrophages were chemotactically attracted to the infarcted region; and (4) the IF/HF ratio and arrhythmia score of PES-induced VA was lower after cell transplantation.

Previous spinal and peripheral nerve repair studies have demonstrated that local SC transplantation can promote axonal renewal and remyelination. Furthermore, paracrine secretion has been identified as the main contributor to induce nerve repair. SCs are capable of releasing NGF, which serves as a major factor for nerve regeneration. In the present study, NGF release peaked at 1 week and was sustained at a high level for 2 weeks after MI, which was in accordance with a previous report using a canine MI model. There is a chronological trend toward higher expression of NGF in the infarcted myocardium in both groups. However, the implanted SCs augmented the release of NGF at both 1 and 2 weeks after MI.

Aside from release of NGF, SCs demonstrate neurogenesis functions through other ways, such as macrophage chemotactical attraction and stimulation of LN expression, which

**Table 3. Heart Rate Variability After Myocardial Infarction**

<table>
<thead>
<tr>
<th></th>
<th>2 Weeks After MI</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Cell Transplantation</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Time domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDNN (mm)</td>
<td>6.64±0.65</td>
<td>5.97±2.08</td>
<td>0.51</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.89±0.62</td>
<td>3.98±1.22</td>
<td>0.17</td>
</tr>
<tr>
<td>Frequency domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF (%)</td>
<td>3.10±1.95</td>
<td>2.26±0.82</td>
<td>0.41</td>
</tr>
<tr>
<td>HF (%)</td>
<td>2.00±1.14</td>
<td>0.96±0.26</td>
<td>0.08</td>
</tr>
<tr>
<td>LF/HF</td>
<td>1.64±0.32</td>
<td>2.43±0.52</td>
<td>0.02</td>
</tr>
</tbody>
</table>

SDNN, SD of NN-interval; CV, coefficient of variance.

Figure 5. Western blot analysis demonstrating protein expression of NGF (A), VEGF (B), GAP43 (C), Cx43 (D), and LN (E) at various time points. CTx, cell transplantation group; Con, control group. *Comparison between 2 groups at 1 week yielding significant difference \((P<0.05)\). †Comparison between 2 groups at 1 week yielding significant difference \((P<0.05)\).
are important for the facilitation of nerve sprouting.\textsuperscript{16,17} Macrophages are attracted to injured nerve fibers immediately following tissue damage. This chemotactic attraction is pivotal for the removal of cellular debris, ultimately assisting nerve fiber sprouting. In the current study, macrophage infiltration was most predominant 24 hours after MI. However, no differences were noted between the 2 groups at 1 or 2 weeks after MI. These observations indicate that macrophages play a key role in early nerve regeneration following SC injection, although this effect was transient. The expression of LN, which is an important component of basal lamina supporting the long-term survival of SCs,\textsuperscript{16,17} followed the pattern of NGF expression, which was highest at 2 weeks after MI. We also found that Cx43 was higher in the cell transplantation group at 1 and 2 weeks after MI (Figure 5D). This observation might support an underlying mechanism explaining the reduced arrhythmic occurrence rate in the cell transplantation group. Interestingly, it has been reported that Cx43 plays a role in modifying arrhythmogenic conditions after MI.\textsuperscript{18} Moreover, acetylcholine released by parasympathetic nerves helps to restore decreased Cx43 expression levels.\textsuperscript{19}

GAP43, a membrane-bound phosphoprotein, which is generally associated with new axon growth during development and regeneration, was expressed at higher levels after cell transplantation from 1 week onwards (Figure 5C). The outgrowth of sympathetic nerves after MI is associated with arrhythmia and sudden death after MI. Moreover, Ando et al\textsuperscript{18} demonstrated that vagal nerve stimulation could lower the occurrence of arrhythmias. Our study revealed that, although the density of sympathetic and parasympathetic nerve fibers was significantly higher in the cell transplantation group, SC transplantation markedly enhanced the outgrowth of parasympathetic nerves and thus increased the parasympathetic/sympathetic ratio at 2 weeks following cell transplantation. These findings illustrate that a gradual establishment of balance between sympathetic and parasympathetic nerve fiber composition may contribute to the reduction of arrhythmic occurrence.

In the present study, we used dynamic electrocardiography and PES in either conscious or sedated rats to evaluate the antiarrhythmic effects of SC transplantation. Partially owing to the high heart rate of the rats, we did not observe any improvement in R-R interval and reduction of variance after heart rate variability analysis. However, we observed an increase in mean HF\% in the cell transplantation group, indicating the role of Schwann cells in parasympathetic regenesis. The ratio of LF to HF (LF/HF), which is an indicator of parasympathetic nervous activity,\textsuperscript{20,21} was decreased at 2 weeks after SC transplantation. Meanwhile, the PES study also revealed that the susceptibility of induced VA was reduced at 2 weeks after cell transplantation. Overall, the in vivo electrophysiological study strongly indicates that SC transplantation may normalize the irritability of the myocardium following MI.

Compared with previous studies examining cardiac stem cell therapies, our study of SCs transplantation improved VEGF levels in the infarcted myocardium (Figure 5B), but did not restore contractile function. However, clinical and experimental studies have demonstrated the proarrhythmic potential of cell-based therapy. One major complication of cell transplantation is that focally transplanted cells may develop an arrhythmogenic substrate.\textsuperscript{22,23} Although the mechanisms are multifactorial, the engraftment of transplanted cells within the local myocardium seems to be vitally important for the prevention of arrhythmia. Furthermore, it has been demonstrated that cell transplantation with expression of gap-junction proteins, such as Cx43, results in effective engraftment.\textsuperscript{24} In our study, the increased expression of Cx43 in the cell transplantation group might serve as an explanation of why transplanted SCs exert antiarrhythmic benefits instead of being inherently arrhythmogenic. Therefore SCs could serve in combination with alternative cell sources to reduce the potential proarrhythmic complication of cell transplantation in the damaged heart.

In conclusion, we have presented a novel cell-based therapy for the prevention of VA following MI. Two weeks after transplantation, injected SCs secrete multiple biological molecules and increase the ratio of parasympathetic/sympathetic nerve fibers. This study offers a promising cell-based antiarrhythmic therapy.

Sources of Funding
This work was supported by National 863 Project (2006AA02A106), Beijing Nova Project (2006A85), and the Fok Ying Tung Education Foundation (121041).

Disclosures
None.

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_Circulation_. 2010;122:S193-S200
doi: 10.1161/CIRCULATIONAHA.109.922740

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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