Nerve Sprouting and Sudden Cardiac Death

Ji-Min Cao, Lan S. Chen, Bruce H. KenKnight, Toshihiko Ohara, Moon-Hyoung Lee, Jerome Tsai, William W. Lai, Hrayr S. Karagueuzian, Paul L. Wolf, Michael C. Fishbein, Peng-Sheng Chen

Abstract—The factors that contribute to the occurrence of sudden cardiac death (SCD) in patients with chronic myocardial infarction (MI) are not entirely clear. The present study tests the hypothesis that augmented sympathetic nerve regeneration (nerve sprouting) increases the probability of ventricular tachycardia (VT), ventricular fibrillation (VF), and SCD in chronic MI. In dogs with MI and complete atrioventricular (AV) block, we induced cardiac sympathetic nerve sprouting by infusing nerve growth factor (NGF) to the left stellate ganglion (experimental group, n=9). Another 6 dogs with MI and complete AV block but without NGF infusion served as controls (n=6). Immunocytochemical staining revealed a greater magnitude of sympathetic nerve sprouting in the experimental group than in the control group. After MI, all dogs showed spontaneous VT that persisted for 5.8±2.0 days (phase 1 VT). Spontaneous VT reappeared 13.1±6.0 days after surgery (phase 2 VT). The frequency of phase 2 VT was 10-fold higher in the experimental group (2.0±2.0/d) than in the control group (0.2±0.2/d, P<0.05). Four dogs in the experimental group but none in the control group died suddenly of spontaneous VF. We conclude that MI results in sympathetic nerve sprouting. NGF infusion to the left stellate ganglion in dogs with chronic MI and AV block augments sympathetic nerve sprouting and creates a high-yield model of spontaneous VT, VF, and SCD. The magnitude of sympathetic nerve sprouting may be an important determinant of SCD in chronic MI.

Key Words: cardiac innervation • myocardial infarction • nerve growth factor • ventricular tachycardia • ventricular fibrillation

Sudden cardiac death (SCD) remains a major and unresolved public health problem, claiming 300 000 lives a year in the United States alone. Previous myocardial infarction (MI) can be identified in 75% of SCD victims. In most cases, the direct cause of SCD is ventricular fibrillation (VF), which is usually preceded by ventricular tachycardia (VT). The interaction between VT (the “trigger”) and the diseased myocardium (the “substrate”) results in the transition of VT to VF and subsequent SCD. There are a number of experimental models in which ventricular tachyarrhythmias have been provoked in the presence or absence of structural alterations imposed on the myocardium. However, the investigators induced ischemia or provided artificial triggers, such as electrical stimulation or drugs, to induce VT or VF. The only SCD model in which VT occurs spontaneously was in dogs with inherited ventricular arrhythmia. This latter model may not be applicable to the vast majority of patients whose vulnerability to SCD develops after they have suffered an MI. To date, no high-yield animal model exists in which SCD caused by VF occurs spontaneously in chronic MI. Recently, Vos et al reported that complete atrioventricular (AV) block may result in electrical remodeling and enhance the susceptibility to acquired torsade de pointes. It is also known that sympathetic activity is important in the generation of spontaneous ventricular ectopy and SCD after MI and that stimulating the left stellate ganglion enhances cardiac arrhythmogenesis. On the basis of these findings, we hypothesize that induction of increased sympathetic nerve sprouting by nerve growth factor (NGF) infusion to the left stellate ganglion in dogs with complete AV block and MI may increase the frequency of ventricular arrhythmia. A combination of this trigger and the diseased myocardium (the substrate) may result in a high-yield animal model of spontaneous SCD. The purpose of the present study was to test these hypotheses.

Materials and Methods

Induction of Cardiac Nerve Sprouting by NGF Infusion

We first developed a method to induce cardiac nerve sprouting and hyperinnervation. The method was tested in 3 dogs. NGF 7S was infused into the left stellate ganglion via an osmotic pump. One month later, the hearts were removed for immunocytochemical studies of myocardial innervation.

Animal Model of Spontaneous SCD After MI

The experimental (NGF) group underwent survival surgery in which AV block was created by catheter ablation. An implantable cardioverter-defibrillator (ICD, Guidant model 1762 or 1810) was...
implanted. The ICD was programmed to the monitor-only mode with a back-up pacing rate of 40 bpm. During follow-up, the ICD declared VT episodes once the ventricular rate exceeded 100 bpm for 8 of 10 beats. An osmotic pump was implanted to infuse NGF to the left stellate ganglion. The left anterior descending coronary artery (LAD) was ligated below the first diagonal branch to create MI. The dog was allowed to recover for up to 3 months. The control group underwent the same procedures to create AV block and MI. However, we did not infuse NGF in the control group.

Immunocytochemical Studies

Left ventricular tissues from the edge of the posterior papillary muscle, the anterior papillary muscle, and the interventricular septum of the middle sections were used for immunocytochemical studies. We also sectioned the tissues in the AV nodal region for immunocytochemical studies. The nerve markers tyrosine hydroxylase (TH), synaptophysin (SYN), and growth-associated protein 43 (GAP43) were stained.

Statistical Analysis

Student’s t tests were used to compare the means between 2 groups. To quantify the periodic structure of the frequency of occurrence of VT, single and double harmonic regression models were fitted to the data. The null hypothesis was rejected at a value of P < 0.05.

Results

NGF-Induced Nerve Sprouting and Hyperinnervation in Normal Dogs

In normal canine hearts without NGF infusion, the numbers of nerves per mm² that stained positive for TH and SYN were 14.9±2.0 and 22.9±1.7, respectively. The GAP43 stains were negative in these tissues, indicating the absence of nerve sprouting in normal dogs. In comparison, the densities (numbers of nerve fibers/mm²) of nerves that stained positive for TH, SYN, and GAP43 (Figure 1A through 1C) were 24.7±1.6, 37.7±2.3, and 18.4±7.3, respectively, in normal dogs that received NGF infusion to the left stellate ganglion (P < 0.01 for all comparisons). These results indicate that NGF infusion to the left stellate ganglion induced significant cardiac nerve sprouting in normal dogs.

NGF-Induced Nerve Sprouting and Hyperinnervation in Dogs With MI and AV Block

There was significantly greater ventricular sympathetic nerve density (Table) in the experimental (NGF-treated) group (examples in Figure 1D through 1F) than in the control group (examples in Figure 1G through 1I). The presence of GAP43-positive nerves in the control group suggests that a certain degree of nerve sprouting occurs after MI even without NGF infusion.

In all dogs, abundant sympathetic nerves were observed in the region of the AV node. These nerves stained positive for TH, SYN, and GAP43 (Figure 2). These findings indicate that radiofrequency ablation of the AV node did not destroy sympathetic fibers passing through that area.

Spontaneous VT, VF, and SCD After MI

A total of 15 dogs (9 in experimental group and 6 in control group) survived the first surgery. The ventricular escape rate after a successful AV junction ablation was between 38 and 65 bpm in all dogs studied. The dogs were followed for 43±25 days (experimental group) and 68±25 days (control group) before SCD or a second surgery for tissue harvesting. Four (44%) of the 9 dogs in the experimental group died suddenly of VF at days 11, 17, 60, and 25 (Figure 3). In comparison, none in the control group died during follow-up. All dogs developed spontaneous VT after surgery. These episodes persisted for 5.8±2.0 days (phase 1 VT) before subsiding. Spontaneous VT reappeared 13.1±6.0 days after surgery (phase 2 VT) (Figure 4 and Figure 1 online; see http://www.circresaha.org). On average, there was an arrhythmia-free period of 7.3±5.8 days (range, 2 to 20 days) between VT phases 1 and 2. The average number of phase 2 VT episodes within 60 days after first surgery was 10-fold greater in the experimental group (2.0±2.0 per day) than in the control group (0.2±0.2 per day, P < 0.05).

The average heart and body weights in the experimental group (259±37 g and 25±3 kg) were not significantly different from those in the control group (226±25 g and
Among the dogs in the experimental group, the heart weight of dogs that died of SCD (249 ± 48 g) was not statistically different from that of the dogs that did not die of SCD (269 ± 25 g). There were no significant differences between the infarct size in the experimental group (17 ± 4%) and the control group (14 ± 4%).

Evidence for Increased Sympathetic Activity During the Initiation of VT and SCD

The phase 2 VTs may have been triggered by increased sympathetic activity. When the dogs were at rest, the ventricular rate was 60 ± 10 bpm (61 ± 10 bpm for the experimental group and 59 ± 12 bpm for the control group, P = NS). In comparison, immediately before the onset of VT, the ventricular rate was 75 ± 14 bpm (P < 0.01). In 10 animals, atrial activation rate could be determined by visual inspection of the electrogram stored by ICD. The atrial rate immediately before the VT averaged 192 ± 24 bpm. After the VT spontaneously stopped, there was a transient but remarkable reduction of the atrial rate to 132 ± 37 bpm (P < 0.01) (Figure 5), suggesting vagal activation and/or sympathetic withdrawal before VT termination.

The phase 2 VT occurred throughout the day (Figure 6), with the maximum frequency occurring in the morning. In the experimental group, the single harmonic fit to the data was statistically significant (P < 0.05), indicating a periodic nature of VT. For this model, the estimated coefficient for the sine term was significantly different from zero (P = 0.0285), whereas the coefficient of the cosine term was statistically indistinguishable from zero. The R² for this model was 0.30. The single-harmonic equation for the frequency of VT was as follows: VT per hour = 11.6 - 5.1 cos (2πt/24) + 6.4 sin (2πt/24), where t is the time of day in hours. The same analyses for the control group showed no statistically significant periodicity in the occurrence of phase 2 VT.

SCD in 4 dogs of the experimental group occurred at 10:22 AM, 12:33 PM, 1:42 PM, and 5:30 AM. Two SCD episodes were witnessed by technicians in the room. These 2 dogs were not exercising or feeding at the time of death. The other 2 SCD episodes were not witnessed, and the dog’s activities at the time of death were unknown.

Among 148 episodes of phase 2 VT or VF in which the stored electrograms were available, the patterns of onset were premature ventricular contraction on the T wave with long-short coupling interval (type 1) in 46 (31%), premature ventricular contraction without long-short coupling (type 2) in 51 (34.5%), and acceleration of ventricular escape rhythm into the VT zone (type 3) in 51 (34.5%). The mean rates (bpm) were 217 ± 98, 141 ± 28, and 119 ± 22 for VT types 1, 2, and 3, respectively (P < 0.01 for all comparisons). Figure 3 shows that 3 VF episodes had type I onset (dogs 2, 6, and 9), and 1 was preceded by type 2 onset (dog 4).

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**Table: Nerve Density**

<table>
<thead>
<tr>
<th></th>
<th>Experimental Group</th>
<th>Control Group</th>
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<tr>
<td></td>
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</tr>
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<td>2</td>
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<td>34.4</td>
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<tr>
<td>9</td>
<td>51.4</td>
<td>62.0</td>
</tr>
<tr>
<td>Mean*</td>
<td>33.2</td>
<td>48.7</td>
</tr>
<tr>
<td>SD</td>
<td>12.1</td>
<td>18.2</td>
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</table>

*For all 3 stains, the nerve density of the experimental group was significantly higher than the nerve density of the control group. The P value was < 0.02 for comparison of SYN between experimental and control groups. In all other comparisons, the P value was < 0.01.

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Figure 2. Immunocytochemical staining of cardiac nerves around AV node. This example was taken from dog 9 in the experimental group. A, Low-power (×12) view of the AV nodal region. Dark purple area shows calcification (Ca) after radiofrequency ablation of AV node. Arrows point to nerves stained positive for TH in the perivascular area. Endocardium (En) is at right upper corner. B through D, High-power (×66) view of the AV nodal artery region (near left arrow in A) with nerves stained positive for TH, SYN, and GAP43, respectively. Arrows point to nerve fibers.
In this study, we have developed a high-yield animal model of spontaneous VT, VF, and SCD after chronic MI. Because the only difference between the experimental group and the control group was the presence of increased nerve sprouting in the former, these results also suggest a direct relationship between sympathetic nerve sprouting and SCD in this model.
Compatible with that hypothesis, Nori et al. demonstrated in refractoriness, resulting in VT to VF transition. We conclude that a combination of the spontaneous ventricular arrhythmia (trigger) and the underlying anatomic and electrophysiological abnormalities (substrate) account for a high incidence of spontaneous VT, VF, and SCD in this animal model.

Mechanisms of SCD

The initiation of spontaneous VT, VF, and SCD depends on the presence of both substrate(s) and trigger(s). The anatomic and functional heterogeneities induced by MI or AV block may serve as the substrates for SCD. However, the presence of MI or AV block alone does not lead to a high incidence of SCD. Hunt and Ross reported a 3% incidence of SCD in 97 dogs that were subjected to LAD ligation. Vos et al. reported a low incidence of spontaneous SCD (1 of 7) in dogs with AV block. These findings suggest that MI and AV block created a substrate for SCD. However, because of the absence of the spontaneous triggers, these dogs had a low incidence of SCD.

Functional studies by other investigators showed that increased sympathetic tone may provoke ventricular arrhythmia both in the animal models and in human patients. In our study, dogs in the experimental group had significantly more frequent VT episodes than dogs in the control group. When VT occurs with either type I or type II AV block, the fast rates of these VT episodes may induce spatiotemporal heterogeneity of action potential duration and onset, the fast rates of these VT episodes may induce

Figure 6. Phase 2 VT episodes within 60 days of the first surgery in the experimental group (A) and the control group (B). These episodes were totaled across days and animals. The data were then plotted according to the time of occurrence throughout the 24-hour period.

Nerve Sprouting and SCD Syndrome in Humans

It is known that MI results in cardiac nerve injury. Because peripheral nerve injury is often followed by nerve sprouting, it is possible that nerve sprouting occurs after MI. Compatible with that hypothesis, Nori et al. demonstrated in a rat model that necrotic myocardial injury resulted in denervation followed by sympathetic regeneration. Also compatible with that hypothesis, abnormal patterns of neurilemma proliferation have been documented in infarcted human hearts and that sympathetic scintigraphy demonstrated both denervation and reinnervation after MI. Others reported that the nerve fibers in human hearts are usually TH-positive.

To demonstrate a relationship between ventricular arrhythmia and nerve sprouting in humans, we recently completed a study using the pathological specimens collected from 53 native hearts of cardiac transplant recipients. We also reviewed the history to determine whether or not there were ventricular arrhythmias. Results showed that the density of nerve fibers in patients with arrhythmia was significantly higher than that in patients without arrhythmia (19.6 ± 11.2/ mm² versus 13.5 ± 6.1/mm², P < 0.05). The nerve density in patients with arrhythmia overlaps with the nerve density in the experimental group of the present study (Table). For example, dogs 1, 2, 7, and 8 in the experimental group have sympathetic nerve densities within 1 SD from the mean nerve density in human patients with ventricular arrhythmia. These results suggest that cardiac nerve sprouting may occur after MI even without exogenous NGF. Infusion of NGF accelerated and intensified the development of nerve sprouting, resulting in a high incidence of SCD. Depending on the quantity and timing of nerve sprouting, ventricular arrhythmia and SCD may occur at different times after the MI. This sequence of events is similar to that observed in injury-related epilepsy, which is associated with abnormal nerve sprouting in the central nervous system after brain injury.

Clinical Implications

The results of our study may explain the efficacy of β-blockers in the prevention of SCD. It may also explain the finding that sotalol, a drug with β-blocking effects, is an effective antiarrhythmic agent in patients with chronic MI. In contrast, d-sotalol, a drug without significant β-blocking activity, increased mortality in patients with MI. One clinical implication of this study is that future development of antiarrhythmic interventions should target not only the electrical remodeling but also the neural remodeling (sympathetic nerve sprouting and hyperinnervation) after MI.

Left stellectomy is effective in the prevention of SCD after MI both in animal models and in humans. The present study provides mechanistic insights into the efficacy of left stellectomy in the prevention of SCD.

Summary

The present study reports a high-yield model of spontaneous VT, VF, and SCD in dogs with AV block and chronic MI. The physiological and pathological evidence supports a causal relationship between enhanced sympathetic nerve sprouting and occurrence of SCD. This model may also be useful in future investigations of the mechanisms of SCD and in testing novel methods for prediction and prevention of SCD syndrome.

Acknowledgments

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Methods

The research protocol was approved by the Institutional Animal Care and Use Committee of the Cedars-Sinai Medical Center and followed the guidelines of the American Heart Association. All surgeries were performed under general anesthesia with either isoflurane inhalation (survival surgeries) or intravenous pentobarbital (non-survival surgeries). Sufficient analgesics were given in the postoperative period to minimize the pain. No antiarrhythmic drugs were used.

Induction of Cardiac Nerve Sprouting by NGF infusion

We first developed a method to induce cardiac nerve sprouting and hyperinnervation by NGF infusion. The method was tested in 3 dogs. The chest was opened from the left 4th intercostal space. NGF 7S (0.2 mg/2.2 ml saline solution containing 0.1% bovine serum albumin, Sigma Inc.) was loaded into an Alzet model 2ML4 osmotic pump (Alza Corp) with a 2.2 ml volume and an output rate of 2.5 \( \mu l/\text{hr} \). A 5-mm 28 gauge needle connected to the pump via a plastic tube delivered the contents of the pump into the left stellate ganglion. To identify the sympathetic ganglion, we first visually identified the ganglion based on its location, then applied rapid electrical stimulation to the nerve plexus. An abrupt increase of heart rate of > 20% from the baseline was used as markers of positive identification. The wound was closed and the dog was allowed to recover. One month later, the hearts were removed for immunocytochemical studies of myocardial innervation (see below). For comparison purposes, we also obtained myocardial tissues from 3 normal healthy dogs for immunocytochemical studies.

First (Survival) Surgery to Create an Animal Model of Spontaneous SCD After MI

Because the results of the above 3 dogs showed that NGF infusion resulted in Cao et al, Nerve Sprouting and Sudden Cardiac Death. Online Supplement. Page 1
sympathetic hyperinnervation (see Result Section and Fig. 1A-C), we performed the following experiments to test the hypothesis that NGF-induced sympathetic hyperinnervation can induce SCD by VF in dogs with complete AV block and chronic MI. The experimental (NGF) group underwent survival surgery in a sterile surgical suite. The blood pressure was monitored by a cuff on the left forelimb. Under fluoroscopy guidance, an 8-Fr ablation catheter with a 4-mm tip was inserted via the right femoral vein to register the His bundle potential at the AV nodal region. Radiofrequency energy (20-30 Watts) was then applied to create complete AV block. An Endotak lead (Guidant, St Paul, MN) was inserted via the jugular vein to the right ventricular apex. The pacing threshold was < 0.2 mA in all dogs. We then connected the lead to an implantable cardioverter-defibrillator (ICD, Guidant model 1762 or 1810). All hardware was then implanted in a subcutaneous pocket on the back of the neck. The ICD was programmed to the monitor-only mode with a back up (inhibited ventricular mode) pacing rate of 40 beats per minute (bpm). During follow-up, the ICD declared VT episodes once the ventricular rate exceeded 100 bpm for 8 of 10 beats. We reviewed stored ICD data to confirm the episode classification. The chest was then opened via the left 4th intercostal space. The left stellate ganglion was identified by visual inspection and by electrical stimulation (up to 10 mA) to observe the acceleration of the escape rhythm and the increase of blood pressure. An osmotic pump was implanted to infuse NGF to the left stellate ganglion. The pericardium was opened and the left anterior descending coronary artery (LAD) was ligated (single-stage, no reperfusion) below the first diagonal branch to create anterior wall MI. The chest was then closed and the dog was allowed to recover for up to 3 months under the care of veterinarians.

The control group underwent the same procedures to create AV block and MI. However, we did not perform electrical stimulation of the left stellate ganglion or NGF infusion in this group of dogs.

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After the dogs recovered from surgery, they were allowed to exercise 2-3 times per day, and were fed between 10 a.m. and 11 a.m. The cages were cleaned twice daily, once around 8 a.m. and once around 3 p.m. The lights were off between 7 p.m. and 7 a.m. daily. The dogs were followed for up to 3 months.

**Immunocytochemical Studies and the Infarct Size Measurement**

The hearts were removed as soon as possible when dogs died of SCD. For dogs that did not die SCD, the hearts were removed during a second (non-sterile) surgery under pentobarbital general anesthesia. The hearts were fixed in 4% buffered formalin for 1 hour. Afterwards they were preserved in 70% ethanol. The ventricles were sectioned horizontally into 3 equal portions. Left ventricular tissues from the edge of the posterior papillary muscle, the anterior papillary muscle and the interventricular septum of the middle sections were embedded in paraffin wax for immunocytochemical studies. The sampling sites were consistent in all dogs studied. To study the effects of AV node ablation on adjacent sympathetic fibers, we also sectioned the tissues in the AV nodal region for immunocytochemical studies.

The nerve markers tyrosine hydroxylase (TH), synaptophysin (SYN) and growth associated protein 43 (GAP43) were stained on 5 μm transmural sections using a modified immunocytochemical ABC method. The primary antibodies used in this study were: monoclonal mouse anti-rat TH (Boehringer Mannheim Biochemica, Indianapolis, IN. Working concentration 0.2 μg/ml), rabbit anti-human SYN (DAKO, dilution 1:100) and monoclonal mouse anti-rat GAP43 (working concentration 2 μg/ml, Boehringer Mannheim Biochemica, Indianapolis, IN). The sections were incubated with serum-free protein block (DAKO Corporation, Carprinteria, CA) for 10 minutes, then washed in 0.1 M phosphate buffer solution (PBS) and reacted with 3% hydrogen peroxide to inactivate endogenous peroxidase, followed by a final 5-min wash with PBS. The slides were then treated with Target Unmasking Fluid.
(Signet Laboratories, Inc., Dedham, MA) for 10 min at 90°C in a microwave oven and washed with PBS after being cooled to room temperature. Sections were incubated with primary antibody for 1 hour and with biotinilated secondary antibodies (DAKO) and then with ABCComplex/HRP (DAKO) for 30 min each at room temperature. Sections were thoroughly washed with PBS between each staining. The immunoreactive products were visualized by incubating tissue sections for 2-3 min with diaminobenzidine tetrahydrochloride. The sections were then counter-stained with dilute hematoxylin. Slides from each sampling sites were coded and were examined under a microscope by an investigator (W. W. L.) blinded to the results of the study. For each slide, 3 fields that had the highest number of nerves were used for analyses. The nerve density was the ratio between the total number of nerves and the total area examined.

For measuring infarct size, the ventricles were further sectioned horizontally into 6 equal portions, with the thickness of approximately 1 cm per section. The ratio between total length of the arc of infarct and the total length of the left ventricular circumference of all sections was used for the infarct size estimation.

**Statistical Analysis**

Student's t tests were used to compare the means between two groups. To quantify the periodic structure of the frequency of occurrence of VT, single and double harmonic regression models were fitted to the data. The period of oscillation was taken to be 24 hrs. Model goodness to fit was evaluated by two-tailed t tests on the estimated coefficients and the F tests on the overall model. The null hypothesis was rejected at a value of $P \leq 0.05$.

**RESULTS**
**Figure 1 Online.** Examples of spontaneous VT episodes in the experimental group. ICD declared VT when the ventricular rate exceeded 100 bpm for 8 of 10 beats. The vast majority of recorded VT episodes showed consecutive fast beats (upper 5 panels). Occasionally, two episodes of short runs of VT occurred back to back, resulting in ICD registration of a single episode of VT (bottom panel).

**References**

Non-sustained VT in Dog 9, 5:24 am, Day 25
VT in Dog 8, 4:33 pm, Day 26
VT in Dog 7, 5:08 am, Day 34
VT in Dog 5, 2:36 am, Day 19
VT in Dog 3, 12:49 pm, Day 22
VT in Dog 1, 10:05 am, Day 20