Relationship Between Regional Cardiac Hyperinnervation and Ventricular Arrhythmia

Ji-Min Cao, MD; Michael C. Fishbein, MD; Jay B. Han, MD; William W. Lai, BS; Angela C. Lai, BA; Tsu-Juey Wu, MD; Lawrence Czer, MD; Paul L. Wolf, MD; Timothy A. Denton, MD; I. Peter Shintaku, PhD; Peng-Sheng Chen, MD; Lan S. Chen, MD

Background—Sympathetic nerve activity is known to be important in ventricular arrhythmogenesis, but there is little information on the relation between the distribution of cardiac sympathetic nerves and the occurrence of spontaneous ventricular arrhythmias in humans.

Methods and Results—We studied 53 native hearts of transplant recipients, 5 hearts obtained at autopsy of patients who died of noncardiac causes, and 7 ventricular tissues that had been surgically resected from the origin of ventricular tachycardia. The history was reviewed to determine the presence (group 1A) or absence (group 1B) of spontaneous ventricular arrhythmias. Immunocytochemical staining for S100 protein, neurofilament protein, tyrosine hydroxylase, and protein gene product 9.5 was performed to study the distribution and the density of sympathetic nerves. The average left ventricular ejection fraction was 0.22 ± 0.07. A total of 30 patients had documented ventricular arrhythmias, including ventricular tachycardia and sudden cardiac death. A regional increase in sympathetic nerves was observed around the diseased myocardium and blood vessels in all 30 hearts. The density of nerve fibers as determined morphometrically was significantly higher in group 1A patients (total nerve number 19.6 ± 11.2/mm², total nerve length 3.3 ± 3.0 mm/mm²) than in group 1B patients (total nerve number 13.5 ± 6.1/mm², total nerve length 2.0 ± 1.1 mm/mm², P<0.05 and P<0.01, respectively).

Conclusions—There is an association between a history of spontaneous ventricular arrhythmia and an increased density of sympathetic nerves in patients with severe heart failure. These findings suggest that abnormally increased postinjury sympathetic nerve density may be in part responsible for the occurrence of ventricular arrhythmia and sudden cardiac death in these patients. (Circulation. 2000;101:1960-1969.)

Key Words: nervous system ▪ tachycardia ▪ death, sudden ▪ cardiomyopathy

Increased sympathetic nerve activity is important in the generation of ventricular arrhythmia and sudden cardiac death.¹² However, previous studies on this subject have focused primarily on the physiological consequences of sympathetic nerve stimulation. No data are available regarding the relation between the distribution of cardiac nerves and spontaneous cardiac arrhythmias. Cardiac conditions such as myocardial infarction (MI) may result in nerve injury.³⁴ A consequence of peripheral nerve injury is that it triggers regeneration via nerve sprouting.⁵ Excessive regeneration could lead to hyperinnervation of the myocardium. Compatible with this prediction, Vracko et al⁶ demonstrated abnormal patterns of neurilemma proliferation in the scars of human myocardium. However, only nonspecific staining (S100 protein) was used in that study. Even with the assumption that all of the positive stains represented nerves, it was still unclear whether the quantity of the abnormal nerves correlated with the occurrence of ventricular arrhythmias or sudden cardiac death (SCD). In the present study, we examined the nerve fiber density in the ventricles of the native hearts of the heart transplant recipients. The nerve fiber density was then compared with the clinical history of ventricular arrhythmia to test the hypothesis that regional sympathetic hyperinnervation in the ventricles secondary to myocardium injury is correlated with the occurrence of spontaneous ventricular tachyarrhythmia or SCD.

Methods

The research protocol was approved by the institutional review board. Ventricular tissues were obtained from the following groups of patients or tissues (Table): Group 1 (n=53) included ventricular tissues that were obtained from the native hearts of the transplant recipients. Paraffin-embedded tissue...
blocks were collected retrospectively (n=41) from January 1994 to June 1996, and fresh ventricular tissues were obtained prospectively (n=12) from December 1996 to February 1998. The fresh tissues were obtained at the time of transplant surgery and were processed immediately. Among group 1 patients, 25 had ischemic heart disease and 28 had idiopathic dilated cardiomyopathy. The group 1 patients were divided into 2 subgroups: patients with a history of VT or SCD (group 1A, n=30) and patients without a history of VT or SCD (group 1B, n=23). Patients with a history of VT or SCD were those who had documented spontaneous sustained VT, nonsustained VT (>3 beats), or SCD requiring either medical therapy or the implantation of an implantable cardioverter-defibrillator.

Group 2 (n=5) included ventricular tissues that were obtained from 5 patients who underwent an autopsy between December 1996 and February 1998 and who died of noncardiac causes and had no history of heart diseases or ventricular arrhythmia. Causes of death were breast cancer (n=1), ovarian cancer (n=1), hepatic failure (n=2), and motor vehicle accident (n=1). The tissues were harvested 8 to 16 hours after death.

Group 3 (n=7) included paraffin-embedded tissue blocks that were collected from 7 patients with coronary artery disease who underwent intraoperative computerized mapping and surgical ablation for sustained monomorphic VT between 1988 and 1991. Tissues excised from the origin of VT were used for the study.

**Immunocytochemistry**

**Tissue Fixation**

All ventricular tissues of the 41 retrospective patients were formalin fixed for ≥48 hours, dehydrated with graded alcohol, cleared in xylene, embedded in paraffin wax, and stored for ≥2 years before immunocytochemical studies. No frozen sections were obtained.

For tissues that were collected prospectively, both frozen and paraffin-fixed sections were obtained. To prepare frozen tissue sections, transmural tissue slices of 2- to 3-mm thickness were cut, embedded in OCT compound (Tissue Tek; Sakura Finetechnical Co, Ltd), and snap-frozen with Cytocool II Tissue Freezing Aerosol (Stephens Scientific).

**Immunostaining Procedures**

Transmural sections of 5 µm were cut perpendicular to the epicardium from either a frozen or a paraffin-embedded tissue block and mounted onto slides. Before being processed for immunostaining, paraffin sections were deparaffinized and rehydrated. Frozen sections were prefixed with 10% buffered formalin for 2 hours because S100 is a highly soluble protein. The slides were then reacted with a 3% hydrogen peroxide/methanol solution to inactivate endogenous peroxidase, followed by a final 5-minute wash with PBS. For S100 and NF staining, paraffin sections were treated with a trypsin solution for 10 minutes in 37°C at pH 8.1. For TH staining in paraffin sections, slides were treated with Target Unmasking Fluid (Signet Laboratories, Inc) for 10 minutes at 90°C in a microwave oven and then washed with PBS after being cooled down at room temperature. For PGP 9.5, slides were incubated in Retrieve-All (Signet Laboratories, Inc) in a circulating water bath maintained at 90°C. To reduce nonspecific staining, sections were incubated with Serum-Free Protein Block (DAKO Corp) for 10 minutes. Sections were incubated with primary antibodies for 1 hour and with biotinylated secondary antibodies (DAKO Corp) and then with an avidin/biotin/alkaline phosphatase (ABComplex/AP; DAKO Corp) for 30 minutes, with sufficient washing with Tris buffer solution between steps. The sections were then developed with a fast red chromogen system to allow visualization of the nerves. The fast red chromogen system yielded a yellow-brown stain, whereas the DAB chromogen system yielded a yellow-brown stain. The sections were then lightly counterstained with hematoxylin, rinsed with an acid/alcohol solution, and mounted with aqueous mounting medium.

**Sources of Antibodies**

Primary antibodies used in this study were rabbit anti-cow S100 (recognizing S100A and S100B, 1:1000 dilution; DAKO Corp), monoclonal mouse anti-human NF protein (1:300 dilution; DAKO Corp), monoclonal mouse anti-rat TH (working concentration 0.2 µg/mL; Boehringer Mannheim Biochemica), and rabbit anti-human receptor trkA (1:100 dilution; Santa Cruz Biotechnology, Inc). PGP 9.5 antibodies (1:4000 dilution) were obtained from Chemicon International Inc.

**Negative and Positive Controls**

For each protein marker, negative and positive control stains were performed in the same staining session. Negative controls were obtained by either replacing primary antibodies with rabbit anti-human von Willebrand factor (factor VIII–related antigen, 1:500 dilution; DAKO Corp) or omitting primary antibodies. Positive controls for S100, NF, and TH were obtained by immunostaining the tissue sections from schwannoma, cerebellum, and sympathetic ganglia, respectively.

**Measurement of the Density of Nerve Fiber and Statistics**

The density of S100-labeled nerve fibers was calculated through computer-assisted image analysis with the MCID imaging processing system (Imaging Research Inc). The procedure was performed by an investigator (W.W.L.) who was blinded to the patient’s clinical history.

For each section, 2 areas of myocardium were studied: (1) the periphery of necrotic or fibrotic myocardium and (2) the perivascular regions. If the periphery of injured myocardium was not easily identified or no myocardium injury existed, nerve fiber density was measured in the non-necrotic myocardium that contained most nerve structures. For each area, 6 fields that contained most nerve fiber structures were chosen visually to calculate the total number and the total length of nerve fiber structures. Under a ×10 objective, 1 image was digitized into a 639×479-pixel array and equaled an area of 0.953 mm².

The nerves were differentiated from the other structures by the use of the grain count program within the MCID. Briefly, all of the immunostained structures were highlighted with an arbitrarily set threshold. The chosen threshold included all nerve fibers that could be easily recognized by microscopy at 10×10 magnification. Shape criteria were used to discount structures based on the degree of roundness. A circle is 1°, and a line is 0°. In most circumstances, a cutoff of 0.4° excluded all of the structures other than nerve fibers (eg, myocytes, blood vessel, fibroblasts, or artifacts). Size criteria were used to discount any nonspecific staining of <1×10⁴ mm².

The nerve fiber density for each patient is expressed as the mean of the total number of nerve fibers per millimeters squared and the mean of the total length (mm) of nerve fibers per millimeters squared, which were calculated from all chosen fields. The data for each patient were calculated from 1 to 3 sections.

**Results**

Nerve fibers immunoreactive to S100 protein (S100) and tyrosine hydroxylase (TH) were easily observed between the myocytes and along blood vessels in group 2 (normal) hearts (Figures 1A and 1B). The longitudinal nerve fibers appeared to be parallel to the orientation of myocardial fibers. The nerve fibers were evenly distributed in the ventricular myocardium. The protein gene product 9.5 (PGP 9.5) stained positive in all 4 diseased tissues. The nerve that stained
positive for PGP 9.5 also stained positive for S100 (Figures 1C and 1D).

Double immunostaining of TH and S100 colocalized the same structures (Figure 2). Immunostaining for S100, TH, and neurofilament protein (NF) conducted on serial sections from frozen tissues also showed that these 3 markers labeled the same structures (Figure 3). These observations suggest that the S100 immunoreactive nerve fiber–like structures contain not only Schwann cells but also nerve axons and that the majority of these fibers are likely sympathetic nerves.

Figure 1. Colocalization of S100 and specific nerve markers in human cardiac nerves. A and B, Normal myocardium stained with antibody to S100 or TH, respectively. S100- and TH-positive nerve fibers (arrowheads) are located between unstained myofibers and are oriented on same longitudinal axis as that of the myofibers. C and D, Positive stains of S100 and PGP 9.5, respectively, in a patient with coronary artery disease, congestive heart failure, and ventricular arrhythmia.

Regional Increase of Innervation in Injured Myocardium
No tyrosine kinase A (trkA) immunoreactivity was observed except in the blood vessel walls. The lack of trkA immunoreactivity in the cardiac tissue is consistent with the observation reported by others.7 Large nerve trunks and small nerve fascicles immunoreactive to S100 were consistently observed in all tissue sections. Consistent immunostaining for NF and TH was obtained only in frozen tissue sections (12 explant hearts and 5 autopsy hearts) and not in sections from paraffin-embedded tissue blocks (41 explant hearts and 7
Figure 2. Double staining with antibodies to S100 and TH shows colocalization of 2 marker proteins on same nerve structures. A, TH stained with alkaline phosphatase/fast red system, which gives a purple-red color. B, Double staining of TH and S100, which shows a brick-red color. C, S100 stained with peroxidase-DAB system, which yields a brown color.
surgically resected tissues), which was likely due to long formalin fixation and storage times.

The distribution of nerves was much less homogeneous in the ventricles with myocardial injury (groups 1A and 1B) than in the ventricles from normal hearts (group 2). The S100-immunopositive nerve fibers were most abundant at the periphery of necrotic tissues (Figure 4A) or in the perivascular regions (Figure 4B). In some sections, nerve fascicles were scattered in a “swarm-like” pattern at the junction between necrotic and surviving myocardium (Figure 4C).
Clusters of S100-labeled small nerve twigs were also found in some injured areas with fibrosis and revascularization (Figure 4D). In contrast to the periphery of damaged myocardium, no S100-reactive nerve fibers were seen in the central zone of necrotic tissues or dense fibrotic tissues. TH-positive nerve fibers were observed in the injured areas or around the coronary arteries in both patients with ischemic cardiomyopathy (Figure 5A) and patients with idiopathic dilated cardiomyopathy (Figure 5B).

In group 3, the ventricular tissues resected from the origin of ventricular tachycardia (VT) showed fibrosis intermingled with normal-appearing myocytes. Similar to those seen in explanted hearts, S100-immunopositive nerve fibers were abundant and easily identified in the tissue fragments from 6 patients (Figure 6A), and TH-positive nerves were detected in 3 patients (Figure 6B).

Taking these observations together, it appears that ischemic or nonischemic myocardial injury results in inhomogeneous distribution of nerve fibers. A regional increase of sympathetic nerves was commonly observed in the injured myocardium. The tissues from arrhythmogenic sites had abundant sympathetic nerve fibers.

Figure 4. Regional cardiac hyperinnervation in patients with cardiomyopathy and ventricular tachyarrhythmias as demonstrated with S100 staining (arrowheads). A, Increased density of S100-positive nerve fibers at border of myocardial injury as revealed by infiltration of inflammatory cells. Compared with relatively “normal” myocardial area (marked by M), injured area had higher nerve density. B, Colony of small nerve twigs around coronary artery. C, S100-positive nerve fascicles scattered in swarm-like pattern at junction between necrotic and survived myocardium. D, Clusters of S100-positive small nerve twigs in some injured areas with fibrosis and vasculogenesis. Innervation patterns shown in A to D were not observed in cardiac tissues of patients who died of noncardiac causes.

Relationship Between Density of S100-Immunoreactive Nerve Fibers in the Ventricles and History of Ventricular Tachyarrhythmias

The density of nerve fibers (Table) was measured only in tissues stained with S100 because the immunoreactivities to TH or NF were not consistently observed in 41 retrospectively collected paraffin-embedded tissues. There was no difference in the nerve densities between patients with ischemic and nonischemic cardiomyopathy. Therefore, the 53 patients who underwent heart transplantation were classified only according to history of VT. All patients with VT were treated with antiarrhythmic drugs, an implantable cardioverter-defibrillator, or both. Group 1A had a higher nerve density than the control group (group 2). In contrast, the density of nerves did not differ significantly between group 1B and group 2. This quantitative result demonstrates that an increase in nerve fiber density in the myocardium is associated with a history of VT in the patients with severe organic heart disease.

Discussion

The main observations of this study are (1) that there is inhomogeneous distribution of sympathetic nerve fibers in the myocardium in patients with cardiomyopathy and ventricular tachyarrhythmias; (2) that there is a regional increase of sympathetic nerves in injured myocardium; and (3) that the density of sympathetic nerve fibers is associated with a history of VT in patients with severe organic heart disease.
the ventricles with myocardial injury, which is characterized by regional hyperinnervation in the perivascular regions or in the periphery of injured myocardium and regional denervation in the regions with necrosis or dense fibrosis. (2) The density of S100-immunoreactive nerve fibers in the ventricles is significantly higher in patients with a history of tachyarrhythmias than in the patients without tachyarrhythmias. These results suggest that there are increased sympathetic nerves after MI and that the density of these sympathetic nerves directly correlates with the occurrence of life-threatening ventricular arrhythmia. In addition to these 2 observations, the present study demonstrated that visualization of nerves in the autopsied heart is now possible as a routine procedure.

Visualization of Nerves in Autopsied Heart
According to Hirsch et al,8 the first descriptions of cardiac innervation were made in the mid-19th century by Lugwig9 and Bidder10 in studies of the frog heart. Since then, many investigators have studied cardiac nerves with the use of conventional histological techniques.8 Recent advancements in immunocytochemical techniques have allowed investigators to study the sympathetic and parasympathetic nerve distributions in autopsied hearts.11–15 Chow et al16 studied the
effects of death on the immunofluorescence of autonomic nerves that supply the human ventricular myocardium. Percutaneous myocardial samples were obtained at 5 to 11 days after death. The authors demonstrated that depending on the type of antibodies used, immunohistochemical techniques can be used on human hearts obtained within 6 days of death. The results of these studies and the present study indicate that visualization of nerves in the autopsied heart is now possible as a routine procedure.

**Myocardial Injury Is Associated With Inhomogeneous Innervation With Regional Hyperinnervation, Most Likely of Sympathetic Nerve Origin**

Consistent with other reports, the present study demonstrates that the distribution of nerve fibers in the ventricles is altered after myocardial injury resulting from either ischemia or cardiomyopathy. The necrotic myocardium is associated with denervation, whereas the peripheries of myocardial scar and

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**Figure 6.** S100- and TH-positive nerve fibers in VT origins of patients with coronary artery disease who underwent surgical ablation of VT. A, S100-positive nerve fibers located in perivascular area and areas with fibrosis. B, TH-positive nerve fibers around coronary artery.

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perivascular regions are richly innervated.\textsuperscript{6,17} Regional hyperinnervation is related with myocardial injury because nerve fiber density was significantly higher in patients with organic heart disease than in patients without heart disease. Animal models of myocardial ischemia indicated that the increase in nerve fibers at the borders of injured myocardium resulted from the proliferative regeneration of Schwann cells and axons.\textsuperscript{18} Whether the increased nerve fibers are all sympathetic nerves cannot be concluded in the present study because the parasympathetic nerves were not stained. However, it is well known that ventricular tissue is innervated mainly by sympathetic nerves. Parasympathetic nerve fibers also innervate the ventricles, but they are much less numerous.\textsuperscript{19} We have shown that the majority of cardiac nerves that stained positive for S100 are immunoreactive to TH and that colocalization of S100 and TH is consistently observed. It is likely that a significant portion, if not all, of regenerated nerves in the perinecrotic or perivascular region are sympathetic nerves. This notion is supported by the demonstration of sympathetic denervation and reinnervation in myocardium injuries with the use of \textsuperscript{125}I-labeled metaiodobenzylguanidine scanning.\textsuperscript{20}

The significant increase in nerve growth factor (NGF) activity had also been observed in the Schwann cells of the sympathetically denervated rat iris.\textsuperscript{21} In addition, it has been demonstrated that interleukin-1 released by macrophages plays a role in the augmented local production of NGF after peripheral nerve injury.\textsuperscript{22} Insults that damage myocardium can also injure nerves (denervation) and cause inflammation with macrophage infiltration. An induction of NGF synthesis in non-neural cells that results from myocardial injuries may provoke sympathetic nerve regeneration. We propose that the increased quantity of sympathetic nerves may be secondary to injury-related nerve sprouting. However, whether nerve sprouting actually was responsible for these observations could not be determined in this retrospective study.

S100-Immunoreactive Structures in Ventricles Are Mostly Sympathetic Nerve Fibers

S100 protein has been used as a maker of cardiac nerve fibers.\textsuperscript{6,17,18} S100 families consist of homodimers or heterodimers of S100\textalpha~ and S100\textbeta~ subunits.\textsuperscript{23,24} S100A (\textalpha\beta) is expressed predominantly in neurons, S100B (\textbeta\beta) is expressed predominantly in glial cells and neurons, and S100A0 (\textalpha\alpha) is expressed predominantly in skeletal and heart muscle\textsuperscript{24} and in neurons.\textsuperscript{25} The anti-S100 antibody used in the present study is polyclonal rabbit anti-cow S100 specific for S100A and S100B, which strongly cross-reacts with human S100A and S100B but does not cross-react with S100A0. Furthermore, the expression of S100 by Schwann cells depends on the axon (ie, the Schwann cell–axon contact).\textsuperscript{24} Immunostaining of serial sections and double immunostaining in the present study show that S100-labeled structures are also labeled by NF, PGP 9.5, and TH. Small nerve fascicles immunoreactive to TH were easily observed in frozen sections of myocardium. These data suggest that most S100-immunonegative nerve fiber–like structures demonstrated in the present study are sympathetic nerves.

An Increase in Nerve Fiber Density Is Associated With a History of Ventricular Tachyarrhythmia

The second major finding of the present study was that an increase in cardiac sympathetic innervation observed in our patients was significantly associated with a history of ventricular arrhythmia. It is well known that ventricular arrhythmia and SCD occur in patients with poor ventricular function of various causes.\textsuperscript{26} Although patients with ischemic heart disease may have arrhythmias triggered by ischemia and anatomic conduction block due to myocardial scarring, the mechanism of arrhythmogenesis in patients with idiopathic cardiomyopathy is unclear. The enhanced spatial inhomogeneity of cardiac innervation may be associated with the occurrence of ventricular arrhythmia in patients with idiopathic cardiomyopathy. As sympathetic nerve activation exerts significant effects on electrophysiological properties such as automaticity, refractoriness, and conduction velocity of myocardial cells,\textsuperscript{27,28} an enhanced spatial inhomogeneity in cardiac innervation might amplify the spatial inhomogeneity of these electrophysiological properties and therefore facilitate the initiation of ventricular arrhythmia. In the present study, we observed that abnormally increased nerve density not only occurred in all of our patients with congestive heart failure but also was an independent factor associated with the occurrence of ventricular arrhythmia. It is therefore reasonable to propose that regional hyperinnervation may play a significant role in arrhythmogenesis in patients with chronic severe congestive heart failure.

Study Limitations

A major limitation was the retrospective nature of this work. Most tissues were fixed in formalin and stored in paraffin-
embedded tissue blocks for 1 to 2 years before they were stained. This may have accounted for the negative TH and NF stains in some tissues. However, in freshly collected and fixed tissues, we have shown that S100, TH, and PGP 9.5 stained the same nerves. Because S100 staining was positive in all retrospectively collected tissue blocks, we were able to quantify the nerves in each tissue without difficulty. This limitation should not invalidate the conclusion of the study.

In summary, the present study demonstrated that a regional increase in sympathetic nerves in the ventricles was present in patients with severe organic heart diseases and that the density of nerve fibers is significantly associated with history of ventricular tachyarrhythmias. The data suggest that abnormal distribution of sympathetic nerves in the ventricle may contribute to the occurrence of VT in patients with severe organic heart disease.

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