Vein to Artery Grafts

An Experimental Study of Reinnervation of the Graft Wall

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Iliolumbar vein to iliac artery grafts were placed in 21 rats by microsurgical techniques. Graft innervation was examined at five time intervals between 1 and 32 weeks after surgery. Nerve fibers were demonstrated microscopically by formaldehyde-induced fluorescence of catecholamines. The morphology and degree of graft innervation were assessed, semiquantitatively, relative to the contralateral iliac artery (control) within each animal. Nerves were seen in the graft region as early as 2 weeks, but it was not until 4 weeks that they were present along its length (5 mm). The formation of a nerve plexus in the adventitia surrounding the graft was evident at 8 weeks. By 16 weeks the degree of innervation in the graft had increased to a level that was greater than the control iliac artery in three of four animals examined. Grafts at 32 weeks were also hyperinnervated. However, the morphology of this innervation was different from the control arteries; nerve fibers were finer, not varicosed, and were located at a greater distance from the outer layer of smooth muscle cells. The origin of the nerves appeared to be collateral sprouts from nerves supplying the adjacent iliac vein and also from invading vasa vasorum. The host iliac artery nerve plexus did not contribute to graft innervation.

THE REPLACEMENT or bypass of sections of diseased arteries with autologous vein segments has become firmly established. This technique of "vein grafting" is used most frequently in the now-common coronary artery bypass operation for the treatment of ischemic heart disease.

It has now been established that the transplanted vein segment becomes "arterialized" by the rapid establishment of an endothelial lining and the development of a smooth muscle layer in the wall.[1] However, this muscle layer is unlike the media of a normal muscular artery because it develops subendothelially in the intimal region; there is no real distinction between the intima and the media. Thus the terms neo-intima, intimal thickening, and intimal hyperplasia have been used to describe the muscle layer of a vein graft.[2] In some cases the arterialized vein develops an excessively thick intimal muscle layer that may ultimately obliterate the lumen and cause graft failure. The histogenesis of smooth muscle in vein grafts and the pathogenesis of excessive intimal hyperplasia are not well understood. There is some evidence that mural ischemia, altered arterial hemodynamics, delayed reendothelialization, and enhanced permeability to macromolecules are factors involved in intimal hyperplasia.[3]

One aspect of vein graft research that has received scant attention is the sympathetic reinnervation of the intimal muscle layer. This question was raised by the authors because there is evidence of a trophic interaction between smooth muscle cells and autonomic nerves. Smooth muscle, and other autonomic effector tissues, have the ability to attract and stimulate autonomic nerves, the action probably being mediated through nerve growth factor (NGF).[4] It has also been shown that the presence of sympathetic nerves among smooth muscle cells *in vitro* inhibits cell division and promotes differentiation.[5] Conversely, sympathetic nerves (*in vivo*) have a proliferative effect on vascular smooth muscle in growing animals.[6]

Although the trophic interaction between smooth muscle cells and sympathetic nerves is not fully understood, it is likely that the relationship between the two may be significant in the development of intimal hyperplasia in vein grafts. Therefore, the current study was designed to examine the time course and development of sympathetic nerve endings in experimental vein grafts. This was to provide baseline data upon which the possible role of sympathetic nerves in intimal hyperplasia might be determined in future studies.

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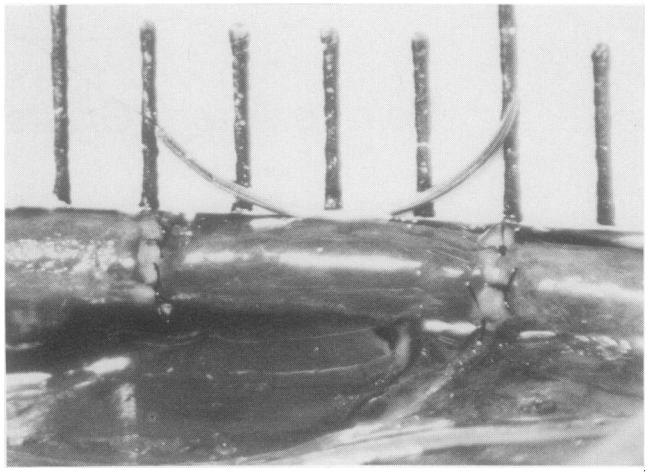

FIG. 1. The vein graft at the time of insertion. The scale is in mm and shows a B.V 3 (130 μm) needle with a 10–0 (22 μm) Ethilon suture.

Materials and Methods

Grafting Procedure

In 21 young adult male Wistar rats, anesthetized with Pentobarbitone (40 mg/kg body weight), 5-mm segments of iliolumbar veins were dissected out and soaked in heparinized saline for approximately 10 minutes. The left common iliac arteries were mobilized and cut between two 1-mm Scovell-Lewis vascular clamps. The divided ends were irrigated with heparinized saline and the vein graft was sutured end-to-end to the artery using 10.0 nylon (Ethilon) microsutures (Fig. 1). The diameter of the autograft was approximately 1 mm and it was ischemic for 60 minutes. Upon removal of the clamps, blood flow was immediate and hemostasis was achieved in 3 to 5 minutes.

Graft Samples

The grafted animals were divided into groups to be killed at different postoperative periods. At 1 and 2 weeks, two and three rats were examined, respectively. At 4, 8, 16, and 32 weeks, four rats for each time period were examined.

At the time of death the animals were anesthetized (as above) and were perfused with a solution that would enhance the demonstration of catecholaminergic nerve endings by fluorescence microscopy.[7] This consisted of a 50-ml solution of 2% glyoxylic acid (monohydrate), 0.5% formaldehyde and 26% $MgSO_4$ in phosphate buffer (pH 7.0). The perfusion was done rapidly (1–5 min) through the left ventricle of the heart. Major arteries to the head and upper limbs were clamped to direct all the perfusate towards the trunk and lower limbs.

The grafts were dissected out carefully in order to maintain the adventitial covering intact. The graft was divided transversely in the center; one-half was used for fluorescence microscopy and the other for routine histology. At the same time, samples of the opposite (ungrafted) iliac artery and the two femoral arteries were removed to act as controls.

Fluorescence Microscopy

Once excised, the samples for fluorescence microscopy were frozen rapidly in liquid Freon 12 (−40 C) and were freeze dried for 2 days at −30 C and 10^{-3} mmHg vacuum. They were then exposed to formaldehyde vapor at 80 C for 1 hour and vacuum embedded in paraffin wax (56 C melting point). This is in accordance with the standard Falk-Hillarp method for the fluorescent demonstration of catecholamines.

Serial cross sections 10 μm thick were cut through the grafts from the center towards the anastomosis. An equivalent length of the control iliac artery was sectioned. Sections at 100 μm intervals were taken for analysis with the fluorescence microscope. Random sections of the femoral artery were taken to check on the fluorescence technique: this artery has a well-established dense plexus of sympathetic nerves. Sections for fluorescence microscopy were examined under uv light with the appropriate filters (excitation: BG 38, BG 12; barrier: K470). The degree of innervation of the graft was assessed relative to that of the corresponding control iliac artery in the same animal. The density of fluorescent profiles was judged as being less than, equal to, or more dense than the control. In all, five categories of innervation were recorded according to established criteria.[8] The five categories were:

0—no innervation
1+—scant innervation
2+—less than control innervation (hypoinnervated)
3+—normal innervation (control artery level)
4+—more than control innervation (hyperinnervated)

Results

The Control Iliac Artery

In the rat this has a lumenal diameter of 1 mm and a wall thickness of 85 μm.[9] It contains alternating layers of elastin and smooth muscle. Fluorescence microscopy revealed a sparse plexus of nerve profiles at the adventitial border close to the adjacent media. No nerves were seen in the media. The fluorescent nerves appeared as bright profiles, 1 to 5 μm in diameter. They were seen in both longitudinal and cross section, the former displaying varicosities (the catecholamine-releasing sites) along their length (Fig. 2a).

The Normal Iliolumbar Vein

The normal donor vein *in situ* had an equivalent diameter to the iliac artery (1 mm) and had a very thin

internal elastic lamina (1 μm thick) with a media consisting of collagen and smooth muscle one or two cells thick. The vein had a nerve plexus of fine, varicosed fibers, about 1μm in diameter. In fact, the density of innervation was greater than that in the control artery. Once grafted, however, the donor vein becomes necrotic and is subsequently replaced by local tissues.

Innervation of the Graft

1 Week. The graft was a thin, fibrous tube with patches of necrotic material unevenly distributed throughout the wall. A discontinuous internal elastic lamina, 1 μm thick, was clearly visible. The surrounding connective tissue contained large numbers of inflammatory cells.

Nerves were absent along one of the grafts while the other revealed two small nerve profiles near the center. These were at a greater distance from the graft lumen than those found in the control iliac artery (Table 1). They were fine nerve fibers (<1 μm diam.), without varicosities and had a fluorescent intensity that was less than in the controls. The area surrounding the anastomosis was completely devoid of nerves but further along the host iliac artery, nerve density gradually increased and by 400 μm it was normal. However, as in the graft, these nerves were not as bright, were finer (<1 μm diam.) and were at a greater distance from the adventitial-medial border than those innervating a normal control iliac artery. No evidence of the normal plexus was seen.

2 Weeks. There were nerves present within the grafted vein. However, these did not extend along the length of the graft. The degree of innervation increased towards the anastomosis and along the host iliac artery (Table 1). The nerves within the graft were similar to those in the host iliac artery at 1 week. They were fine fibers (<1 μm diam.), without varicosities and were located at a greater distance from the outermost smooth muscle cells than nerves within the control iliac artery. The fluorescent intensity of the nerves appeared to be brighter than those seen at Week 1, suggesting an increase in noradrenaline content of the nerves between Weeks 1 and 2. Nerve profiles were seen only in longitudinal section, *i.e.,* arranged circumferentially around the graft. In general, profiles were irregularly distributed around the grafted vein, although one graft (No. 1) revealed a slight predominance of fibers on its inferomedial aspect, near the iliac vein.

Nerve profiles were found at the anastomosis and also along the host iliac artery. These were similar both in appearance and position to those seen throughout the adventitia of the grafts and to those seen at Week 1. Innervation density reached normal proportions in the host iliac artery 400 μm from the anastomosis (Fig. 2b).

4 Weeks. Nerves were present along the length of the four grafted veins examined at 4 weeks. In general, the

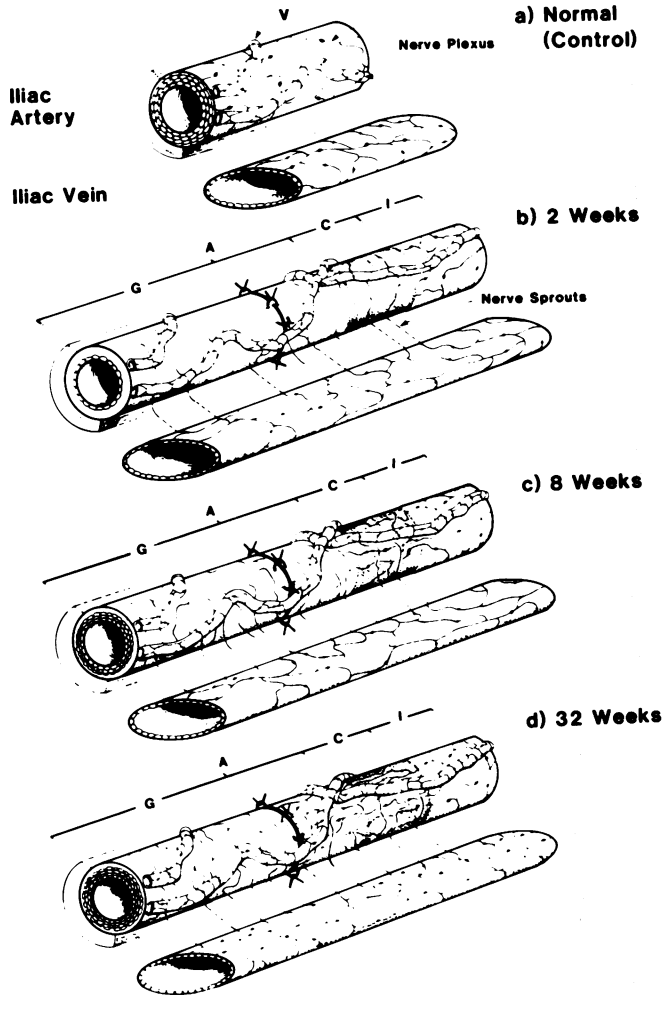

FIG. 2. A diagrammatic representation of vein graft reinnervation. The normal iliac artery and vein (a) is compared to stages of graft innervation at 2, 8, and 32 weeks (b, c, and d, respectively). Hyperplasia of smooth muscle in the graft intima is shown. Vasa vasorum (v) are fully established by 2 weeks[9] and contribute to graft reinnervation (A–anastomosis; G–graft; I–iliac artery; C–clamp region).

degree of innervation was similar to that of the respective control iliac arteries. Different cross-sections through the same graft, however, revealed both areas of hyper- and hypoinnervation (Table 1). The nerve profiles were similar to those of Weeks 1 and 2. An occasional nerve fiber had varicosities although in most cases they were not evident. In sections at Weeks 1 and 2, nerves were seen only in longitudinal section, thereby appearing to form a discontinuous ring of nerves in the outer adventitia of the graft. However, at 4 weeks some nerves were also seen in cross-section. The presence of both longitudinally and cross-sectioned fibers in the same section may have represented an early stage of plexus formation. Although in most cases nerve profiles were irregularly distributed throughout the outer adventitia of the graft wall, some cross-sections through the graft revealed a slight concen-

TABLE 1. *The Degree of Graft Innervation Assessed at 100 μm Intervals from the Center of the Graft, Across the Anastomosis, and Along the Host Iliac Artery*

	Host Iliac							Graft					
Time	600	500	400	300	200	100	Anastomosis	100	200	300	400	500	600
1 week													
Rat 1	*	2+	3+	3+	1+	0	0	0	0	0	0	0	0
Rat 2	3+	3+	3+	1+	2+	0	0	0	0	0	0	0	1+
2 weeks													
Rat 1	*	3+	3+	2+	1+	1+	2+	3+	3+	1+	0	0	0
Rat 2	3+	3+	3+	3+	3+	3+	2+	2+	2+	2+	2+	1+	0
Rat 3	*	3+	3+	2+	1+	1+	0	0	0	0	0	0	0
4 weeks													
Rat 1	4+	4+	4+	3+	3+	2+	3+	3+	3+	4+	4+		
Rat 2	*	4+	4+	3+	3+	2+	3+	3+	4+	3+	3+		
Rat 3	*	*	*	3+	3+	3+	3+	4+	4+	2+	3+	3+	
Rat 4	*	*	4+	4+	3+	2+	2+	3+	3+	3+	2+	3+	3+
8 weeks													
Rat 1	3+	4+	4+	4+	3+	3+	3+	4+	4+	3+	3+	3+	3+
Rat 2	*	4+	4+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+
Rat 3	*	*	4+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Rat 4	3+	3+	3+	3+	3+	3+	2+	3+	3+	4+	4+	4+	4+
16 weeks													
Rat 1	*	*	4+	4+	4+	3+	2+	3+	3+	3+	3+	3+	3+
Rat 2	*	*	3+	4+	4+	3+	4+	4+	4+	4+	4+	4+	4+
Rat 3	*	3+	4+	4+	4+	3+	3+	4+	4+	4+	4+	4+	4+
Rat 4	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+
32 weeks													
Rat 1	3+	4+	4+	4+	3+	3+	3+	3+	3+	4+	4+	4+	3+
Rat 2	*	*	*	*	*	*	*	4+	4+	4+	4+	4+	4+
Rat 3	*	3+	4+	3+	3+	3+	3+	4+	3+	3+	4+	3+	4+
Rat 4	*	4+	4+	4+	3+	3+	3+	3+	4+	3+	3+	3+	

All assessments were made relative to the control iliac artery on the opposite side in the same animal.
0—no innervation
1+—scant innervation
2+—less than control innervation
3+—normal innervation (control artery level)
4+—greater than control innervation (hyperinnervated)
*—artifacts prevented reliable assessment

tration of nerves down the inferomedial aspect in close proximity to the iliac vein. Well-innervated vasa vasorum were also seen in intimate contact with the graft wall.

In one of the four grafts (No. 2) some larger nerve bundles approximately 2 μm in diameter were present on the host iliac artery, close to the adventitial-medial border 500 μm from the anastomosis. Apart from these fibers, which probably represented regeneration of the original plexus across the clamp-damaged region, the innervation of the host vessel was similar to that seen at Weeks 1 and 2.

8 Weeks. The length of the grafted vein was innervated in all four cases and there appeared to be extensive regions of hyperinnervation, which varied from between 200 to 500 μm in length (Table 1). There were no regions in any of the grafts that showed a degree of innervation less than that of the control iliac artery.

The pattern of graft innervation was essentially the same as that described at Week 4. The nerve profiles seen were of fine fibers (<1 μm diam.) and were located further out in the adventitia than those seen in control arteries. The fluorescent intensity of the nerve profiles appeared to be normal, indicating that the concentration of noradrenaline within the nerve fibers had reached control levels. However, the nerve fibers had no varicosities.

Indications of early plexus formation seen at Week 4 are also present at Week 8. Although nerve profiles were predominantly seen in longitudinal section, some fibers cut cross-sectionally were also observed in all aspects of the four grafts examined. Three of the four grafts examined at Week 8 exhibited large (2 μm) nerve profiles on the host iliac artery, similar to those at Week 4. In one graft they were as close as 300 μm to the anastomosis. Cross-sections that displayed these nerve fibers also retained the fine fibers in the outer adventitia such that the host iliac became hyperinnervated (Fig. 2c).

16 Weeks. Three of the four grafts examined were hyperinnervated. The morphology of innervation was similar to that seen in previous weeks (Table 1). Varicosities were not identifiable. Nerves were seen in approximately equal proportions of both cross- and longitudinally sectioned profiles, indicating an established nerve plexus around the graft. Cross-sections through the host iliac artery revealed an innervation similar to that

at Week 8. Nerve profiles were seen both as fine fibers (1 μm) located in the outer adventitia and larger fibers (2 μm) adjacent to the adventitial-medial border. The larger profiles, probably representing regrowth of the host vessel's original plexus, extended as far as, but did not cross, the anastomosis.

32 Weeks. In general, the grafts were hyperinnervated along their length (Table 1). The pattern of innervation was similar to that seen in previous grafts. Nerve profiles were predominantly fine fibers (1 μm diam.) and were at a greater distance from the outer layer of muscle cells than nerves within the control artery. Fluorescence intensity appeared normal but varicosities were not identifiable (Fig. 2d).

One of the grafts (No. 2) displayed a continuous line of fluorescent profiles along its entire inferomedial aspect. The nerves were a mixture of both cross- and longitudinally sectioned profiles of large and fine fibers. They were found as close as 8 μm to the outer muscle layer, a distance comparable to the control iliac artery.

Discussion

This study was undertaken to determine the time course and morphology of reinnervation in experimental vein grafts. In summary, the results show that nerves appeared within the graft, in appreciable numbers, by 2 weeks after surgery and occupied the length of the graft by Week 4. The degree of innervation of the grafted vein increased over the time course of the study so that by Week 16 the graft was hyperinnervated relative to the control iliac artery within the same animal.

Graft Reinnervation

Although nerves were present within the graft region from 2 weeks onwards the morphology of this innervation was different from that of the control iliac artery and from conventional descriptions of arterial innervation.[10] The graft's nerve fibers were finer than normal (about 1 μm or less in diameter) and were not varicosed. The small size of the fibers and their lack of varicosities (the functional releasing sites for neurotransmitter) may be a reflection of their immaturity. It has been shown, in both myelinated and unmyelinated nerves, that following a crush injury a large proportion of regenerating axons fail to regain their normal diameter.[11,12] It has been suggested that this may be due to either a lack of synaptic connections, or the fact that a neurone can support only a certain volume of axoplasm, so that the number and size of axonal sprouts is limited.

In addition to nerve fibers within the graft being smaller than normal, they were also at a greater distance from the outer layer of smooth muscle cells in the graft than in the control artery. This intervening space (the diffusion distance) was packed with collagen and elastin that was more dense than that found in the adventitia of the normal control artery. Collagen can act as a barrier to the free diffusion of noradrenaline released from adrenergic nerves and it even has the capacity to bind the neurotransmitter.[13]

The relative immaturity of the nerve fibers seen surrounding the grafts, together with the greater than normal diffusion distance and the presence of dense collagen between the nerve fibers and muscle cells in the graft, raises the question of the effectiveness of this innervation. If noradrenaline is released from these terminals, would it be in a large enough concentration, after having diffused through dense connective tissue, to initiate contraction upon reaching the smooth muscle cells?

Origin of Nerves

There is evidence to suggest that the nerves seen around the graft were derived from a collateral supply consisting of the iliac vein, invading vasa vasorum (involved in revascularization), and possibly, nearby paravascular nerves. Vasa vasorum, which were well innervated, were seen in close association with the wall of the graft. Moreover, cross-sections through the grafts frequently revealed a slightly higher concentration of nerve profiles on the inferomedial aspect, which is in close proximity to the iliac vein, a vessel that is richly innervated. In addition, portions of the graft classed as hyperinnervated were often bounded on either side by areas of less dense innervation. This indicated that reinnervation did not involve a progression of nerves along the graft's length but rather was the result of random collateral sprouting.

Olson and Malfors[14] have shown that the outgrowth of autonomic nerves is not confined to the organ that they normally innervate. They found that a variety of tissues, such as femoral artery, portal vein, vas deferens, kidney, pancreas, and many more, when transplanted into the anterior chamber of the eye in rats, were reinnervated by nerves growing out from the adrenergic plexus of the host iris. In addition, transplants of sympathetic ganglia onto a denervated iris resulted in axonal sprouts from the ganglia to the host iris. They also found that mild infections accelerated and enhanced reinnervation. Therefore, it is not improbable that, in response to some stimulus—possibly inflammation, nerves of the vasa vasorum and iliac vein developed sprouts that reinnervated the nearby grafted vein. Axonal sprouts can occur within 2 to 4 days following a crush injury to unmyelinated nerves,[11] and once developed can grow at a rate of 1 to 2 mm per day.[15] Malmfors and Olson[14] showed nerves within a tissue transplant to the anterior eye chamber in rats (see above) within 4 days following transplantation. It was not unexpected, therefore, to find nerves in the graft region by Week 2, since the distance between the graft and invading vasa vasorum and iliac vein is only a matter of micrometers. The stimulus for sprouting from the nerves of the vasa vasorum and iliac vein was probably

due to inflammation of the surrounding connective tissue following surgery. This conclusion is supported by evidence from other studies: for example, it has been shown that there is an increase in nerve numbers within human inflammatory dental periapical lesions.[16] There are similar results in psoriatic skin as a result of chronic inflammation.[17] In addition, an active role for proliferating adrenergic nerves in tissue responses to injury and inflammation has been proposed, when it was found that biogenic amines (noradrenaline and dopamine) stimulated the revascularization of corneal lesions.[18]

Studies on the revascularization of grafted veins show that a significant number of invading vasa vasorum are derived from the host artery.[19] However, the normal nerve plexus of the host artery, in this study, does not appear to make a contribution to the reinnervation of the graft. At no stage could fibers, seen within the graft, be followed across the anastomoses and along the host iliac artery. There is probably a three-fold reason for the failure of growing nerves from the host vessel plexus to cross the anastomosis: there is considerable scarring and a consequent dense accumulation of collagen and elastin at the suture site; the neurilemmal tubes of Schwann cells, which provide a low resistance pathway for regenerating axons, are completely disrupted at the anastomosis and the site is at least 4 mm from the nearest nerve cell body, a distance which may be close to the limit over which it can sustain continued axonal growth. In fact, it was 4 weeks before any nerves of the original plexus were seen to have crossed the lesion on the vessel caused by the application of the vascular clamp. Any nerves seen in association with the host vessel prior to 4 weeks were probably a result of collateral sprouting. This is in agreement with the results reported for the carotid artery (a similar elastic artery to the iliac) where it took 3 weeks for a clamp-injured region to be reinnervated with only a variable contribution from nerves growing across the scar.[8] The relatively long time taken by regenerating nerves to cross the "clamp lesion" is probably due to the formation of scar tissue, which can act as a barrier to axonal regeneration.[20] It has been shown that the growth of regenerating axons in response to a clean, incised wound of the brain is always hampered, and in some cases prevented, by the formation of tightly packed collagen bundles around the wound.[20] If this is the case, and collagen can act as a major physical barrier to nerve growth, then it would explain why nerves, sprouting from either vasa vasorum, the iliac vein, or paravascular nerves, and growing towards the graft region, remain at a fixed distance from the graft lumen (greater than that in the control iliac artery). Moreover, it would also explain why they fail to reestablish normal innervation of that part of the host artery denervated during the surgical procedure. This is because following graft insertion, inflammatory and repair responses produce dense layers of collagen around the grafted vein and adjacent segment of the host vessel. The high-pressure gradient set up in the graft wall by placing a vein into an arterial environment might also contribute in this respect, particularly early on, prior to the establishment of an intimal muscle layer.[21]

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