Neuromatous Regeneration as a Nerve Response After Catheter-Based Renal Denervation Therapy in a Large Animal Model Immunohistochemical Study

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Background—Renal denervation (RDN) emerged as a therapeutic option for resistant hypertension. Nerve regrowth after RDN has been questioned. We aimed to characterize the nerve response after RDN.

Methods and Results—Swine underwent bilateral RDN and were followed up for 7, 30, and 90 days and evaluated with S100 (Schwann cell), tyrosine hydroxylase (TH; efferent nerves), and growth-associated protein 43 (neurite regeneration) markers. At 7 days, nerve changes consisted of necrosis associated with perineurial fibrosis and distal atrophy with inflammation. At 30 days changes were substituted by healing changes (ie, fibrosis). This response progressed through 90 days resulting in prominent neuroma formation. Immunohistochemistry at 7 days: TH staining was strongly decreased in treated nerves. Early regenerative attempts were observed with strongly TH and growth-associated protein 43 positive and weak S100 disorganized nerve sprouts within the thickened perineurium. Distal atrophic nerves show weak staining for all 3 markers. At 30 days, affected nerves show a weak TH and S100 staining. Evident growth-associated protein 43+ disorganized neuromatous tangles in the thickened perineurium of severed nerves were observed. At 90 days, some TH expression was observed together with prominent S100+ and growth-associated protein 43+ neuromatous tangles with disorganized architecture. The potential for regenerative activity is unlikely based on the disrupted architecture of these neuromatous tangles at the radiofrequency lesion sites.

Conclusions—This study is the first documentation that a progressive regenerative response occurs as early as 7 days after RDN, resulting in a poorly organized neuromatous regeneration. This finding is of paramount importance to further establish the potential functional significance of a regeneration after RDN. (Circ Cardiovasc Interv. 2015;8:e002293. DOI: 10.1161/CIRCINTERVENTIONS.114.002293.)

Key Words: models, animal ■ nerve regeneration ■ renal denervation

Thirteen percent of global mortality has been associated with arterial hypertension. Approximately 34% of the total adult population worldwide is hypertensive, and 13% of this segment of the population is further categorized as having resistant hypertension (RHTN).\(^1\) Criteria for the diagnosis of RHTN are the following: any patient requiring \(\geq 3\) antihypertensive drugs, including a diuretic, and still maintaining a blood pressure (BP) >140/90 mm Hg.\(^2\) RHTN has been previously described as a multifactorial phenomenon involving multiple biological mechanisms; however, the hyperactivity of the sympathetic nervous system plays a paramount role in the onset, maintenance, and progression of RHTN.\(^3\) The renal sympathetic nervous system, composed of afferent and efferent nerves, courses immediately adjacent to the wall of the renal artery.\(^4\) The afferent renal sensory nerves, with neuronal cell bodies located in the ipsilateral dorsal root ganglia, modulate the central sympathetic outflow by providing sensory information from mechanoreceptors and chemoreceptors in the renal tissue. Renal injuries (ie, hypoxia) increase afferent sensory signals, resulting in an increase in efferent sympathetic nerve activity, peripheral arterial vasoconstriction, and subsequent increase in arterial BP. The efferent renal sympathetic nerves transmit signals from the central sympathetic nervous system to the kidneys (ie, renal vasculature, tubules, and juxtaglomerular apparatus). Efferent renal sympathetic activity is moderated by an inhibitory renorenal reflex and central sympathetic nervous system outflow. Elevated efferent renal sympathetic activity increases sodium reabsorption and renin release and causes renal arterial vasoconstriction, leading to hypertension.\(^4\)

Catheter-based ablation of afferent and efferent sympathetic nerves surrounding the renal arteries has been proposed...
WHAT IS KNOWN

- Renal denervation (RDN) emerged as a therapeutic option for resistant hypertension.
- RDN has demonstrated safety in clinical trials.
- Nerve regrowth after RDN has been questioned.
- The porcine model is the preferred large preclinical model to evaluate RDN technologies.

WHAT THE STUDY ADDS

- A progressive regenerative response occurs as early as 7 days after RDN.
- The resulting regenerative response is a poorly organized neuromatous regeneration.
- Growth-associated protein 43 expression associated with neurite regeneration indicates progressive nerve sprout growth from 7 to 90 days after RDN.
- Distal nerve atrophy is the only histological evidence of effective ablation.

Methods

The care and use of animals for this preclinical study were reviewed and approved by the contract research organization’s Institutional Animal Care and Use Committee (IACUC; T3 Laboratories, Translational Testing and Training Laboratories, Inc, Atlanta, GA) before the start of the study. Treatment of the animals was in accordance with relevant site procedures, which use the regulations outlined in the US Department of Agriculture (USDA) Animal Welfare Act (9 CFR, Parts 1, 2, and 3) and the conditions specified in The Guide for Care and Use of Laboratory Animals, Eighth Edition (ILAR publication, 2010, National Academy Press) as a guide.

Catheter-Based RDN Device Description

The Iberis Renal Denervation System (CE Marked) is a highly flexible and 6F guide catheter compatible device with unique single point of contact energy delivery capabilities. Iberis is designed to deliver low-level radiofrequency energy through the wall of the renal artery for RDN. The system consists of 2 major components: a sterile single use ablation catheter and a proprietary generator, which delivers radiofrequency energy sufficient for ablating sympathetic nerves adjacent to the treatment site.

Animal Model

A total of 18 juvenile female domestic Yorkshire swine were included in this study and were maintained on a standard chow diet. Animals were fasted at least 12 hours before the interventional procedure. On the day of treatment, 4 mg/kg of Telazol in combination with 0.5 mg/kg IM of Xylazine was given before the procedure. All animals underwent endotracheal intubation and were maintained with a continuous inhalation of 5% isoflurane in oxygen. Buprenorphine 0.005 to 0.01 mg/kg and atropine 0.04 mg/kg IM was given for preoperative analgesia. Femoral arterial access was obtained under general anesthesia using a percutaneous Seldinger technique. All animals received 325 mg of aspirin single dose daily for 1 day before the initial procedure. This same dose was administered on the day of the procedure and daily until the completion of the study. Anticoagulation with heparin was achieved during the procedure (75 U/kg IV) to maintain an activated clotting time ≥250 seconds. After baseline angiography of the renal arteries, radiofrequency treatment was applied within the main stem of the renal artery at 4 to 6 evenly distributed sites, from the first renal artery bifurcation to the renal artery ostium. Radiofrequency ablation was applied for 120 seconds at each

fibrous connective tissue, and thickening of the epineurium and perineurium at 6 months postradiofrequency therapy. Renal arteries displayed medial and adventitial fibrosis with neither smooth muscle hyperplasia nor inflammatory infiltration. Despite the extensive preclinical work performed to evaluate the safety of renal nerve radiofrequency ablation, the morphology of the short- or long-term responses of nerves to the application of radiofrequency ablation therapy is not well characterized. Mazor et al observed an increase in the average number of total nerves per plane examined in treated arterial segments at 180 days after renal radiofrequency ablation, suggesting potential plasticity of nerve growth as a compensatory effort for target organ reinnervation. These data suggested the possibility of a regenerative process, but did not characterize the time course or histological details of the response through special stains or immunohistochemistry. In this study, our goal was to characterize the acute, subacute, and chronic nerve response after renal radiofrequency therapy from a catheter system. Evaluations were performed in a porcine model using both standard histology and immunohistochemistry techniques.

In the preclinical setting, numerous large-scale studies have been performed to determine the safety of delivering radiofrequency therapy to the renal nerves. Steigerwald et al described the following changes at 45 minutes postradiofrequency therapy: loss of endothelium in the renal artery, coagulative necrosis of the adventitial connective tissue, and edematous response. These findings resulted in fibrous scarring in the tunica media and both vasculogenesis and inflammatory infiltrates in the tunica adventitia. This pattern was maintained ≤6 months. Rippy et al demonstrated that injury to renal nerves involved nerve fibrosis, replacement of nerve fascicles with

as a therapeutic alternative for patients with RHTN. To this day, multiple procedures for renal nerve ablation have been studied; however, transcatheter renal radiofrequency ablation of the afferent and the efferent nerves remains the most commonly investigated technique in preclinical and human studies. Early clinical trials suggested a significant and sustained reduction in systolic BP in patients with RHTN, based on in-office BP measurement with an attenuated difference when ambulatory BP monitoring was considered. Recently, the field of renal denervation (RDN) experienced a setback when the SYMPLICITY HTN-3 trial was a regulated clinical study, designed as a US-based multicenter, prospective, randomized, rigorously blinded trial evaluating safety and efficacy of renal. SYMPLICITY HTN-3 reached its safety end of renal denervation in Patients With Uncontrolled Hypertension [Symplicity HTN-1] and Renal Denervation in Patients With Uncontrolled Hypertension [Symplicity HTN-2], but failed to show a statistical difference in the reduction of office systolic BP and ambulatory BP in patients with RHTN and 12 months after renal-artery denervation as compared with a sham control. Further investigations of the therapy, the delivery techniques, and research into new devices and approaches are ongoing.

In this study, animal models were fasted at least 12 hours before the interventional procedure. On the day of treatment, 4 mg/kg of Telazol in combination with 0.5 mg/kg IM of Xylazine was given before the procedure. All animals underwent endotracheal intubation and were maintained with a continuous inhalation of 5% isoflurane in oxygen. Buprenorphine 0.005 to 0.01 mg/kg and atropine 0.04 mg/kg IM was given for preoperative analgesia. Femoral arterial access was obtained under general anesthesia using a percutaneous Seldinger technique. All animals received 325 mg of aspirin single dose daily for 1 day before the initial procedure. This same dose was administered on the day of the procedure and daily until the completion of the study. Anticoagulation with heparin was achieved during the procedure (75 U/kg IV) to maintain an activated clotting time ≥250 seconds. After baseline angiography of the renal arteries, radiofrequency treatment was applied within the main stem of the renal artery at 4 to 6 evenly distributed sites, from the first renal artery bifurcation to the renal artery ostium. Radiofrequency ablation was applied for 120 seconds at each
ablation point. After final angiography, isoflurane was discontinued and the animal was exsanguinated when the gas reflex returned. Rimadyl 2 to 4 mg/kg IM is given as immediate postop analgesic and once on the day after procedure. Animals received cefazolin as prophylaxis (1 g IM). At the terminal time point (7, 30, and 90 days) animals were heparinized and renal angiography was performed before euthanasia.

Renal Ablation Therapy Delivery
The radiofrequency ablation therapy was performed according to common protocols of catheter-based RDN. In brief, there were no renal arteries excluded based on anatomy (diameter, <4.0 or >7.0 mm; length, <20 mm or amount of taper across target length greater than =30%). The test catheter was advanced into the right or left renal artery and a standard treatment at the target location was performed using the following ablation settings: power, 8 W; duration, 120 seconds. Once the ablation was completed, the catheter was repositioned to locate the next ablation site =5 mm proximal to the previous ablation site. Ablation was repeated for up to 6 ablations per artery. If anatomy did not allow 6 ablations, the number of ablations was dictated by the available length of the renal artery.

Tissue Harvesting
For the extraction of the area of interest, the abdominal cavity was accessed and the intestines removed to expose the retroperitoneum. The abdominal aorta was cannulated and flushed with physiologic saline solution to clear all blood. When the vessels were clear of blood the abdominal aorta cannula was flushed with 10% neutral buffered formalin solution. The renal arteries were pressure perfused at a height that provided 100 to 120 mmHg for ≈10 minutes (=1 L of 10% neutral buffered formalin). The treatment area and surrounding tissues were extracted en-block to include the following tissues: abdominal aorta; renal arteries; both kidneys and surrounding tissue (retroperitoneal connective tissue, renal capsule, perirenal fascia, and peritoneum), underlying posterior psoas and sublumbar muscles, adrenal glands, and ureters (Figure 1). Tissue explants were immersed in 10% neutral buffered formalin while supported by a solid cord backing to ensure stability and structural integrity of the tissue block.

Tissue Preparation for Histological Analysis
After no <24 hours of 10% neutral buffered formalin fixation, the aorta was bisected to expose the renal ostia. The renal artery and ≈1.5- to 2-cm radius of the surrounding retroperitoneal connective tissues and fasciae were isolated by removing excess tissue to create an ≈3-cm-wide renal stump centered on the renal artery (Figure 1). Equidistant sections were taken from the ostium to the hilus of the renal stump, resulting in 5 cross-sections along the length of the treated renal artery that included associated soft tissues (lymph nodes, soft connective tissue, veins, and nerves) and underlying skeletal muscle. The ureters and adrenal glands were also included when present within the block of tissue presented for sectioning. The resulting tissue slices were dehydrated in ascending concentrations of alcohol and embedded in paraffin. Serial 5-μm sections were stained with hematoxylin and eosin (H&E) and elastin trichrome.

Immunohistochemistry
For each time period (7, 30, and 90 days), 2 representative paraffin tissue blocks were selected based on the presence of direct radiofrequency changes in the sympathetic nerves and stained for expression of S100 for Schwann cells (SCs), tyrosine hydroxylase (TH) for efferent nerve fibers or growth-associated protein 43 (GAP43) to specifically label the neuronal sprouting. GAP-43 has been associated with axonal growth and its overexpression correlates with the spontaneous formation of new synapses and enhanced sprouting after nerve injury. Slides were pretreated using heat-activated antigen retrieval in Diva Decloaker (Biocare Medical, Concord, CA). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in distilled water for 5 minutes. After a protein block using Background Sniper (Biocare Medical), slides were incubated with rabbit polyclonal anti-S100 (Abcam, Cambridge, MA), rabbit polyclonal anti-TH antibody (Millipore, Billerica, MA), or rabbit polyclonal anti-GAP43 (Novus Biologicals, Littleton, CO) for 60 minutes at room temperature at a dilution of 1:1200 (S100) or 1:300 (TH and GAP43), respectively. Slides were then incubated with a biotinylated antirabbit secondary antibody for 30 minutes at room temperature. Detection was performed using VECTASTAIN Elite ABC (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, followed by incubation with Sigma Fast DAB (Sigma, St. Louis, MO). Slides were
counterstained with hematoxylin. The resulting slides were examined via light microscopy and were described based on the presence of positive nerve fibers or cells in normal and affected nerves.

Results

Seven Days
Under routine stains (H&E and elastin trichrome stain), the changes in the targeted renal nerve bundles were primarily and most conspicuously characterized during the acute phase (7 days) by hyalineization of collagen (thermal denaturation) and coagulative necrosis of the nerves (endoneurium and perineurium). Initially, there was swelling of nerve fibers (spheroids) with varying degrees of mononuclear inflammatory cell response and calcification of necrotic endoneurium. The endoneurium became disorganized, showing varying proportions of swollen axons, mononuclear cells, mineralized debris, and thin bundles of fibroblastic mesenchymal cells (early endoneurial fibrosis). A perineurial response was visible at day 7, in the form of a reactive, slightly thickened, and hypercellular perineurial sheath. Immunohistochemical staining at this time point revealed uneven TH staining that was in general strongly decreased in nerves directly affected by radiofrequency treatment, with the exception of strong TH staining of the dilated and degenerate axons (spheroids).

Most importantly, the thickened perineurium contained disorganized nerve sprouts that were strongly TH positive. These perineurial nerve sprouts were also strongly positive for GAP43 and weakly positive for S100 staining. These changes were consistent with early regenerative activity within the fibrous tissue response at the sites of direct radiofrequency nerve damage. Atrophic nerves present distally in these 7-day sections showed sparse and weak staining for all 3 markers further documenting the endoneurial depletion occurring distal to radiofrequency nerve lesions (Figure 2B).

Thirty Days
The acute phase response observed at 7 days was followed at 30 days by a gradual healing response. The amounts of fibrous connective tissue around and within the targeted nerves gradually increased over time. At 30 days, H&E staining of the renal artery showed segmental residual hyalinization in the media (an area of unremodeled coagulative necrosis), surrounded by multifocal areas of adipose tissue necrosis. Nerve bundles with a thick layer of maturing fibrous connective tissue expanding the perineurium were present. There were small clusters of vacuolated cells detected by H&E in the perineurial reaction (consistent with regenerative SCs based on immunohistochemistry staining).

Immunohistochemical staining of the renal artery site at this time point demonstrated weak to absent TH staining and S100 staining in necrotic nerves, except for fibers and regenerative sprouts in the thickened perineurium of nerves directly within the radiofrequency lesion. However, all nerves, except distal atrophic nerves, showed strong GAP43 staining. GAP43 staining was particularly prominent in perineurial sprouts and perineural neuromatous tangles within the fibrous perineurial response. These findings are consistent with a neuromatous regenerative response (Figure 2C).

Ninety Days
By 90 days, H&E staining revealed a uniform arterial media with slight fibrosis, slight periarterial fibrosis, and no evidence of residual necrosis. Targeted nerve bundles were surrounded by a thick layer of fibrous connective tissue that expanded the perineurium to ≈200 to 500 μm. The radiofrequency-damaged nerves resembled a fibroneuroid scar and were characterized by large amounts of fibrous connective tissue encapsulating a largely sclerotic residual endoneurium, surrounded by numerous small neuroid tangles within the fibrous perineurium. The morphology of these fibroneuroid scars was reminiscent of amputation or traumatic neuromas and was referred to as fibroneuromatous regenerative response.

At 90 days after renal radiofrequency nerve ablation therapy, there was some degree of recovery of TH expression on immunohistochemically stained section in the periarterial nerves when compared with earlier time points. However, staining remained uneven and somewhat weaker than in the untreated controls. There were also prominent neuromatous tangles that demonstrated strong S100 staining (S100) and regenerative activity (GAP43), with highly disorganized architecture (analogous to amputation neuromas).

Immunohistochemistry staining for S100 protein, TH, and GAP43 at 90 days after renal radiofrequency nerve ablation showed some nerves with normal morphology and nearby atrophic nerves with a marked decrease in TH staining and a lesser decrease in S100 staining. It is speculated that the atrophic nerves were affected by the radiofrequency therapy at the initial time of treatment (0 days) at a more proximal location along the artery, whereas the normal nerve was not likely affected.

A critical and newly described finding in renal nerves after radiofrequency ablation therapy was the observation of atrophic nerves distal to the therapy site. This change presented on H&E staining as reduced nerve size together with endoneurial hypercellularity (increased nuclear density) with or without endoneurial edema, prominent endoneurial vasculature, and low grades of eosinophilic granulocyte infiltrate within the endoneurium and perineurium. Distal nerve atrophy was detectable as early as 7 days post ablation and generally still conspicuous at 30 days. Interestingly by 90 days, atrophy was often less conspicuous as distal nerves seem to be populated by plumper SCs (Figure 2D) with uneven staining for efferent nerve fibers (TH).

Study Limitations
The present study is the first to describe in detail the nerve response after radiofrequency ablation as early as 7 days post therapy and its evolution over time. Although the histopathologic presentation suggests the development of a neuromatous lesion, the functionality of these regenerative structures is beyond the scope of this study and in need of assessment. Furthermore, this study was followed up ≤90 days. Our laboratory has observed that this response progresses ≤180 days retaining the fibroneuromatous appearance suggestive of abortive regeneration after ablation (data not presented). Longer time points including evaluation of functionality such as tissue norepinephrine concentration or tracing metaiodobenzyl-guanidine may allow some insights in the degree of nerve disconnection or potential for functional restoration.
Discussion

A variety of catheter-based treatment modalities (ie, ultrasound, cryoablation, localized neurotoxin delivery, and radiation) are being investigated to ablate renal sympathetic nerves.21 This preclinical study specifically focused on intraluminally delivered catheter-based radiofrequency ablation technology.

Normal Renal Innervation

The kidney is densely innervated with both efferent adrenergic and somatic afferent neurons. Efferent renal sympathetic nerve activity is a product of the integration of several sensory inputs arising from the kidney. The innervated effector units of the kidney, including the tubules, the vasculature, and the renin-containing juxtaglomerular apparatus, regulate the homeostasis body fluid volume (Figure 2A).

Sympathetic (efferent) nerves innervate both vascular and tubular structures throughout the kidney, except in the inner medulla.22 The entire renal vasculature is innervated, with the greatest density seen along the afferent glomerular arterioles. Sympathetic nerve fibers have also been found in the wall of the renal pelvis, although in much less abundance than the sensory nerves.23 Importantly the sympathetic nerve fibers in the pelvic wall were in close association with the sensory nerves. Likewise, sympathetic and sensory nerve fibers have been identified intermixed within the renal nerves coursing along the renal artery.21 The majority of afferent sensory fibers, containing the neuropeptides substance P and calcitonin

Figure 2. A. Untreated normal renal nerve (clear arrows in Aa, hematoxylin–eosin [H&E] and Ab, elastin trichrome [ET]) showing intact ganglion cells (darker blue areas at the top and along the edge of the larger nerve). Immunohistochemistry (IHC) shows S100-positive Schwann cells in the endoneurium (Ac, clear arrows) and strong tyrosine hydroxylase (TH) staining in normal nerves (Ad). Untreated nerves show weak expression of GAP43 (Ae); B) 7-day follow-up. The dotted line (Ba, H&E) shows the extent of the renal denervation treatment area (Bb, ET) and necrotic nerve with thickened reactive perineurium (Ba, clear arrow). There is evidence of localized media hyalinization of the arterial wall (Ba and Bb). S100 showed weak staining in the endoneurium (Bc, clear arrow inside dotted line) and sparse S100-positive cells (Bc, solid arrowhead) in the thickened perineurium. TH demonstrated heterogeneous staining with moderate to strong positive staining in the endoneurium (Bd, clear arrow, endoneurium-dotted line) contrary to strong staining of growth-associated protein 43 (GAP43; Be, clear arrow) with disorganized tangles of TH and GAP43-positive fibers (Bd and Be, solid arrowheads) and TH and GAP43-positive sprouts emerging through in the thickened fibrous perineurium (Bd and Be, solid arrow). C, Thirty-day follow-up. Postradiofrequency treatment. Nerves (Ca, clear arrows) show endoneurial and perineurial fibrosis (Ca, H&E and Cb, ET). S100 IHC shows strong staining in the endoneurium (Cc, clear arrows) and moderately sparse S100-positive cells in the thickened perineurium (Cc, solid arrowheads). A significant decrease of TH (Cd, clear arrows) and increased GAP43 (Ce, clear arrows) staining was observed in the endoneurium of the treated nerve (dotted line). TH-positive fibers in the thickened perineurium are rare and show weak staining (Cd, solid arrowhead). However, GAP43 showed an increased of disorganized nerve tangles in the thickened perineurium (Cd and Ce, solid arrow) and increased number of sprouting nerves emerging from the endoneurium (Ce, solid arrowheads) and around an arteriole (Ce, clear arrowheads). D, Ninety-day follow-up. Increased endoneurial and perineurial fibrosis (Da, clear arrow and arrowheads) with a prominent perineurial neuromatous regenerative response (Da, clear arrowheads and Db, ET). S100 show a strong labeling (Dc, clear arrows) in the endoneurium (Dc, dotted line). There is a clear and strong TH (Dd, clear arrows) and GAP43 (De, clear arrows) signal in the endoneurium accompanied by extensive disorganized tangles of TH (Dd, solid arrowheads) and GAP43 (De, solid arrowheads) positive fibers in the thickened perineurium. A indicates renal artery.
Figure 3. A. Schematic representation of renal denervation therapy. The radiofrequency (RF) treatment is delivered along the trajectory of periarterial nerves. The region of the nerve proximal to RF ablation remains normal (top right) and the region of the nerve that receives the RF ablation shows complete coagulation necrosis (top center). The distal region of the nerve shows evidence of atrophy, which provides clear evidence of effective denervation (top left). Via immunohistochemistry, the nerve response demonstrates consistently over time evidence of a failed attempt at regeneration in the RF-exposed area. There is an increase nerve sprouting out of the perineurium forming disorganized tangles of nerve fibers. This response has been found to be progressive with slight but gradual increase in size and in the number of nerve tangles with an increase in fibrous tissue. The neuromatous response starts as early as 7 days after treatment (bottom left) and continues at 30 days (bottom middle) and progresses ≤90 days (bottom right). Reproduced with permission from Alizee Pathology. B. Delivery of radiofrequency ablation showed to be safe to the vascular wall. At 7 days showed medial necrosis (hyalinization) in exposed area (dotted line) that eventually evolves at 90 days to a fibrous healing (left). Different degrees of distal compromise can be found in the same histological section. In this representative image (tyrosine hydroxylase [TH] stain) there is an intact nerve (short solid arrows) and a nerve that was affected proximally to this section displaying a low TH signal (long solid arrows).
Figure 4. Schematic representation of the neuromatous response to radiofrequency (RF) therapy. **Top**, The nerves are represented in 4 areas along the treated artery (horizontally) before treatment (top) and at 7, 30, and 90 days vertically. **Bottom**, Schematic histology of cross-section and corresponding histological sections at low and high magnification at various levels along the artery and across time periods. The normal nerve before therapy delivery is depicted at left showing the normal distribution of the renal nerve; Schwann cells host multiple axons with no myelin formation. There is virtually no connective tissue between them. Seven days after therapy, the proximal normal nerve segment remains normal. The segment at the proximal edge of the ablation (proximal regeneration nerve segment) shows an increase in fibrous connective tissue separating the Schwann cells and associated nerve fibers. There are early tangles of nerve fibers and nerves sprouting, which extend beyond the perineurium. The ablated nerve segment shows swelling and necrosis. The distal nerve is reduced in size and Schwann cells show reduced number of or are devoid of axons. The neuromatous response in the segment immediately proximal to the nerve segment progresses at 30 and 90 days with increased fibrous tissue and an increase in number, size, and extension of the nerve tangles around the fibrous perineurium. At 90 days, the distal nerve segment recovers its size but evidence of atrophy remains.
gene–related peptide, are found in the renal pelvic wall, in keeping with their mechanosensory functions.24

Atherton et al25 reported in a human cadaveric study that 75% of the nerves surrounding the renal artery remain within a distance of 0.5 to 1.5 mm from the lumen of the renal artery wall.25 In contrast, Sakakura et al26 concluded that despite a considerable variability (<1 to >10 mm), the majority of nerves were ≈4.28 mm from the renal artery lumen, still within reach of endovascular treatment. Tellez et al27 reported that the renal nerves surrounding the renal arteries in porcine models follow a similar distribution to that of humans in regards to location and size, supporting the relevance of the porcine model to safety and efficacy evaluations of catheter-based RDN technologies.4

Peripheral Nerve Regeneration After Nerve Damage

Based on the observations of Waller,27 the distal portion of a damaged nerve undergoes progressive degeneration. It has been described that axonal degeneration and subsequent processes after nerve injury occur in the peripheral nervous system as early as 7 to 14 days after injury.28 In vivo animal models, one of the primary responses after axonal injury is the influx of calcium by a vesicle-mediated process29 as early as 30 minutes after injury leading to a process known as acute axonal degeneration.30 After the acute axonal degeneration, microtubules, neurofilaments, and other cytoskeletal components disassemble in a stage described as granular disintegration of axonal cytoskeleton.31 This granular disintegration may occur proximally (retrograde) or distally (anterograde) along the axon, depending on the type of nerve injury,32 over variable periods of time dependent on the species, the distance from neuronal cell body, and the diameter of the axon (axons of smaller caliber such as in the autonomic system degenerating faster than larger caliber axons).33 In humans, the time between nerve injury and detectable axon degeneration is several days.34 SCs respond to axonal injury by phagocytizing and presenting debris to macrophages35,36 and by rapid proliferation7,38 as early as 3 days after injury.37 At the same time, SC and macrophages are stimulated by cytokines (macrophage-derived interleukin 1) to produce nerve growth factor.39 In healthy nerves, nerve growth factor is produced in small amounts. However, between 3 and 14 days after nerve injury, a 5- to 7-fold increase in nerve growth factor mRNA expression has been demonstrated in murine models.40 At the distal end, a wide variety of neurotrophic molecules are produced (ie, nerve growth factor, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, ciliary neurotrophic factor, leukemia inhibitory factor, insulin-like growth factor, fibroblast growth factor),31 promoting SC proliferation and axonal regeneration. In addition to growth factors, SC also provide a scaffold for structural guidance of axons in their attempt for regrowth.41 Peripheral motor nerves are myelinated and their degeneration is accompanied by disintegration of the myelin sheath with phagocytosis of cellular debris (digestion chambers); after clean-up a neurolemma or neural tube remains and guides regrowth of the new sprouting axon.42 Peripheral sympathetic nerves are comprised in their postganglionic tract of nonmyelinating SCs, each SC hosting and supporting a cohort of individual nerve fibers in membrane-bound groves or invaginations of its cytoplasm. On degeneration, the axons swell (spheroids), subsequently disintegrate and cellular debris are processed by the hosting SC. As such digestion chambers typical of the Wallerian degeneration of myelinated nerves does not occur in postganglionic sympathetic nerves and there are no neural tubes left to guide new axons on regeneration.

The regeneration of renal nerves after chemical or surgical denervation has been extensively studied in experimental animals,43,44 and there are indications of at least partial reinnervation of the kidneys. However, Hansen et al47 demonstrated that the nerves supplying a transplanted kidney do not achieve functional innervation,47 thus implying that evidence of microanatomical regeneration does not equate functional recovery nor appropriate reconnection of the neural cell, receptors/effectors, and neural fiber networks.48

Histological Renal Nerve Response After Catheter-Based Radiofrequency RDN

Animal models have demonstrated the safety of catheter-based radiofrequency RDN. The acute and chronic findings by Steigerwald et al14 described the renal nerve injury as primarily characterized by nerve fibrosis, replacement of nerve fascicles with fibrous connective tissue, and thickening of the epineurium and perineurium at 6 months after treatment.15 The increase in the total nerves found by Mazor et al16 after 180 days post renal radiofrequency ablation suggests potential plasticity of nerve growth. Arterial healing was characterized by a fibrous response in the tunica media without smooth muscle hyperplasia or inflammatory infiltration.49

In our study, we observed under H&E staining a fibromatous response at 90 days that resembles a neuroma. This response was characterized by a tangle of neural fibers and SC within sclerotic fibrous connective tissue. This lesion was highly reminiscent of the tissue response associated with amputation or trauma-induced neureomas and represents the attempt of an injured or severed nerve to seek the distal nerve segment, failing to complete nerve repair. The neuroma represents an exaggerated healing response to nerve injury, resulting in localized reactive hyperplasia at the point of injury.49 Our immunohistochemical observations demonstrate that nerve fibers are a prominent element of the neuromatous response observed at the site of radiofrequency injury. At the 90-day time point, the original delineation of the nerve is attenuated and is surrounded by the neuromatous reaction. The potential for the regenerative activity to restore function remains unclear but is likely low in consideration of the much disrupted architecture of the neuromatous tangles at the radiofrequency lesion sites. Earlier evaluation at 7 and 30 days demonstrated that this disorganized SC, nerve fiber, and fibrous tissue response has an early onset and is detectable as early as 7 days after renal radiofrequency denervation using appropriate immunohistochemical stains, becoming gradually more prominent over time. S100 and TH staining showed that the nerve regrowth extends beyond the perineurium within a densely fibrous perineurial scar.

Immunohistochemistry also confirmed the observation of nerve atrophy distal to radiofrequency lesions at 7 days, providing a useful confirmatory marker of appropriate nerve ablation. There was early and sustained pronounced decrease in TH and S100 staining

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in nerves that were morphologically atrophic under H&E staining and there was little evidence of regenerative activity (no to weak GAP43 immunostaining). In our experience the extent of distal nerve atrophy is an indicator of treatment effectiveness. Further studies are necessary to assess the progression and sustainability of this effect over the long term (Figures 3 and 4).

Conclusions
Clinical data are currently in debate and early studies suggest that the BP-lowering effect of catheter-based RDN is maintained for ≤3 years.60 However, and in contrast, the latest clinical data suggest that the effect was not statistically different from a sham control group.10,11 In renal transplantation, functional innervation is not established despite apparent attempts for renal reinnervation.47 In our study, we characterized the nerve response after catheter-based RDN in a large animal model. This study demonstrated that treatment-exposed nerves show a general decrease in TH staining at 7 and 30 days, most pronounced in nerves directly in the radiofrequency lesion and more evident at 30 days than at 7 days. At 90 days, there was partial recovery of TH staining in perirenal nerves. There was also evidence of regenerative activity (increased GAP43 staining) as early as 7 days after treatment, most prominently at sites of direct radiofrequency injury and in association with fibrous perineurial thickening where disorganized TH and GAP43-positive fibers formed variably sized sprouts and tangles of nerves becoming analogous overtime to amputation neuromas. The potential for the regenerative activity to restore function remains unclear, but is likely low in consideration of the disrupted architecture of the neuronautic tangles at the radiofrequency lesion sites (neuromatous regeneration). It is also important to note that the sympathetic nerve distribution around the renal artery is not a simple linear structure but rather a network of nerves that fuse and depart from each other in their trajectory around the renal artery. It is potentially possible that nerves seen in a specific cross-section may also fuse with other nerves that not necessarily innervate the renal artery or kidney distally. Our observations also offer the first recognition of the distal nerve atrophy that occurs as a result of successful ablation more proximally, being evident at 7 and 30 days and becoming gradually less evident afterward. Further characterization of the distal nerve healing response and persistence or reversibility of distal nerve atrophy may provide clues on the effectiveness of the nerve healing process and long-term sustainability of denervation treatment effects on RHTN.

Disclosures
A. Sakaoka is a full time employee of Terumo Corporation. The other authors report no conflicts.

References


Neuromatous Regeneration as a Nerve Response After Catheter-Based Renal Denervation Therapy in a Large Animal Model: Immunohistochemical Study

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