The ligament of Marshall as a parasympathetic conduit


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Submitted 2 February 2007; accepted in final form 31 May 2007


The ligament of Marshall as a parasympathetic conduit. Am J Physiol Heart Circ Physiol 293: H1629–H1635, 2007. First published June 1, 2007; doi:10.1152/ajpheart.00139.2007.—The objective of the study was to investigate the morphology, distribution, and electrophysiological profile of the autonomic fibers that innervate the ligament of Marshall (LOM). Gross anatomical dissections were performed in 10 dogs. Sections of the left vagus nerve, left stellate ganglion, and the LOM were immunostained to identify adrenergic and cholinergic nerves. Hearts were also stained for acetylcholinesterase to identify epicardial cholinergic nerves. In vivo electrophysiological studies were performed in another 10 dogs before and after LOM ablation. The anatomical examination revealed that the LOM is innervated by a branch of the left vagus. Immunohistochemistry confirmed that these nerve bundles are predominantly cholinergic (cholinergic-to-adrenergic ratio of 12.6:3.9:1). Cholinergic nerves originating in the LOM were found to innervate surrounding left atrial structures, including the pulmonary veins, left atrial appendage, coronary sinus, and posterior left atrial fat pad. Ablation of the LOM significantly attenuated effective refractory period shortening at distant sites, such as pulmonary veins and left atrial appendage, in response to vagal stimulation (vagal-induced ERP decrease in the left atrium: baseline vs. postablation = 17 vs. 4%; \(P = 0.0056\)). In conclusion, the LOM contains a predominance of cholinergic nerve fibers. Cholinergic fibers arising from the LOM innervate surrounding structures and contribute to the electrophysiological profile of the left atrium. These findings may provide a basis for the role of the LOM in the genesis and maintenance of atrial fibrillation.

ablation; arrhythmia (mechanisms); autonomic nervous system; innervation; supraventricular arrhythmia

IN 1850, JOHN MARSHALL DESCRIBED a “vestigial fold of the pericardium” that is the developmental vestige of the embryonic left superior vena cava (14). This fold has since become known as the ligament of Marshall (LOM). The LOM contains the vein of Marshall and numerous other small blood vessels, fibrous bands, and nervous filaments. Subsequent work by Scherlag et al. (25) demonstrated that the LOM was the terminal end of an inferior interatrial pathway in the canine heart, and that double potentials (defined as discrete activations separated by an isoelectric interval) recorded near the LOM during sinus rhythm could be associated with striated cardiac muscle within both the LOM and the left atrium. Later studies by Kim et al. (10) expanded on this work, finding that the LOM in humans contains striated cardiac muscle bundles, known as Marshall bundles, that have multiple reinsertions into the coronary sinus (CS) and the left atrial wall. This study also demonstrated that the human LOM contains multiple sympathetic nerve fibers. These findings suggest that the LOM could play a role in the genesis and maintenance of adrenergic atrial fibrillation and have made the LOM a target of many subsequent electrophysiological studies that attempted to identify the source of atrial fibrillation (3, 7, 16, 27). However, little attention has been given to the study of parasympathetic innervations of the LOM. Since vagal stimulation (VS) has been shown to trigger atrial fibrillation (21, 23), we hypothesized that parasympathetic activity originating in the LOM may help promote atrial fibrillation. A more complete description of the autonomic components present within the LOM is essential to better understand the contributions of the LOM to adrenergic or cholinergic arrhythmias.

MATERIALS AND METHODS

Anatomical dissection. The experimental protocol was approved by the Animal Care and Use Committee of Northwestern University and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996).

Gross anatomical dissections were performed on 10 normal mongrel dogs. Before euthanization, heparin (100 U/kg iv) was administered to prevent the formation of blood clots within the heart. After euthanization, the neck was opened, and both vagus nerves were identified. The ribcage was then removed to fully expose the thoracic cavity. The stellate ganglia were located, and the branches of the stellate ganglia that communicate with the vagus nerve were identified. The term “vagus node” is used to refer to the point of junction between the branches of the stellate ganglia and the vagus nerve. After the left and right vagus nodes were located, the two vagus nerves were traced down toward the heart. All branches of the vagus nerves that innervate the heart were located, and the vagus branch that innervates the LOM was identified. The heart and the cardiac branches of the left vagus were then removed to allow for more detailed analysis.

Immunohistochemical analysis. The LOM was procured from the heart and was frozen rapidly in liquid nitrogen. After freezing, serial circumferential cross sections were cut along the length of the LOM. In addition, control specimens were taken from the vagus nerve (positive control for parasympathetic nerve fibers) and stellate ganglia (negative control for parasympathetic nerve fibers, but positive control for sympathetic nerve fibers). Transverse sections of the left vagus were taken above the left vagus node, at the node, and below the node. Control specimens were stained using a hematoxylin-phloxine-saffron stain (Sigma, St. Louis, MO) that stains muscle tissue red, collagen and fibrous tissue yellow, and cell nuclei blue.

Sections of the left vagus nerve, stellate ganglia, and the LOM were immunostained to identify adrenergic and cholinergic nerve fibers. Sections were air dried and fixed in acetone for 10 min, then washed in Tris-buffered saline. Hydrogen peroxidase block (Dako, Carpinte-
ria, CA) was placed on the sections for 10 min, and then the slides were again washed in Tris-buffered saline. Protein block was placed on the sections for 30 min. Primary antibodies were then incubated overnight at 4°C. Antibodies for dopamine β-hydroxylase (DBH) (Chemicon, Temecula, CA) were used to stain sympathetic nerves, while antibodies for choline acetyltransferase (ChAT) (Chemicon) were used to stain parasympathetic nerves. The specificity of DBH for sympathetic nerve fibers was confirmed by the use of a second sympathetic marker, i.e., tyrosine hydroxylase (Chemicon); tyrosine hydroxylase was noted to stain the same nerve elements that were being stained by DBH (data not shown). Similarly, the specificity of parasympathetic staining was confirmed by the use of second parasympathetic marker, i.e., acetylcholine esterase (ACE) (Chemicon); ACE was found to stain the same nerve elements that were being stained by ChAT (data not shown). Some sections were double-stained for both ChAT and DBH. After incubation, the slides were washed in Tris-buffered saline, and the appropriate secondary antibody (Chemicon) was then placed on the sections for 30 min. The sections were again washed in Tris-buffered saline, and the appropriate chromogen solution was added to each specimen. Cell nuclei were marked by placing the specimens in methyl green (Dako) for 10 min. The specimens were then dehydrated in alcohol, mounted, and examined under light microscopy. Similar techniques have previously been used to successfully characterize the autonomic innervation of other tissues (24).

Epicardial nerve stain. A novel histochemical method for ACE was used to identify epicardial cholinergic nerves in eight whole canine hearts. The hearts were incubated for 24 h in a buffer at pH 5.6. The composition of the buffer was 50 mM dibasic sodium phosphate, 170 mM monobasic sodium phosphate, 40 mM glycine, 5 mM acetylthiocholine iodide, and 8.25 mM CuSO4. This is an adaptation of the method pioneered by Karnovsky and Roots (8). The acetylthiocholine iodide in the buffer precipitates with acetylcholinesterase, staining cholinergic nerve fibers a bright white color.

Electrophysiological studies. Ten additional normal mongrel dogs were used to correlate the anatomic and immunohistochemical findings with in vivo electrophysiological data. These experiments were performed under general anesthesia (2.5% inhaled isoflurane; preanesthesia with intravenous propofol, 4.5 mg/kg). The body temperature was maintained at ~37°C, and blood pressure was monitored and maintained within a physiological range. The left cervical vagus was isolated in the neck. The chest was opened via a median thoracotomy. The pericardium was opened, and epicardial bipolar electrodes were sutured onto the region of LOM, the left superior pulmonary vein (PV), the left inferior PV, the right atrial appendage (LAA), the left atrial appendage (LPA), the left pulmonary artery; LV, left ventricle; LIPV, left inferior pulmonary vein; LSPV, left superior pulmonary vein; PFP, posterior left atrial fat pad; RSPV, right superior pulmonary vein; SVC, superior vena cava; PA, pulmonary artery.

Data analysis. The number of cholinergic and adrenergic nerve fibers found in sections of the left vagus nerve and left stellate ganglion was counted manually. In addition, the number of cholinergic and adrenergic fibers was counted manually within 15 randomly selected nerve bundles from the LOM. To account for variation in nerve/nerve bundle size, the ratio of cholinergic to adrenergic nerve fibers (averaged for the 15 randomly selected bundles as well as for sections from the vagus nerve and the stellate ganglion) was taken as an estimate of the relative distribution of cholinergic vs. adrenergic nerve fibers in each region. Data are expressed as means ± SE.

Since multiple ERPs were obtained at each site and in each animal, the mean ERP was calculated for each site in an individual animal. The mean (and standard deviation) of these site means was then calculated for all dogs. ERP changes in response to the above-mentioned maneuvers were compared among the different stages using the Student t-test or analysis of variance, as appropriate. Values of P ≤ 0.05 were considered statistically significant.

RESULTS

Anatomical dissection. Both vagus nerves and stellate ganglia were located, and the left and right vagus nodes were identified in each animal (Fig. 2A). Distal to the left vagus node, cardiac branches of the left vagus were found to innervate the posterior surface of the left atrium, while the cardiac branches of the right vagus innervated the superior vena cava and the anterior surface of the right atrium. As the left vagus approaches the posterior surface of the heart, it divides into several discrete branches. One of these branches runs the length of the LOM along the vein of Marshall, passing between the left PVs and the LAA (Fig. 2, B–D). This branch is the only significant innervation of the LOM. After exiting the LOM, this vagus branch travels along the CS toward the right atrium. After gross staining of fresh hearts for ACE, many smaller fibers were observed to leave this primary vagus branch and
innervate surrounding tissues (Fig. 3). Specifically, small cholinergic nerve fibers originating in the LOM were found to innervate both left PVs. Small cholinergic nerve fibers splitting off from the primary vagus branch along the CS were also found to innervate the PFP. Some fibers that innervated the PFP were observed to continue to both the left and the right inferior PVs. Additionally, cholinergic nerve fibers from the LOM were seen to descend to the posterior surface of the left ventricle. The observed anatomy was consistent in each of the dogs.

Immunohistochemical analysis. Above the left vagus node, ChAT and DBH staining of the left vagus revealed a high concentration of cholinergic nerve fibers with a minimal concent-

Fig. 2. Gross anatomical examination of the innervations of the LOM. A: the junction (i.e., vagus “node”) between the left stellate ganglion (LSG) and the left vagus nerve (LVN). The left subclavian artery (LSA) and the left carotid artery (LCA) are labeled for reference. B: a branch of the LVN enters the LOM along the LSPV. The vagus branch descends adjacent to the LAA and then continues along the CS. C: detailed view of the entrance of the vagus branch into the LOM. D: the LOM is dissected away from the posterior surface of the left atrium. An instrument is inserted into the CS, allowing the junction between the vein of Marshall (VOM) and the CS to be observed. A branch of the vagus nerve is seen to continue across the CS to the posterior surface of the LV. RA, right atrium.

Fig. 3. The posterior surface of the left atrium stained for acetylcholinesterase. A: the cholinergic fibers (CFs) of the vagus nerve are observed to enter the LOM along the LSPV. A small nerve branch (N) originating in the LOM is seen to innervate the LSPV. B: white CFs originating in the LOM are seen to innervate the LSPV and the LIPV. C: CFs from the PFP can be seen to innervate the LIPV. D: a small number of cholinergic nerve fibers from the PFP can be seen to innervate the RIPV.
tration of adrenergic nerve fibers (Fig. 4A). Conversely, ChAT and DBH staining of the stellate ganglia demonstrated a high concentration of adrenergic nerve fibers with a minimal concentration of cholinergic nerve fibers (Fig. 4B). Sections of the left vagus nerve taken below the left vagus node exhibited a greater concentration of cholinergic fibers than adrenergic fibers, with a ratio of $57 \pm 6.7$ cholinergic fibers per adrenergic fiber (Fig. 4C).

ChAT staining of sections taken from the LOM revealed that the LOM contains large nerve bundles that are primarily parasympathetic in nature (Figs. 5 and 6). DBH staining of these sections revealed that sympathetic nerve fibers also exist within these nerve bundles. The cholinergic nerve fibers significantly outnumber the adrenergic nerve fibers, with a ratio of $12.6 \pm 3.9$ cholinergic fibers per adrenergic fiber ($P < 0.05$). A consistent distribution was observed throughout the length of the LOM. Muscle bundles and the vein of Marshall were also clearly identifiable in the LOM.

**Electrophysiological data.** ERPs were measured at baseline with propranolol, as well as in the presence of VS in the left-sided PVs, LAA, proximal and distal CS, PFP, and the LOM, for a total of seven sites measured for each maneuver in each experiment. Overall, VS produced ERP shortening at all sites (Fig. 7A), with a mean decrease of 17% ($P < 0.05$).

After the first LOM ablation, the vagal-induced ERP shortening was less pronounced, with an overall shortening of 7% ($P < 0.05$). After the second LOM ablation, the magnitude of vagal-induced ERP shortening decreased further to 4% ($P = 0.01$). Vagal-induced ERP shortening was significantly reduced after the two ablations compared with the basal state with $\beta$-blockade ($P < 0.05$).

Figure 7B shows the changes in ERP for each of the investigated sites (with and without LOM ablation). Vagal-induced ERP shortening was most significantly attenuated in the PVs ($P < 0.05$) and the LAA ($P < 0.05$). Although similar trends were observed at the other sites, they did not reach statistical significance.

**DISCUSSION**

The major new finding of the present study is that the LOM contains predominantly parasympathetic nerves and that these nerves originate from the left vagus, travel through the LOM, and innervate a number of structures in the posterior left atrium. Notably, ablation of the LOM severely attenuated vagal effects on refractoriness in the posterior left atrium. Although sympathetic nerves were found within the LOM, as previously described, these nerves were much less abundant than the parasympathetic nerves.

The immunohistochemical stains used in the present study have been previously utilized by a number of investigators. The ratio of sympathetic and parasympathetic nerves found in the vagus and in the stellate ganglion confirm the accuracy of the identification of the type of nerve structures using the techniques employed in the present study. In addition, two separate stains (for sympathetic as well as parasympathetic nerves) yielded similar results. Thus the finding that parasympathetic nerves predominate by a ratio of more than 10 to 1 in the LOM is not an artifact of the technique used. While the whole heart ACE staining technique has not been previously used extensively in the atrium, histological and anatomic correlations confirm its identification of parasympathetic nerves. The electrophysiological studies performed as part of this study further confirm the parasympathetic origin of these fibers.
The PVs are the region of the heart that are most frequently associated with the initiation of atrial fibrillation. Haissaguerre et al. (6) initially found that 94% of ectopic foci responsible for the initiation of paroxysmal atrial fibrillation were located within the PVs. However, other studies have shown a higher incidence of arrhythmias of extrapulmonary origin, especially in patients with chronic atrial fibrillation or in those with vagally mediated atrial fibrillation (12, 17). In addition, treat-
ment of atrial fibrillation by radio-frequency isolation of the PVs has not been consistently successful (2, 4–6, 15, 26). These results indicate a possible role for extrapulmonary structures in the genesis of atrial fibrillation. Prior studies have demonstrated that, in some patients, the LOM may initiate atrial fibrillation (3, 7, 27). However, these studies have focused on the electrophysiological origin of atrial premature beats in the muscle bundles of the LOM, rather than the effects of innervation of the LOM on cardiac electrophysiology. Recently, Pappone et al. (19) have suggested that the elimination of vagal reflexes during ablation of atrial fibrillation enhances the long-term benefit of the procedure. Although the sites at which vagal reflexes were found did not exhibit entirely consistent anatomical localization, some may have originated from the region of the LOM.

Our dissection demonstrated that a branch from the vagus nerve is the only major innervation of the LOM. This vagus branch is seen to run the entire length of the LOM. Thus we would expect the composition of nerve bundles observed within the LOM to reflect that of the vagus nerve. The results of histological studies are consistent with this hypothesis. Staining of sections taken at several locations throughout the LOM reveals that the LOM contains predominantly parasympathetic fibers. This result contrasts with prior studies, which have shown only that sympathetic nerve fibers are present in the LOM and have speculated that the LOM may be a source for adrenergic atrial fibrillation (10).

Although sympathetic fibers were found in the present study, they were only one-tenth as common as parasympathetic fibers. In addition, cholinergic fibers originating in the LOM were found to innervate the left PVs, the CS, and the PFP. A small number of fibers were also seen to innervate the right inferior PV. Other studies have implicated all of these structures as potential sources of atrial fibrillation (9, 11, 12, 22). Recent studies have shown that wide area circumferential ablation that involves the LOM may be more effective at treating vagally mediated atrial fibrillation, suggesting that the vagal inputs of the LOM may be important in the pathogenesis of this arrhythmia (18). We postulate that these observed arrhythmogenic effects are modulated by parasympathetic branches that originate in the left vagus and pass through the LOM. The electrophysiological data presented in this study further support an important role for the LOM in mediating vagal effects in the left atrium. In addition, a recent study in human autopsies revealed the presence of parasympathetic ganglia located at the junction between the LOM and CS (13). However, in that study, the authors also found a predominance of sympathetic fibers near the LOM. The lack of a specific parasympathetic immunohistochemical marker in that work and different tissue preparations might explain this disparity.

Heterogeneity of vagal responses in the left atrium. In our study, LOM ablation caused an unequivocal attenuation of vagal-induced ERP shortening in the left atrium, thereby verifying the presence and the prominence of parasympathetic fibers in the LOM. Since the effect of ablation was more pronounced in the left PVs and in the LAA than in the rest of the left atrial sites that were tested, the LOM appears to contribute more significantly to parasympathetic effects of the lateral left atrium than to other left atrial structures, such as the CS and posterior left atrium. Acetylcholine release from parasympathetic nerve endings activates muscarinic receptors on the atrial myocytes, which, in turn, activate the G protein-gated potassium inward current ($I_{K,ACH}$), producing a shortening in refractoriness; as a result, heterogeneity at one or more levels of this cascade, i.e., parasympathetic nerve density, muscarinic receptor distribution, G protein concentration, or $I_{K,ACH}$ expression, may account for the preferential effects of LOM ablation on the left-sided PVs and LAA. Each of these possible explanations needs to be further explored to fully understand the contribution of the LOM to the autonomic physiology of the left atrium and PVs.

Physiological and clinical implications. Alterations in autonomic activity in the atria have been suggested as a cause for atrial fibrillation. Although a substantial body of work has
examined the intrinsic nervous system of the atria and defined some of the pathways involved in autonomic innervation, the precise localization of the physiology of sympathetic and parasympathetic trafficking in the left atrium has not been fully elucidated. The findings of the present study have both physiological and clinical implications. They suggest that the LOM has physiological importance as a conduit for parasympathetic activity in substantial regions of the heart. This is unlikely to be a nonspecific effect, since the attenuation of vagal-induced ERP shortening was greater in the PVs and LAA than in other areas. There are also clinical implications to these findings. Since activity within the PVs may be important to the genesis of atrial fibrillation and since ablation of the LOM may lead to an attenuation of vagal-induced ERP shortening, the results support a role of LOM ablation in the cure of atrial fibrillation.

Study limitations. This study was performed in canine hearts; there may, therefore, be inherent limitations in the applicability of these findings to humans. However, the fresh staining techniques utilized in the present study to identify epicardial innervation would not have been possible in autopsy specimens.

Our study did not assess the relationship between the LOM and the branches of the sympathetic trunk that supply the posterior surface of the heart (1, 20). One large nerve that supplies the posterolateral surface of the heart is the ventrolateral cardiac nerve; this nerve arises from the caudal cervical sympathetic ganglion and is mostly composed of sympathetic fibers (1, 20). The LOM, on the other hand, as shown in the present study, is innervated by a branch of the vagus nerve and is composed primarily of parasympathetic fibers. Further studies are necessary to understand the relative roles of these two nerves (as well as the interactions between them) in the creation of substrate for atrial fibrillation.

GRANTS
This study was supported by The Fannie Penikoff and Everett O’Connor Trusts and National Heart, Lung, and Blood Institute Grant 5K08HL074192. R. Villuendas is supported by a grant from the Spanish Society of Cardiology.

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