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Department of Plastic Surgery
Helsinki University Hospital
University of Helsinki
Finland

THE DISAPPEARENCE AND REGROWTH OF ADRENERGIC INNERVATION IN ARTERIAL GRAFTS IN RATS

A MICROSURGICAL EXPERIMENTAL STUDY

by

Heikki Penttilä

To be publicly discussed with the permission of the Faculty of Medicine of The University of Helsinki, in the main auditorium of Töölö Hospital, Helsinki University Hospital, Topeliuksenkatu 5, Helsinki, on 10th February 2012 at 12 noon

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Supervised by
Docent Timo Waris
Chief of Plastic Surgery (ret.)
Oulu University Hospital
Oulu, Finland

Reviewed by
Docent Outi Kaarela
Department of Surgery
Oulu University Hospital
Oulu, Finland
and
Professor Seppo Soinila
Department of Neurology
Turku University Hospital
Turku, Finland

Opponent
Docent Kalervo Werkkala
Department of Thoracic Surgery
Helsinki University Hospital
Helsinki, Finland

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to by their Roman numerals:

I. DISAPPEARANCE OF CATECHOLAMINE FLUORESCENCE FROM THE ADRENERGIC NERVES IN ARTERIAL GRAFTS IN RATS: AN EXPERIMENTAL FLUORESCENCE HISTOCHEMICAL STUDY. Heikki K.T. Penttilä, Karl A.J. von Smitten and Timo H. Waris. Scand J Plast Reconstr Hand Surg 35: 123–128, 2001.

II. NORADRENALINE LEVELS RISE SIGNIFICANTLY IN RATS' ARTERIAL GRAFTS AT 6 HOURS POSTOPERATIVELY AND TAKE 48 HOURS TO COME DOWN. Heikki Penttilä, M.D., Pirkko Huttunen Ph.D., Karl von Smitten, M.D., Ph.D., Timo Waris, M.D., Ph.D., Nureddin Ashammakhi, M.D., Ph.D., F.R.C.S.Ed.. Microsurgery 24: 139–142, 2004

III. REINNERVATION OF ARTERIAL GRAFTS BY ADRENERGIC NERVES OCCURS IN RATS AS INDICATED BY INCREASED LEVELS OF NORADRENALINE. Heikki Penttilä, M.D., Pirkko Huttunen, M.Sc., Karl von Smitten, M.D., Ph.D., Nureddin Ashammakhi, M.D., Ph.D., FRCSEd., Timo Waris, M.D., Ph.D. Plast Reconstr Surg 113: 2057–2060, 2004.

IV. DEGENERATION AND REGENERATION OF PERIVASCULAR INNERVATION IN ARTERIAL GRAFTS Penttilä Heikki, MD; Waris Timo, Professor; Ashammakhi Nureddin, FRCSEd; Vanhatalo Sampsa, MD, PhD. The Journal of Craniofacial Surgery 15: 570–581, 4 July 2004

ABBREVIATIONS

ACh	acetylcholine
AR	adrenaline
ChAT	choline acetyltransferase
CNS	central nervous system
GABA	gamma-amino butyric acid
GAP-43	growth-associated protein 43
HPLC	high performance liquid chromatography
IR	immunoreactivity
NA	noradrenaline
NF	neurofilament
NO	nitric oxide
NOS	nitric oxide synthase
eNOS	endothelial NOS
iNOS	inducible NOS
nNOS	neuronal NOS
ta	tunica adventitia
TH	tyrosine hydroxylase
tm	tunica media
VIP	vasoactive intestinal peptide

ABSTRACT

In many fields of surgery, such as coronary bypass, microvascular free-flap, organ transplantation, vascular and hand surgery, the function of transplanted arteries is of crucial importance. In all these fields of surgery vasospasm is regarded as a serious threat to a successful outcome. It can be caused by many factors such as mechanical handling of the recipient artery, drugs, perioperative trauma and changes in temperature. We still do not fully understand the exact mechanisms behind vasospasm and there is great variety in the expression of this phenomenon between different individuals from almost nil to very profound, as in Raynaud's disease. The factors behind vasospasm have been studied in connection with operative trauma in venous grafts, but not in arterial grafts.

When an artery is dissected from its surrounding tissues it loses its sympathetic innervation. The early disappearance of catecholamines from vein grafts has been shown previously. However, the fate of adrenergic nerves in arterial grafts has not been studied.

To investigate the changes in adrenergic nerves in transplanted arterial grafts in rats, female laboratory rats of 3 to 40 weeks old were operated on. Femoral arterial grafts were anastomosed to carotid arteries and compared with control non-operated femoral artery segments.

In our first experiment the disappearance of catecholamine fluorescence from noradrenalin-containing sympathetic nerve fibers after arterial transplantation was studied using femoral artery grafts. In the first study glyoxylic acid-induced fluorescence was used to demonstrate adrenergic nerves histochemically. In the second and third studies high-performance liquid chromatography (HPLC) with electrochemical detection was used to determine noradrenaline content in grafts and controls. The fourth experiment was performed to characterize neuronal degeneration and regeneration events qualitatively using immunohistochemistry. Specimens were assessed for vasoactive intestinal peptide, neurofilaments, growth-associated protein 43, tyrosine hydroxylase, and nitric oxide synthase isoenzymes.

According to our experimental studies, at six hours postoperatively the network of fibers had started to degenerate, and catecholamine fluorescence from the adrenergic nerves had almost completely disappeared within 24 hours of grafting. Control specimens from normal femoral arteries showed a dense network of fluorescent adrenergic nerves. Immediately after grafting, the noradrenaline content was 76% of the control value, at 6 h 130%, and at 48 h 4% of the control value. The drop in graft noradrenaline content (at 48 h) was statistically significant ($p=0.005$). The difference between 0-h, 12-h, and 24-h groups was statistically insignificant.

Compared with control specimens, grafts contained less noradrenaline after 12 h, and it disappeared almost completely from femoral arterial grafts in rats within 48 h. The operation itself resulted in decreased noradrenaline concentrations in the grafts to 76%of that in the control segments.

One week after the operation, the noradrenaline concentration had fallen to less than 2% of control values and it started to recover thereafter. One month after the operation it was 23%, at 3 months it was 31% and at 20 weeks it was 43% of control values. The decrease from time 0 to 1 week was significant ($p<0.001$), as was the increase from 1 week to 20 weeks ($p<0.004$) Catecholamine levels increased over the follow-up period of 20 weeks as a result of reinnervation of the grafts. In rat femoral arterial grafts transplanted into carotid arteries, NA concentrations had risen to levels that did not significantly differ from those in controls or the day 0 group.

During neuronal degeneration, vasoactive intestinal peptide disappeared within 1 day, transmitter-synthesizing enzymes (nitric oxide synthase and tyrosine hydroxylase) had vanished by day 7, and neurofilaments (cytoskeletal markers) had essentially disappeared after 1 week.

In the regeneration phase, the most robust axonal growth, as visualized by growth-associated protein 43, was observed at 1 month, followed by a gradual increase in neurotransmitter markers at 1 and 3 months, whereas the neurofilaments increased gradually up to the end of the 5-month observation period. Reinnervation started from the proximal carotid (host) trunk and proceeded distally to the graft. Axonal regrowth occurred mainly in arterial adventitia. Innervation density, as visually assessed, was denser in the graft than in the host, which is consistent with the density of neuronal networks in the corresponding arteries in their natural surroundings.

In conclusion, we suggest that the relatively rapid liberation of catecholamines from degenerating adrenergic nerves may have an important role in early vasospasm in microvascular and coronary bypass surgery. Our findings also suggest that the main sequence of degeneration and regeneration follows that reported in other models of neuronal degeneration. Our findings indicate that reinnervation of arterial grafts comes mainly from host arteries, and innervation density in the graft may differ from that in the host, which may suggest target-derived regulation of innervation. An unexpected finding was that there is a rise in noradrenaline levels in grafts six hours after grafting. This may be because of the fact that the nerve fibers do not receive stimuli, which causes them to release noradrenaline to the synaptic void. At the same time the active, energy- and sodium ion-dependent reuptake of noradrenaline occurs in the terminal ends of the fibers.

1. INTRODUCTION

Vasospasm is a major threat to successful outcome of surgery and it has been studied for decades. Blood flow through a vessel is dominated by several factors, pressure gradient over the observed vessel, vessel diameter, vessel length and fluid dynamic viscosity. Poiseuille´s law (Fig. 1) demonstrates the relationships between the different factors in one equation (Sutera and Skalak 1993, Goyton 1991).

$$R = \frac{\pi r^4 p}{8 \eta l}$$

Fig. 1. Poiseuille´s law, R = flow rate (ml/s), r = radius of the pipe, η = dynamic fluid viscosity (Pascal × seconds), p = pressure gradient, l = length of the pipe.

From the equation it can easily be seen that an increase in pipe (vessel) length and increased viscosity (thickness of the fluid) decrease the flow rate, and increasing the diameter (radius) of the pipe (vessel) increases the flow rate to the power of four. Thus, doubling the vessel diameter will increase the flow 16-fold and even a modest increase by a factor of 1.2 will double the flow. Poiseuille´s law as such is applicable to non-compressible homogeneous (Newtonian) fluids. Blood is a shear-thinning non-Newtonian fluid and its flow in the vessels is a more complex phenomenon. In thin vessels with a diameter of about 15 µm blood viscosity falls greatly. This is thought to happen because blood cells will aggregate to the center of the vessel and create a cell-free low-viscosity layer of plasma against the vessel wall (Forbes and Lowe 1986)

Catecholamines freed from nerve endings during and after an operation are possibly a major contributor to smooth muscle contraction and vasospasm. The use of arteries as graft material has increased consistently over the years. Today, arteries are used in free-flap surgery, organ transplantation, vascular surgery, replantation surgery, and coronary bypass surgery. Venous grafts have been studied in regard to viability of the graft wall, perioperative damage, fate of the endothelium, and innervation. In coronary bypass surgery, both the radial artery and the right gastroepiploic artery are well known for their tendency to go into spasm during the operation (Chen et al. 1996, Grandjean et al. 1996). In venous grafts, noradrenaline concentrations increase and reach a steady state within 18 weeks. In mesenteric arteries, reinnervation has been found to be complete by 8 weeks, whereas in carotid arteries it has been thought to take longer (Cowen et al. 1982). The autonomic nervous system is a powerful controller of arterial blood pressure, gastrointestinal secretion and motility, sweating, bladder emptying and

other vital functions of the body. Clinical and histochemical studies have indicated that when open heart surgery is performed, catecholamines freed from heart tissue can be a risk factor. If the heart tissue contains large adrenergic nerves with “droplet fibers“ or “adrenaline bombs“ containing huge local intra-axonal concentrations of catecholamines, mostly noradrenaline, there is an increased risk of arrhythmia and even death (Kyösola et al.1988) Remes and coworkers (Remes et al.1985, Remes et el. 1990) have demonstrated that instrumentation causes crush injury to the vessel wall with loss of metabolic activity and inflammatory cell infiltration.

2. REVIEW OF THE LITERATURE

2.1. AUTONOMIC NERVES

2.1.1. SYMPATHETIC NERVES

Sympathetic control of the circulation was first described in 1851 by Bernard (Bernard 1851, 1852) and by Brown-Sequard (Brown-Sequard 1852), and Gaskell and Langley both described the anatomy of the autonomic nervous system and its effects on end organs (Gaskell 1889 a and b, Langley 1889 a and b).

Sympathetic innervation is mainly controlled by centers in the spinal cord, brain stem and hypothalamus. The limbic cortex, which is part of the cerebral cortex, can also transmit signals to lower centers and therefore control the autonomic nervous system. The paraventricular nucleus (PVN) has been shown to be an important central nucleus as regards control of the sympathetic system. PVN microinjections of excitatory amino acids have been shown to produce non-uniform changes in the level of sympathetic nerve activity. These results support the concept that the PVN is an important component of the central neurocircuitry regulating functional characteristics of efferent sympathetic nerve outflow (Kenney et al. 2003, Lee et al. 2006, Higa-Taniguchi et al. 2007).

The autonomic nervous system controls arterial blood pressure, gastrointestinal secretion and motility, sweating, bladder emptying and other vital body functions. The sympathetic system is a very powerful regulator of these functions; it can double arterial pressure within 10 to 15 seconds, it can increase heart rate to double of normal within three to five seconds, and sweating can start within seconds (Wray et al. 2005, Lee et al. 2006).

The sympathetic nervous system consists of two paravertebral sympathetic chains of ganglia that are located on both sides of the vertebral column and two paravertebral ganglia, the hypogastric and coeliac, and the nerves from these ganglia to the target organs. The sympathetic nerves arise from the spinal column between segments T-1 and L-2. They then pass through the sympathetic chain to the end organs that are regulated by the nerves. The sympathetic neurons rise from the intermediate lateral horn of the spinal column. The afferent fibers pass through the posterior root to the ganglia of the sympathetic chain and synapse to secondary neurons. An alternative route is that they pass through the sympathetic chain and synapse in a peripheral ganglion, and another alternate route is that they pass upwards or downwards in the sympathetic chain and then synapse with the peripheral neurons. The primary neurons use acetylcholine as transmitter and

noradrenaline is used in secondary neurons, except in those leading to sweat glands and pilomotor muscles, which are always cholinergic. Some of the sympathetic neurons pass back from the sympathetic chain through the gray rami into the spinal nerves. These pathways comprise type C fibers and they pass to all parts of the body with the spinal nerves. The fibers control piloerector muscles, sweat glands and blood vessels. About eight percent of nerve fibers of any peripheral nerve are sympathetic, a fact that underlines their importance (Guyton and Hall ed.: Textbook of Medical Physiology, 11th edition, Philadelphia Pa., U.S.A. Saunders 2006.

Sympathetic innervation of blood vessels in different organs varies greatly in density, as shown by Hillarp and by Morgan and it also varies between individuals (Hillarp 1959, Morgan et al. 1983, Chen et al. 1996). Large numbers of sympathetic nerves innervate the arterioles of the skin and the splanchnic vessels, but the sympathetic network in veins in these areas is less dense. The effects of adrenergic innervation have been thoroughly studied and are summarized in Table 1.

Table 1. Effects of sympathetic stimulation on different receptors, organs and tissues (Zaugg and Shaubb 2005).

Adrenergic receptor subtype	**Main signaling pathways**	**Effects on neuronal and cardiac tissue**	**Effects on vascular tissue**
β1	G-protein signaling component-adenyl cyclase-cAMP-protein kinase A	Positive on heart rate inotropy, lusitropy, metabolism, growth, myocyte toxicity	–
β2	G-protein signaling component-adenyl cyclase- cAMP-target-specific serine/theorine protein kinase; G-protein signaling component inhibiting adenyl cyclase; G-protein signaling component- phospholipase C-diacylglycerol-target-specific serine/threonine protein kinase and G-protein signaling components–phosphatidylinositol 3-kinase-PI3K-dependent kinase-target-specific serine/threonine protein kinase	Positive on inotropy, lusitropy, metabolism, growth, myocyte survival	Relaxation of vascular smooth muscle cells
β3	G-protein signaling component inhibiting adenylyl cyclase; G-protein signaling components-phospholipase-C-diacylglycerol-target-specific serine/threonine protein kinase and G-protein signaling component-NOS-NO-guanylyl cyclase- cyclic guanosine monophosphate-target-specific serine/threonine protein kinase	Negative on inotropy and myocyte survival	–
α1A	G-protein signaling component - phospholipase-C- inositol trisphosphate-Ca^{2++}	Positive on heart rate, inotropy, and growth in myocytes	Vasoconstriction
α1B	G-protein signaling component - phospholipase-C- inositol trisphosphate-Ca^{2++}	Positive on heart rate, inotropy, and growth in myocytes	–
α1D	G-protein signaling component - phospholipase-C- inositol trisphosphate-Ca^{2++}	Contraction of smooth muscle cells	Vasoconstriction
α2A	G-protein signaling component-inhibiting adenyl cyclase	Presynaptically reduces sympathetic outflow	Lowers RR
α2B	G-protein signaling component-inhibiting AC, also G-protein signaling component-stimulating adenyl cyclase	Postsynaptically counteracts the effects of α2 A	Peripheral vasoconstriction
α2C	G-protein signaling component-inhibiting adenyl cyclase	Presynaptically reduces sympathetic outflow, postsynaptically lowers cAMP	Lowers RR and participates in vasoconstriction after exposure to low temperatures

2.1.2. PARASYMPATHETIC NERVES

The parasympathetic nervous system (PSNS) is the other of the two main divisions of the autonomic nervous system (ANS). Sympathetic and parasympathetic divisions typically function in opposition to each other. This natural opposition is better understood as complementary in nature rather than antagonistic (Moore and Agur 2007). The transmitter substance is ACh and there are five different types of receptors: M_1 is in the CNS and M_2 in the heart, where they slow down the heart rate and decrease the contractile force. M_3 receptors are located in many places in the body, such as the intestinal system, lungs and blood vessels. M_4 and M_5 receptors have possible CNS effects.

Understanding the effect of M_3 receptors on blood vessels is important in this work. Acetylcholine causes the production of NO and as a result of its action smooth muscle cells relax and vasodilatation occurs.

As in the sympathetic nervous system, parasympathetic nerve signals are carried to their end organs from the central nervous system by a system of two neurons. The first neuron in this pathway is referred to as the preganglionic neuron. Its cell body is located in the central nervous system and its axon usually extends to a ganglion somewhere else in the body, where it synapses with the dendrites of the second neuron in the chain. This second neuron is referred to as the postganglionic neuron. In the CNS the presynaptic neurons are located in specific nuclei, in the pelvic division in the lateral gray horn of the spinal cord. The axons of presynaptic parasympathetic neurons are usually long: they extend from the CNS into a ganglion that is either very close to or embedded in the target organ. As a result, the postsynaptic parasympathetic nerve fibers are very short (Moore and Agur 2007).

Although the parasympathetic nerves cause vasodilatation in visceral organs, it has been reported that the nerves responsible for vasodilatation in the peripheral vessels are sympathetic vasodilator fibers (Charcoudian 2003). This would indicate that the parasympathetic system would then be responsible for vasodilatation only in the visceral organs. However, the author of the report seems to have drawn false conclusions. He states that the transmitter is unknown, while reporting that botulin toxin abolishes the action. He also states that the muscarine receptor blocker atropine does not abolish the effect. From this data it would be more logical to assume that the transmitter is ACh (botulin toxin effect) but the receptor is not a muscarine receptor (lack of atropine effect).

2.2. TYPES OF TRANSMISSION

2.2.1. CLASSICAL TRANSMISSION

In classical transmission, a transmitter substance is synthesized in a neuron and it is then stored in granules in the presynaptic region. Noradrenaline, like other transmitters, goes through the same pathway in the sympathetic neurons. Peptide transmitters are typically synthesized in the soma of the neuron and then transported to the axon by axonal transport. The transmitter is released from the granules after an axon potential enters the synaptic region and causes opening of the Ca^{++} channels. This eventually leads to fusing of the storage granule with the cell membrane and the contents of the granule, i.e. the transmitter substance(s), are released to the synaptic cleft. The transmitter substance(s) then bind to corresponding receptors, causing either permeability changes in the target cell membrane or activation of a secondary messenger system. The first of these is rapid and happens within milliseconds, whereas the second event lasts seconds. In the last phase the transmitter molecule, noradrenaline in the sympathetic system in the end organ synapse is eliminated from the synaptic cleft either by specific enzymes (Cooper et al. 1991) in the synaptic cleft or transported into pre-, postsynaptic or glial cells (Lester et al.1994). There it will be reused in presynaptic cells or inactivated in the postsynaptic or glial cells.

2.2.2. VOLUME TRANSMISSION

In classical transmission, signals are passed from one cell to another through a specialized structure, the synapse, in a quantal manner. This concept was challenged in the late 1970s and in the 1980s by new findings (Burnstock 1976, Kupfermann 1991). There was a mismatch in distribution of receptors and transmitters. This suggested that these receptors either had no role in signal transmission or that there is an alternative form of transmission outside synapses. After that there were findings of neurons with synaptic granules in areas with no synaptic membrane specialization. This kind of transmission has been found in both the central and peripheral nervous systems. In volume transmission the transmitter substances may diffuse long distances from their sources to their targets. Volume transmission gives a totally new concept to neurological transmission in addition to quantal transmission. With volume transmission large populations of neurons can be controlled in an even, tonic manner by a transmitter substance. The most important factors affecting the extent of volume transmission are the amount of transmitter released, the volume and tortuosity of the extracellular space and the time span of activity of the transmitter, this being determined by the activity of the transmitter-

degrading mechanisms (Fuxe and Agnati 1991, Syková 2004). Volume transmission is present in all parts of the nervous system and conditions favoring it, the diffusion of transmitter about the synaptic cleft and to another neuron, are the same as conditions favoring false transmission. Glial cells play an important role in volume transmission by regulating the volume transmission conditions.

Volume transmission is involved in many physiological phenomena such as pain modulation, sensory perception, vision, regeneration, excitability, motor regulation, arousal and attention as well as manic-depressive disease and exitotoxicity.

2.2.3. NONEXOCYTOTIC TRANSMISSION

Quantal release of transmitters, which is also known as exocytotic release of transmitters, was long regarded as the "right" method of transmission. In nonexocytotic transmission, neurons release transmitter substances in a non-quantal manner through reversal of the transmitter transport mechanism. This release is independent of Ca^{++} ions (Attwell et al. 1993). Since this release is carried out by the transport mechanism, it is also called carrier-mediated release. Gaseous transmitters, such as nitric oxide (NO) and carbon monoxide (CO), are released solely by nonexocytotic transmission, which is also non-carrier-mediated. Clinically, nonexocytotic transmission is involved in many pathological states in the central nervous system (CNS) and heart. In the CNS all endothelial and glial transmission is carried out via nonexocytotic transmission (Attwell et al. 1993, Huszti et al. 1995).

2.2.4. COLOCALIZATION AND COTRANSMISSION

There are several criteria that need to be filled to establish that two compounds really are cotransmitters and not only colocalized in the nerves. The universally accepted ones are:

1. The substance must be synthesized and stored in the nerve.
2. The release of the substance is caused by nerve stimulation.
3. There are specific receptors for the substance on postjunctional sites that, when stimulated with the substance, causes changes in postjunctional activity.
4. There is an existing transport system for the substance itself or its breakdown products that takes it/them up and causes renewal of messenger storage in nerve terminals.

Different neurotransmitters are released by different pulse sequences, multiple or single, depending on their molecular size (Bradley et al. 2003, Ventura et al. 2003).

Negative crosstalk means that the cotransmitters inhibit the responses that are evoked by the same transmitters in the postsynaptic receptors. Sometimes

the cotransmitter substances may act as long-term trophic factors, while being neurotransmitters. For example, ATP, after ectoenzymatic breakdown, can act on P2 receptors, or P1 (adenosine) receptors, to promote cell proliferation, motility, differentiation or death, and neuropeptide Y released from sympathetic nerves has cardiovascular trophic effects in end-stage renal disease (Esler 2003).

Although cotransmitters usually have similar actions on postsynaptic cells, there are some examples of cotrans-mitters having opposite actions (Yang et al. 2002, Landis 2002). Cotransmitter plasticity occurs during development and aging, following trauma or surgery and after chronic exposure to drugs, as well as in disease, and it has been reviewed by Burnstock (Burnstock 1990 a, 2004). Evidence was presented in a recent study involving primary cultures of neonatal rat spinal neurons, for the regulation of substance P (SP) (NK1) receptor expression by calcitonin gene-related peptide (CGRP) (Seybold et al. 2003). There have been some outstanding early studies of the factors influencing cotransmitter expression in sympathetic nerves/neurons (Grant and Landis 1991). A recent study provided evidence that neurotrophic factors from three different protein families (glial cell line-derived neurotrophic factor, neurotrophin 3 and ciliary neurotrophic factor) promote cholinergic differentiation in sympathetic neurons, whereas noradrenergic differentiation is promoted by nerve growth factor (Brodski et al. 2002).

2.2.5. FALSE TRANSMISSION

The concept of false transmission came about when it was noted that a neuron may take up, store and release transmitter substance that is not the actual transmitter substance utilized by the neuron (Burnstock 1976, Kupferman 1991). This other substance often structurally resembles the actual transmitter substance. Originally, analogs and metabolites of noradrenaline were identified as false transmitters, but later cholinergic and GABAergic false transmitters were identified and found to be useful research tools for study of the nervous system. False transmission gained a new dimension when it was noted that a genuine transmitter substance may act as a false transmitter in another population of neurons. This is because the transmitter transport systems are not absolutely specific to their transmitters; rather, they have relative specificities for their transmitters. The concentration of transmitter substance in extracellular fluid is an important factor in determining if the transmitter is taken up by a neuron. Since different concentrations of various transmitters are present all the time in the nervous system, false transmission presumably occurs all the time. The effects of a false transmitter on a target neuron resemble those of cotransmission. At the presynaptic neuron level a false transmitter may either interfere with or aid authentic transmission.

A classic example of false transmission is the effect of serotonin on vascular tone in perivascular sympathetic nonadrenergic neurons. Naturally these neurons do not synthesize serotonin but it has been found to be present in them. Serotonin is taken up by the cocaine-sensitive transport system and can be released by electrical stimulation of the nerve. The release of serotonin has been reported to enhance the vasoconstriction caused by noradrenaline, suggesting synergistic action of these substances (Cohen 1985, 1988).

2.3. ADRENERGIC INNERVATION OF THE VASCULAR WALL

2.3.1. INTRODUCTION

Adrenergic neurons can be studied by using many different methods: immunohistochemistry, chemical analysis, light microscopy, electron microcopy and functional studies in living organisms. Each of these methods offers specific advantages and carries certain disadvantages. If anatomic information is sought, the neurons, or tissue, must be fixed in formaldehyde or other fixative, it must by dyed for examination, sliced thinly and mounted on slides. Since the neurons are dead, all functional data is lost. If functional data is sought, the neurons, tissue or living organism is subjected to stimuli and the response, for example heart rate, is measured. Under such conditions anatomic data is not available. Under certain circumstances living neurons can be studied under the microscope but only limited data can be obtained.

2.3.2. LOCALIZATION OF NERVES IN THE VASCULAR WALL

Blood flow distribution within an organ is controlled through complex interactions that regulate arteriolar resistance and define the paths of blood flow. The resistances of the entire arteriolar network culminate to define what is commonly known as organ resistance. The specific resistance of an individual arteriole is controlled by a number of local variables including flow, pressure, metabolites and neural input, while a coordinated network response requires vascular communication. Vascular communication is the process whereby areas of the vascular network adjust to the vasomotor signals that are generated either within the tissue, or generated at other remote locations along the vascular tree. It potentially plays a role in the precise control of intra-organ blood distribution. Although various efforts have been made, the underlying mechanism of this vascular communication is not fully understood (Thengchaisri and Rivers 2005).

There have been many histochemical studies of the relationship between the adrenergic nerves and the blood vessels. Adrenergic fibers normally run to the periphery in the sensory nerves. Sympathetic nerves fibers are located in the tunica adventitia. They terminate in the muscular layer where they perform their regulatory function on the vascular wall. This occurs through α-adrenergic receptors located in the muscularis layer of the vascular wall that cause vasoconstriction and β-adrenergic receptors that cause vasodilatation. This regulatory function is similar in all vascular wall smooth muscles. They have also been found along the vasa vasorum (Ehringer et al. 1966). As a rule, adrenergic innervation is less dense in veins than in arteries (Ehringer et al. 1966) and there does not seem to be any in the capillaries (Ehringer et al. 1966, Furness and Marshall 1974, Waris and Partanen 1975).

Traditionally, catecholamines were specifically demonstrated by means of formaldehyde-induced fluorescence (Eränkö 1955, Eränkö 1967, Falck 1962) and the method was refined by the introduction of glyoxylic acid-induced fluorescence (Axelsson et al. 1972, Lindvall 1974, Waris and Partanen 1975). This method has the advantage of directly demonstrating the transmitter substance itself, whereas immunohistochemical demonstration of adrenergic nerves is based on revealing the enzyme tyrosine hydroxylase (TH). It is the rate-limiting enzyme for catecholamine production in cells but it only shows the location of potential catecholamine production, not the transmitter substance. Therefore, despite the age of the methodology, glyoxylic acid-induced fluorescence can be regarded as a valuable research tool and it still holds its place in histochemistry. It is not a quantitative method, however. In precise measurements of the amount of catecholamines in tissues, high- performance liquid chromatography (HPLC) has proven to be an invaluable tool (Taylor 1983, Cheng and Kuo 1995;Taylor 1983).

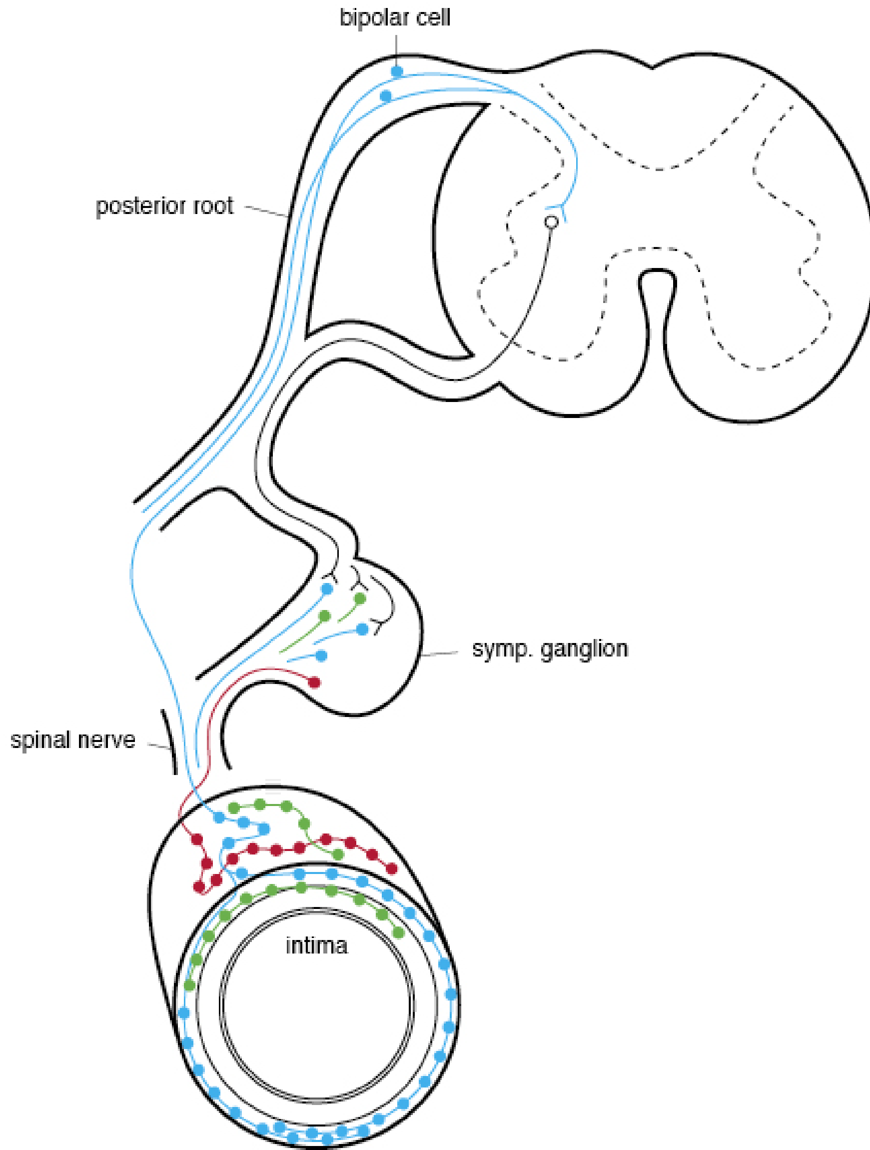

Figure 2: Schematic representation of adrenergic innervation of the vascular wall. These two differ from each other significantly. Both fibers leave the spinal column through the posterior root. The fibers that are destined to go to the blood vessel leave the spinal column and traverse the sympathetic ganglia and enter the spinal nerves that take them to their end organ. Red = cholinergic, green = VIPergic, blue = adrenergic fibers (posterior root sensory fibers). Modified from Guyton and Hall, Textbook of Medical Physiology, 11th edition, Saunders 2006.

2.3.3. Transmitter substances

Noradrenaline is synthesized from tyrosine in a three-enzyme pathway through the intermediates 3,4-dihydroxy-phenylalanine (DOPA), and dopamine. These reactions are catalyzed by th+e enzymes tyrosine hydroxylase, amino acid decarboxylase and dopamine β-hydroxylase. Tyrosine hydroxylase was first characterized by Nagazu, Levitt and Udenfriend (Nagazu et al. 1964). It is the rate-limiting enzyme in the pathway and was the last to be characterized. The second enzyme in the pathway, amino acid decarboxylase, was initially described as 3,4-dihydroxyphenylalanine decarboxylase. It removes the carboxyl group from the side-chain of the molecule to produce dopamine from DOPA. These reactions take place in the cytoplasm of the nerve endings. The third enzyme, dopamine β-hydroxylase, converts dopamine to noradrenaline by adding a hydroxyl group to its side

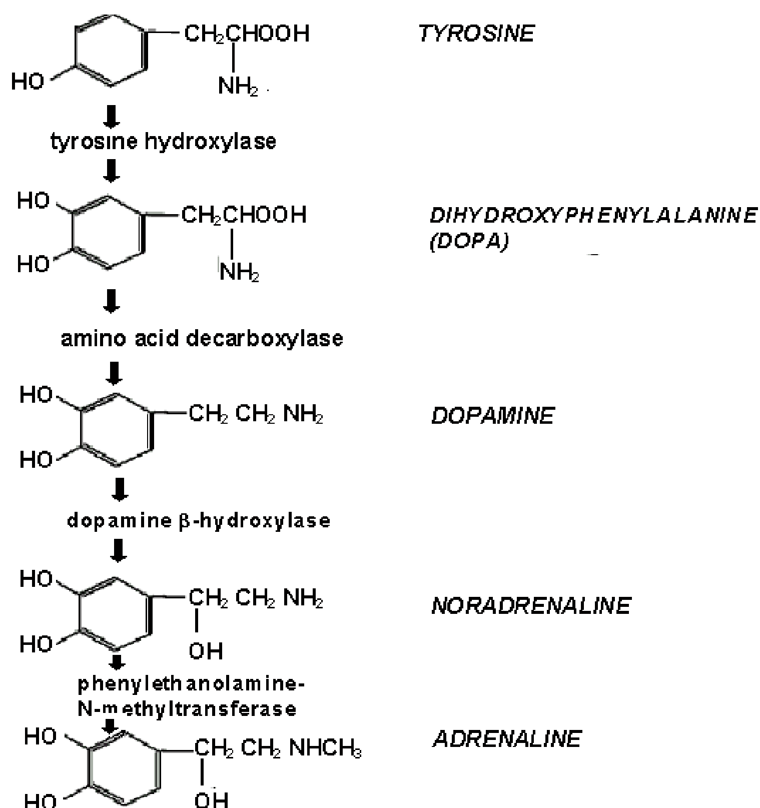

Fig. 3: Synthesis of noradrenaline (and adrenaline) from their common precursor tyrosine through enzymatic steps where tyrosine hydroxylase is the rate-limiting enzyme. Tyrosine hydroxylase also needs oxygen, tetrahydrobiopterin and NADPH for the conversion of tyrosine to DOPA (Kanagy 2005).

chain. This latter synthesis takes place in electron-dense vesicles that are 0.05 to 0.2 µm in diameter. These vesicles are found in the nerve endings of the adrenergic nerves. They also contain ATP, ascorbic acid and encephalin precursor peptides. Only about half of the dopamine formed in the cytoplasm is captured by the vesicles; the rest is deaminated to 3,4-dihydroxyphenylacetic acid and then o-methylated to homovanillic acid.

Vasoactive intestinal peptide (VIP) is a 28-amino acid peptide first isolated by Said and Mutt (Said and Mutt 1969, 1972). It belongs to a family of peptides called the glucagon/secretin superfamily with its homologous peptides PACAP (pituitary adenylate cyclase activating peptide) and PHI (peptide with N-terminal histidine and C-terminal isoleucine amide) (Fahrenkrug 1993). PHI shares the same precursor peptide with VIP, the prepro-VIP from which both can be cleaved. A longer variant of PHI, PHV, is biologically almost as active as VIP as a smooth muscle-relaxing molecule, whereas PHI is less potent (Fahrenkrug and Hannibal 2004). The normal actions of VIP include vasodilation, bronchodilation, anti-inflammatory actions, immunosuppression, hormone secretion, cell proliferation and smooth muscle relaxation (Gozes and Furman 2004). Its receptors can be found in almost all human cells, including cancer cells, and the $VPAC_2$ receptor is found in smooth muscle of various organs including the vascular wall. VIP and ATP are cotransmitters with acetylcholine in parasympathetic nerves (Burnstock 2004). VIP immunoreactivity has been found to be co-localized with the acetylcholine-synthesizing enzyme, choline acetyltransferase (ChAT) (Lundberg et al. 1980). Other studies on the rat SPG (sphenopalatine ganglion) have demonstrated that ChAT-immunoreactive fibers in the middle cerebral arteries originate in the SPG (Suzuki et al. 1988, 1990). Studies of the SPG of the rat have provided further evidence that VIP and ACh are co-localized in the same cells in the SPG, and that the SPG is also a major source of VIPergic innervation (Yu et al. 1998). In the middle cerebral arteries of the pig, VIP-I and ChAT-I fibers have also been found to be coincident with NOS-I and NADPHd fibers, suggesting that VIP, nitric oxide and ACh are co-localized in the same axons in the perivascular nerves of these blood vessels (Yu et al. 1998).

Nitric oxide and nitric oxide synthase (NOS) isoenzymes

Nitric oxide (NO) is a major secretory product of mammalian cells, with critical functions in homeostasis and host defense. It is surprising that so simple, fleeting, and indiscriminate a reactant can convey enough information in a regulated manner to help control vital servomechanisms such as vascular tone and neurotransmission (Nathan 1992).

There are three nitric oxide synthase (NOS) isoforms, neuronal NOS (nNOS; type I), inducible NOS (iNOS; type II) and endothelial NOS (eNOS; type III). These

isoforms differ from each other not only in location but also in regulation, catalytic properties, inhibitor sensitivity, structure and locations in the genome. All isoforms are dimeric in their active form but require two calmodulins (calcium-binding proteins that play an important role in eukaryotic intracellular signaling), forming, in effect, a tetramer. This protein complex then binds flavin adenine dinucleotide (FAD) and flavin mononucleotide/riboflavin phosphate (FMN) and iron protophorphyrin IX (haem). In the reaction in Fig. 4 NADPH donates two electrons and becomes NADP, and L-arginine is converted to citrulline and NO.

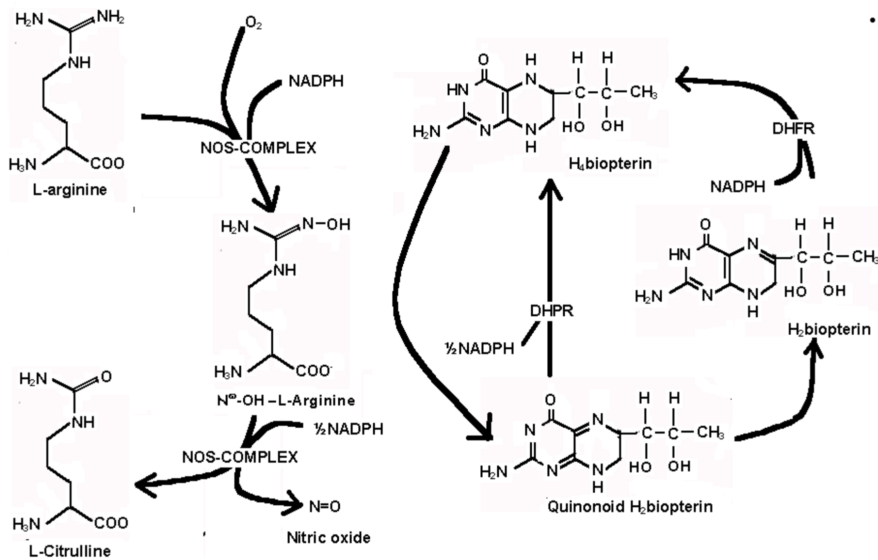

Fig. 4: Nitric oxide (NO) is synthesized by five-electron oxidation of a guanidino nitrogen of L-arginine to yield the gaseous radical (NO) via an N^{ω}-hydroxyl-L-arginine intermediate. Two electrons are donated by NADPH for the formation of this intermediate, plus one electron for its oxidation. Both steps are catalyzed by nitric oxide synthase (NOS). Both NO and the ureido group of citrulline incorporate molecular oxygen in the process. Sub-stoichiometric amounts of tetrahydrobiopterin are also needed with respect to NO generated, if tetrahydrobiopterin can be regenerated from its oxidized form, quinonoid dihydrobiopterin. Regeneration of tetrahydrobiopterin proceeds through two enzymes, the methotrexate-sensitive enzyme dihydrofolate reductase (DHFR) and the methotrexate-resistant enzyme, dihydropteridine reductase (DHPR) (Xie and Nathan 1992).

Nitric oxide can also be generated by direct nonenzymatic reduction under the acidic and highly reduced conditions that may occur in ischemia.

Receptor activation by bradykinin or acetylcholine, or shear stress in the vascular endothelium causes an influx of calcium. The increase in intracellular calcium stimulates the nitric oxide synthase enzyme complex. The nitric oxide formed by this enzyme diffuses to nearby smooth-muscle cells, resulting in enhanced synthesis of cyclic guanosine monophosphate (c-GMP). This increase in c-GMP in the smooth-muscle cells leads to their relaxation and vasodilatation (Moncada and Higgs 1993).

Nitric oxide also inhibits platelet aggregation by way of a c-GMP-dependent mechanism and synergizes with prostacyclin, which, by increasing the concentrations of cyclic AMP inhibits the aggregation of platelets, but unlike prostacyclin, nitric oxide also inhibits platelet adhesion. Platelets themselves also generate nitric oxide, which acts via a negative-feedback mechanism to inhibit platelet activation. Thus, platelet aggregation in vivo may be regulated by platelet-derived nitric oxide as well as by nitric oxide and prostacyclin released from the vascular endothelium. Therefore, nitrovasodilators in combination with prostacyclin analogs may provide a useful form of antithrombotic therapy (Moncada and Higgs 1993).

2.4. OTHER NEURONAL ELEMENTS

2.4.1. NEUROFILAMENTS

Neurofilaments (NFs) are structural proteins found in all neurons. They were described as early as in the late 1800s (Apathy 1897, Cajal 1903) after the silver-staining method was discovered. After the invention of electron microscopy in Germany by Ernst Ruska and Max Knoll in 1931, neurofilaments were described as filaments of roughly 10 nm in diameter and present only in neuronal cells. There are three groups of cytoskeletal proteins, microfilaments (MFs), roughly 6–8 nm, intermediate filaments (IFs), roughly 10 nm and microtubules (MTs), roughly 24 nm in diameter. Microfilaments belong to the type-five IF group. Neurofilaments share common structures with peripherin, α-internexin and nestin. They consist of an α-helical 310-amino acid central rod that is flanked by a non-α-helical carboxyterminal sidearm ending and a globular N-terminal region. These subunits are assembled in polymers where light subunits (NFLs) are paired with either medium (NFMs) or heavy (NFHs) subunits, so that in every pair there is one NFL and one of the others (Liu et al. 2004). These pairs are formed in a head-to-end manner so that the N-end of the NFL is against the C-end of the other subunit. The flanking C- and N-terminal side-arms stick out of this assembly and the 310-amino acid helical portions of the subunits form the pairs where the rods stay parallel to each other (Perrone Capano et al. 2001). The pairs are then grouped into bigger units comprised of 16 such pairs that form the final neurofilament. The formation of the typical IF of 10 nm diameter is determined by the correct assembly of the NFL, NFM and NFH subunits. Assembly of the three NF subunits is dependent on the N-terminal head region. NFL is known to polymerize on its own in vitro, whilst NFM and NFH cannot (Petzold 2005). The exact assembly mechanism is not yet fully understood (Liu et al. 2004). How NFs are transported into different parts of the neuron is unclear (Liu et al. 2004), but there have been some recent advances (Baas

and Buster 2004) in clarifying the concept of "slow axonal transport" – formerly thought to be responsible for transport of these proteins, either as polymers or as subunits, to the periphery of the neuron. Microtubules and actin filaments also act as railways for the motor-based transport of subcellular organelles. This "slow transport" actually seems to take place in short, rapid bursts followed by periods of immobility. During the past 10 years data has accumulated showing additional mechanical and non-mechanical roles of nuclear and cytoplasmic IFs in positioning organelles and contributing to their shape and function and targeting proteins to their proper subcellular compartments (Toivola et al. 2005).

2.5 VASCULAR GRAFTS IN A CLINICAL CONTEXT

2.5.1. VENOUS GRAFTS

Venous grafts have been studied, for example, to assess the viability of graft walls, perioperative damage and collagen metabolism (Remes et al. 1986). These investigators studied cytochrome oxidase and succinate dehydrogenase in order to assess aerobic activity of the grafts, and lactate dehydrogenase to measure anaerobic activity. Glucose-6-phosphate dehydogenase was measured to assess the hexose monophosphate shunt. In the study innervation was not investigated but it was discovered that a vein transplanted to an artery suffered transient metabolic depression for three days and then recovered (Remes et al. 1986).

Waris et al. (1988) studied aortic patch grafts in rats, using three kinds of material as grafts. These materials were syngeneic right atrium of the heart, syngeneic supradiaphragmatic vena cava inferior and supradiaphragmatic vena cava inferior which had been treated with 2% glutaraldehyde for two hours. The findings in this study were that deficient and patchy innervation was present in the venous grafts and right atrial grafts after the observation period of 24 weeks, but the glutaraldehyde-treated venous grafts remained without any adrenergic innervation.

Hukki et al. (1989) studied the noradrenaline content of venous grafts. They used inferior vena cava transplanted into the abdominal aorta of rats. They discovered that noradrenaline concentrations in the grafts fell significantly compared with control veins and they remained low. However, they had reached levels comparable to those in the aorta 35 weeks after the operation.

2.5.2. ARTERIAL GRAFTS

Vasospasm may be caused by various factors, such as mechanical handling of the recipient artery, drugs, or perioperative trauma (Benmilloud et al. 1963, O'Brien et al. 1990, Kroll et al.1996). The ultimate mechanisms behind post- and perioperative vasospasm in arterial grafts remained unexplored. Arterial diameter, and hence arterial blood flow, is regulated by both neural and endothelial factors. Thus, the potential cause of vasospasm may be connected with degeneration of the perivascular innervation and/or altered endothelial function.

Arteries have been studied in coronary by-pass surgery, where they are known for their tendency to go into spasm (Cate et al. 1996, Akar et al. 1997, Girerd et al. 1998, Tauolis et al. 1999). Other workers have shown that sympathetic nerves disappear from transplanted irides within 28–36 hours (Seiger et al. 1984). Vasoconstriction results from the stimulation of α-adrenergic receptors in arterial smooth muscle by noradrenaline (He and Yang 1998, Gustafsson et al. 1999, Segal et al. 2000). In coronary by-pass surgery both the radial artery and right gastroepiploic artery are well known for their tendency to go into spasm during the operation (Cate et al. 1996). In transplanted tissues there is initial degeneration of neural axons within the graft, followed by regeneration of nerve fibers from the surrounding tissues. Cowen et al. studied the effect of denervation in carotid and mesenteric arteries (Cowen et al. 1982). They studied crush injury (and experimental denervation) and discovered that denervation occurred rapidly, in three days. This was complete in the carotid artery and partial in the mesenteric artery. Reinnervation of the mesenteric artery was found to be complete by eight weeks, while in carotid arteries it was thought to take longer.

Crush Injury and operative trauma have been studied by Remes and coworkers. They used rats in which the aorta was clamped with two types of clamp for 30 and 60 minutes. The investigators measured lactate dehydrogenase (LD) and succinate dehydrogenase enzyme activities as indicators of metabolic activity of the arterial wall. They found that after 30 minutes of clamping, enzyme activities were decreased for 3 days postoperatively and after 60 minutes of clamping, enzyme activities were diminished for one and three days, thus demonstrating injury to the aortic wall. However, this injury was not found in all specimens. This was thought to be because the clamps they used caused only mild injury to the aortic wall. This was further emphasized by the fact that one week after injury, enzyme activities had returned to normal (Remes et al. 1990).

Godden and co-workers studied the effect of microvascular anastomosis on sympathetic innervation. They raised groin flaps in rats and two to 12 days postoperatively sacrificed the animals. They measured vascular tone when the specimen arteries were exposed to increasing concentrations of phenylephrine, a sympathetic agent. This sensitivity was blocked by phentolamine and the

investigators interpreted this as a sign of sympathetic denervation (Godden et al. 2000).

Kaarela and coworkers studied rabbit forepaws (Kaarela 1991a). Perivascular sympathectomy was carried out on one or two forepaw arteries in rabbits. Blood flow was measured by means of Tc^{96m}-angiography before the initial operation and five days after it. No statistically significant difference was found between the operated left side and the right side, which served as control (Kaarela et al. 1992). Kaarela and coworkers also studied the effect of perivascular sympathectomy in monkey hands (Kaarela et al. 1991b). They performed perivascular sympathectomy on common digital arteries of *macaca arcroides* monkeys and studied glyoxylic acid-induced fluorescence in the sympathectomized and opposite, control hand digital arteries. There was no difference in the distal adrenergic nerve network after the procedure and the investigators suggested that therefore the operation should be called adventitectomy instead of sympathectomy. The same kind of result was obtained in connection with the rabbit ear central artery, where sympathectomy lowered noradrenaline concentrations but did not abolish it from the tissue (Kaarela et al. 1991c).

Similar phenomena are present in transplanted organs. They are completely detached from their surroundings and reanastomosed to a new site. Under these circumstances newly formed nerve tissue contacts may be functional and contribute to neuronal control of the organ (Lassmann and Piza 1984, Kawaguchi et al. 1998, Uberfuhr et al. 2000a, Uberfuhr et al. 2000b). However, often the grafted tissue only stimulates robust sprouting of nerve terminals from the host tissue into the graft, and only limited or no functional innervation may be found (Schroeder 1978, Takachi et al. 1995, Doering et al. 1996). Enhancing functional reinnervation of denervated tissues, either after neuronal trauma or after tissue transplantation, constitutes a major challenge in many fields of surgery (Kiyochi et al. 1995, Fansa et al. 1997, Kjaer and Secher. 1999).

3. AIMS OF THE STUDY

Neuronal regulation of vascular tone is important for the control of blood flow in vascular grafts, yet there have been few studies on the basic features of the changes occurring in postoperative innervation patterns in arterial grafts. Therefore, we set out to study degeneration and regeneration of perivascular innervation in transplanted femoral arteries using an experimental rat model. We used a number of different histochemical markers to reveal distinct aspects of perivascular innervation.

Aims of the current study:

1. To qualitatively examine the disappearance of noradrenaline from arterial grafts.
2. To quantitatively measure the rate of disappearance of noradrenaline from arterial grafts.
3. To quantitatively measure the regeneration of adrenergic nerves in arterial grafts.
4. To study histochemical changes in both degeneration and regeneration of adrenergic nerves in arterial grafts

4. MATERIALS AND METHODS

4.1. MATERIALS

Experimental animals (I, II, III and IV)

Inbred A-O/Ks:OC 4-week to 11-month-old female rats were used. They were kept in the vivarium of the University of Helsinki Surgical Hospital. They had free access to commercial rodent fodder, and water was supplied by a drip bottle. They were housed in plastic animal boxes with wire covers in rooms maintained at a temperature of 22 ^{O}C. The weight of the animals ranged from 180 to 373 grams. Three rats were used in each group for our first set of experiments, five to seven in each group for the second and third sets of experiments and two for each observation period in the fourth set of experiments. A total of 82 animals was used for this study. The number of animals used in each of the original studies is summarized in Table 2.

Table 2.

EXPERIMENTAL ANIMALS					
STUDY	**N**	**Age**	**Weight, g**	**Follow-up time**	**Rats/group**
I	9	6 months	330–351	0h, 6h, 24h	3
II	30	3–10 months	220–373	0h, 6h, 12h, 24h, 48h	6
III	31	2–10 months	254–373	0h, 1w, 4w, 12w, 20w	5 to 7
IV	12	2–3 months	180–284	1d, 3d, 7d, 1m, 3m, 5m	2

4.2. METHODS

A variety of methods was used to study the grafts at different time points and they are summarized in Table 3.

Table 3. Methods and time points used in the original studies

Methods and time points	Glyoxylic acid fluorescence	HPLC	HPLC	Immuno-histochemistry
Article	I	II	III	IV
Time				
0 h	x	x	x	x
6 h	x	x		
12 h		x		
24 h/1 d	x	x		x
48 h/2 d		x		
72 h/3 d				x
1 w			x	x
4 w			x	x
12 w			x	x
20 w			x	x

Different methods were employed at different time points depending on their suitability.

4.2.1. SURGICAL METHOD (I, II, III AND IV)

Before the operation the rats were weighed and appropriate areas were shaved. The left femoral artery was exposed in the inguinal region through a longitudinal incision, the subcutaneous fat was lifted cranially and the artery was freed from the femoral vein and nerve. Care was taken not to damage these structures. The artery was then ligated just below the inguinal ligament and just below the branching point of the inferior epigastric vessels. The deep branch of the femoral artery was crushed with a clamp to stop it from bleeding and divided. The segment of femoral artery between the ligatures, about 8 mm in length, was then removed with scissors, rinsed with heparin solution (0.05 ml of heparin solution [Loevens Kemiske Fabrik, Ballenrup, Denmark] and 0.45 ml of sterile 0.9% NaCl) and the ends slightly dilated with microsurgical forceps under an operating microscope. The adventitia was carefully retracted from the ends of the artery. The segment was stored in 0.9% sterile saline. The inguinal incision was then sutured in one layer with 4/0 polyglycolic acid (Dexon).

A transverse incision was then made just cranially of the jugulum. The salivary glands were dissected free caudally and lifted cranially. The left carotid artery was exposed just lateral to the trachea and was carefully freed from the surrounding tissues. A microsurgical approximator clip was placed on the carotid artery and the artery was then cut between the clip jaws. The carotid artery was not resected.

The adventitia was pulled with microsurgical forceps and cut with scissors at both ends in order to keep it from getting between the end of the artery and the graft.

The graft was placed between the ends of the artery and sutured in place with interrupted 10/0 nylon monofilament sutures. First the proximal anastomosis was completed and checked by injecting heparin solution into the graft through the open end. Then the distal anastomosis was completed. After completing the distal anastomosis, retrograde flow was brought about by easing the distal clip of the approximator device. Hence patency and hemostasis were investigated. If necessary, hemostatic sutures were placed at that point, the proximal clip was eased and a patency test performed. When free flow could be demonstrated and there was no bleeding, the jugular wound was closed with cuticular 4/0 polyglycolic acid sutures.

4.2.2. ANESTHESIA (I, II, III AND IV)

The rats were anesthetized with a mixture of Dormicum (midazolam, 1.0 mg/ml, F. Hoffman-La Roche, Basle, Switzerland) and Hypnorm (mixture of phentanyl citrate, 315 mg/ml and flunisole, 10 mg/ml, Jansen Pharmaceutical Ltd, Grove, Oxford, UK). For anesthesia a solution consisting of equal parts of both agents and two parts of water (sterile, distilled pharmaceutical water) was prepared and the rats were given 0.2–0.4 ml of the solution intraperitoneally. Grafts were inserted under a WILD stereo operating microscope (M651, type 32 7733, Heerbrugg, Switzerland). At certain points during the operation and if necessary, 0.1–0.2 ml of anesthetic solution was given intraperitoneally.

4.2.3. GLYOXYLIC ACID-INDUCED FLUORESCENCE (I)

The grafts and control specimens were treated with 2% glyoxylic acid (glyoxylic acid monohydrate dissolved in 0.1 M phosphate buffer, pH adjusted to 7.2 with NaOH). After at least 20 minutes of immersion the specimens were blotted dry, stretched on microscope slides and dried with hot air from a hair dryer. The specimens were then heated for six minutes at 100 ^{o}C and mounted in Entellan (Merck) as described by Waris and Partanen (1975). Samples from one animal were treated as above but glyoxylic acid was omitted from the phosphate buffer. No catecholamine fluorescence could be demonstrated in the samples from this animal.

4.2.4. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (II AND III)

After the specimens were collected they were dissected free of any remaining suture material on a Petri dish resting on a bed of crushed ice. The grafts and control specimens were then dried quickly by blotting on tissue paper and stored in individually marked small plastic bags in a freezer at -80 ^{O}C. They were later transported to the laboratory for analysis in a styrofoam container kept cold with solid carbon dioxide.

For determination of noradrenaline content the weighed sample of artery was homogenized in 200 µl ice-cold 0.1 M HClO4 solution and the homogenate was centrifuged for 20 min at 25000 x g. Noradrenaline from 50 µl of the supernatant was extracted into a solution of 30 mg Al2O3 in 3 ml of tris HCl buffer (pH 8.65) in a 5-ml conical test tube. 3,4-Dihydroxybenzylamide hydrobromide (Sigma, St Louis, MO, USA) was used as an internal standard to correct for absolute recovery variations of noradrenaline. After washing four times with 2 ml H2O, noradrenaline was eluted into 50 µl 0.2 M HClO4 solution. Noradrenaline in the eluate was measured by high-pressure liquid chromatography with an electrochemical detector (ESA Coulochem Multi-Electrode, model 5100A, ESA Inc., Chelmsford, MA, USA). An ESA catecholamine HR-80 column (80 × 4 mm, 3 µm) and methanol phosphate buffer, pH 2.2 (ESA cat-A-Phase reagent) as mobile phase were used. The flow rate was 1.5 ml/min. For calibration purposes, known noradrenaline standards were treated in the same way as samples and the peak height ratio (relative to the peak height of the internal standard) of the unknown noradrenaline concentration in the sample was compared with that of the known synthetic standard (D-2500 Chromato-Integrator, Hitachi, Tokyo, Japan).

4.2.5. MICROSCOPIC EVALUATION (I AND IV)

For glyoxylic acid fluorescence, microscopic observations were made using a Leitz Aristoplan dark field fluorescence microscope (Type 020-503 030) with an automatic Leitz microscope camera. The microscope was equipped with an Osram ultraviolet-emitting bulb, type HBO 102. The filter combination employed for fluorescence microscopy consisted of I-2 450-490 nm (blue) and 510-520 nm (green) fluorescein filters.

For immunohistochemistry the specimens were then examined and photographed using a Leitz Vario-Orthomat (Ernst Leitz GmbH, Wetzlar, Germany) fluorescence microscope. Fluorescence was detected using rhodamine (block N 2.1, excitation 515–560 nm; emission 580 nm) and/or fluorescein (I3, 450–490 nm; 510–520 nm) filters. For microphotography Ilford HP5 and Kodak TMAX 400 ASA films were used.

4.2.6. IMMUNOHISTOCHEMISTRY (IV)

Grafts were collected under anesthesia at preset time points, and they were immediately fixed by way of immersion for 1 to 2 hours in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). For cryoprotection the tissues were then immersed in 20% sucrose, frozen, and cut into 12-μm thick longitudinal cryostat sections. Several sections were stained and analyzed for each marker and time point.

The sections were then incubated with 5% normal swine serum in phosphate-buffered saline (PBS), pH 7.4, then overnight with the primary antibodies (Table 4). The samples were rinsed twice in PBS, followed by incubation for one hour with secondary antibodies. Secondary antibodies were rhodamine- or fluorescein-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoRes. Lab., Inc., West Grove, PA, USA) for the polyclonal antibodies, and fluorescein-conjugated goat anti-mouse IgG (1:300, Jackson ImmunoRes. Lab., Inc., West Grove, PA, USA) for the monoclonal antibodies. Each slide was rinsed twice in PBS and a coverslip applied using glycerol-Na-veronal mounting medium.

Table 4. Primary antibodies used in Study IV

ANTIBODY	SPECIES	DILUTION	SOURCE	REFERENCE
Tyrosine hydroxylase	rabbit, pc	1:1000	In collaboration with Tong Joh	Vanhatalo 1995
neuronal Nitric Oxide Synthase	rabbit, pc	1:1000–4000	Euro-Diagnostica, Malmö, Sweden	Alm 1993
Endothelial Nitric Oxide Synthase	mouse, mc	1:50–100	Transduction Laboratories, USA	commercial product
Inducible Nitric Oxide Synthase	mouse, mc	1:30–100	Transduction Laboratories, USA	commercial product
GAP-43	mouse, mc	1:500–1000	Boeringer-Mannheim, Germany	Schreyer and Skene 1991
NEUROFILAMENT	mouse, mc	1:10–30	Gift from Prof. I. Virtanen	Bäck et al. 1995

mc = monoclonal antibodies
pc = polyclonal antibodies

Neuronal fiber patterns and general changes in them were analyzed by studying neurofilaments immunohistochemically. Immunoreactivity against neuronal growth-associated protein 43 (GAP-43) reveals active axonal growth (Benowitz and Routtenberg 1997, Caroni 1997). Immunoreactivity towards tyrosine hydroxylase (TH) reveals sympathetic nerves. Vasoactive intestinal polypeptide (VIP) and neuronal nitric oxide synthase (nNOS) are both present in parasympathetic nerves and we identified these nerves immunohistochemically (Yun et al. 1997, Morris et al. 1998, Yu et al. 1998). We also studied two other isoforms of nitric oxide synthase:

endothelial nitric oxide synthase (eNOS), which is an important regulator of vascular tone (Iadecola 1993, Goodson et al. 1994, Pluta et al. 1996), and inducible NOS (iNOS), which is often activated during tissue trauma (Nathan 1992, Forstermann 1994).

The specificities of the antibodies used in the present study are described elsewhere, as indicated in Table 5. Specificity information on the commercial iNOS and eNOS antibodies is not available. Although extensive sequence homology between NOS isoforms may result in cross-reactivity of these antibodies, the distribution of staining observed in the present study supports the assumption that the iNOS and eNOS antibodies stain macrophages and endothelium, respectively (IV).

4.2.7. STATISTICAL ANALYSIS (II AND III)

The results are presented as means and standard deviations (SDs). The results were analyzed (after a test of normality) using one-way analysis of variance (Anova). Differences between graft and control samples were analyzed by paired Student's *t*-tests. The level of significance was set at $p<0.05$. Tukey's *post hoc* test was carried out to compare the graft groups with each other. Statistical analysis was performed by prof. P Huttunen in conjunction with the HPLC analysis.

5. RESULTS

5.1 GENERAL

All animals survived the operation. In the 0-hour group there was no time for complications to occur since the animals were sacrificed immediately. In the 6-hour group the animals still appeared to be under the effects of anesthesia. At 24 hours the animals had fully recovered from these effects. In the 6-hour group two grafts showed evidence of thrombosis; these were rejected and new animals were operated upon for this group.

5.2. CONTROLS

In the first set of experiments contralateral inguinal and inferior epigastric vessels as well as dorsal segmental subcutaneous vessels were used as controls. A typical fluorescent network of adrenergic nerves was seen in the femoral arteries and veins. In the arteries the nerves formed a very dense network in the adventitia. In the veins the neural network was less dense and the fibers were spaced at more regular intervals than in the arteries; they were also less tortuous than in the arteries. In the inferior epigastric arteries and veins, as well as in the segmental dorsal arteries and veins the patterns were similar in both types of blood vessel (Fig. 5).

In Studies II and III we used contralateral femoral vessels as controls and the results are shown in Figures 10 a and b, 11 a and b. There was no significant difference between the 0 h, 6 h, 12 h and 24 h control groups (p values > 0.05). In the 48-hour control group there was a statistically significant ($p<0.05$) fall in the noradrenaline content compared with the other control groups.

In immunohistochemistry, samples of carotid (host) and femoral arteries (graft) were analyzed. Immunoreactivity against any of the mentioned markers was very sparse in the carotid artery, indicating a sparse innervation pattern in general. In contrast, there was a markedly denser perivascular nerve plexus in the femoral artery compared with the carotid artery, and it exhibited immunoreactivity against all the markers used in the present study. The visual appearance of fiber densities in the femoral arterial grafts was comparable to that seen in intact femoral arteries.

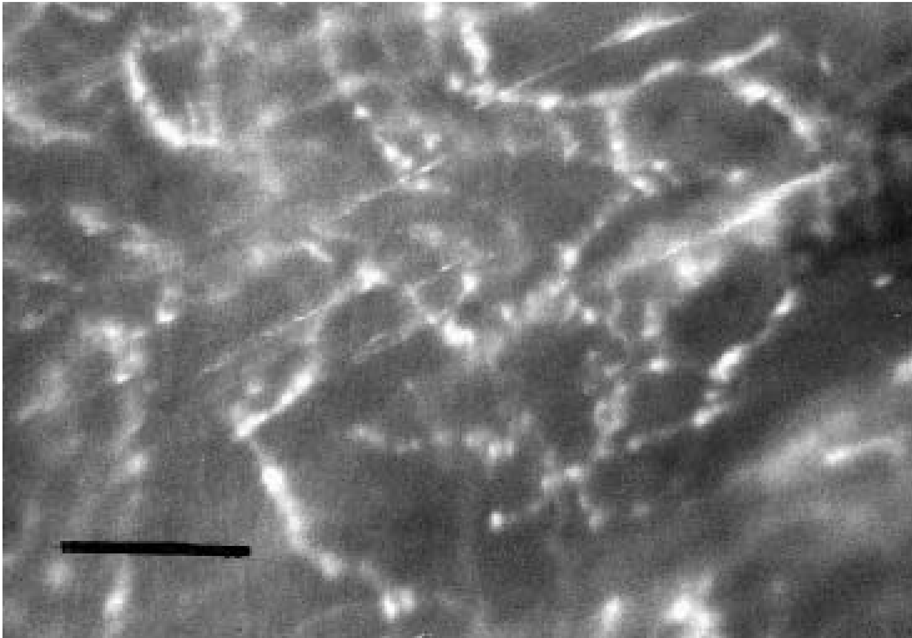

Fig. 5. Control specimen of an non-operated rat femoral artery wall showing an intact neuronal fluorescent network. Black bar, 100 μm.

5.3. GRAFTS

Graft weights

Grafts differed from controls in weight. In all groups the grafts were heavier and the difference was statistically significant in all groups apart from the 0 h group (1 w $p<0.001$, 4 w $p<0.01$, 12 w $p<0.01$ and 20 w $p=0.001$). The grafts were at their heaviest one week after the operation, corresponding to the clinical finding that they were edematous and thick-walled (Table 5).

Table 5. Graft and control weights in milligrams

WEEKS		N	MEAN	ST DEV
CONTR. WEIGHT	0	6	0.9683	0.1477
	1	5	1.1060	0.2281
	4	6	1.0967	0.2443
	12	6	1.2583	0.1578
	20	7	1,1243	0.1875
TOTAL		30	1.1243	0.2039
GRAFT WEIGHT	0	6	0.9567	0.3028
	1	5	2.5620	0.7493
	4	6	1.9017	0.4280
	12	6	1.7550	0.2843
	20	7	1.8829	0.3758
TOTAL		30	1.7890	0.6476

Table 6. Noradrenaline concentrations in the grafts and controls in each experimental animal group shown as ng/mg of tissue, and standard deviations

Noradrenaline content of grafts and controls in this material				
	Mean grafts		**Mean controls**	
Group	NA ng/mg	SD	NA ng/mg	SD
0h	0,7596	0,1963	1,1105	0,3695
6h	1,5575	1,1115	1,1972	0,518
12h	0,5645	0,1828	1,0113	0,1864
24h	0,1963	0,1556	0,9004	0,3594
48h	0,0293	0,05	0,4982	0,0205
1w	0,0176	0,0152	0,1163	0,2978
4w	0,2797	0,2196	0,1411	0,383
12w	0,3653	0,2796	0,1347	0,3187
24w	0,6646	0,4769	0,148	0,4398

0 hours (I–IV)

In the grafts a rich adrenergic network was seen at 0 hours (Fig. 6) by using glyoxylic acid fluorescence (I). This network, however, was not exactly like that of the control femoral arteries (Fig. 5) but seemed to be coarser and some of the filaments seemed to have slightly lost their fluorescence.

In the 0 h group the grafts were collected immediately after operation. In this group the noradrenaline content in the grafts (measured by HPLC) was 76% of the control value, but the difference was not significant (II, III). Since there was no delay after the operation it seems that that operation itself caused this difference

In immunohistochemistry both GAP-43 and NF were present in the tunica adventitia in a dense network of fibers, as well as the parasympathetic markers nNOS and VIP (IV).

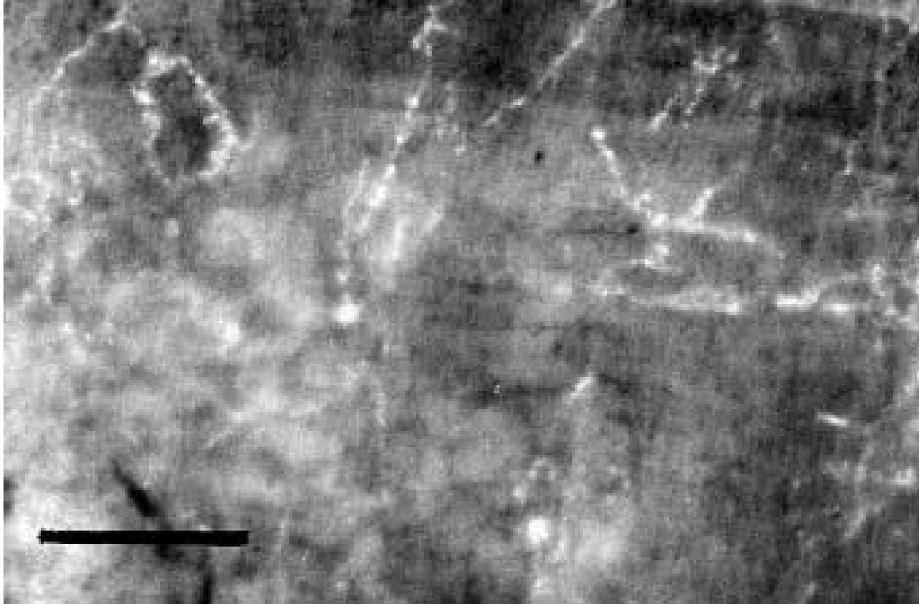

Fig. 6. A graft harvested immediately after operation showing an intact neuronal network

6 hours (I and II)

At 6 hours fewer fluorescent fibers could be visualized by means of glyoxylic acid (Fig. 7). They still formed a network although it appeared to be more diffuse and the fibers interrupted. There was a loss of fluorescence compared with the 0 hour group (I). There was a significant rise in noradrenaline concentration while using HPLC when compared with the 0-hour group ($p=0.05$) (II) (Figs. 9a and b).

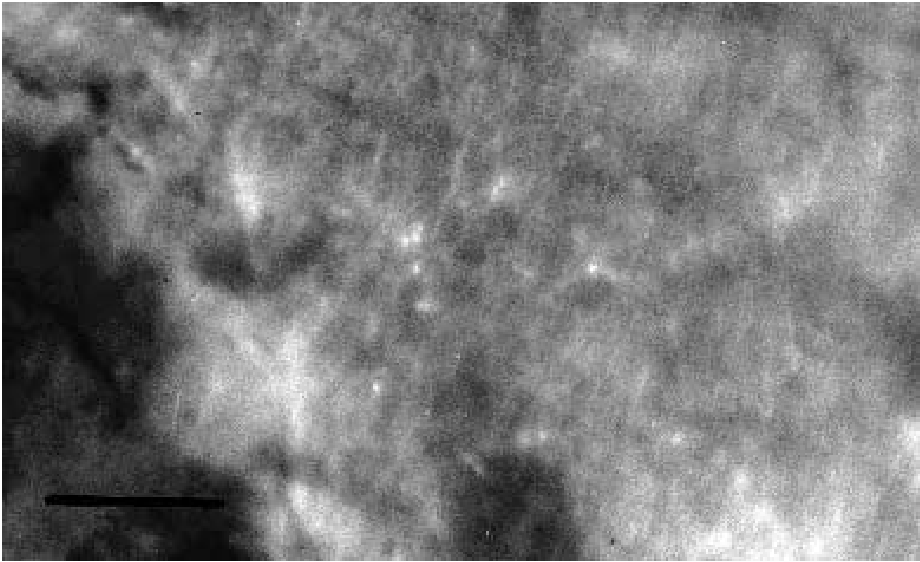

Fig. 7. A graft six hours after operation showing a diminished amount of glyoxylic acid-induced fluorescence. Black bar, 100 μm.

12 hours (II)

At 12 hours HPLC showed that there was a decrease in noradrenalin concentration when compared with the 0 h and 6 h groups (Figs. 9a and b).

24 hours (I, II and IV)

At 24 hours almost all fibers had disappeared and only dots of glyoxylic aid-induced fluorescence were present. These dots were scattered irregularly over the graft and were very dim. An adrenergic neuronal network could no longer be identified (I) (Fig. 8).

There was a significant decline in noradrenaline concentration (in HPLC) when compared with the 6-hour group.

In immunohistochemistry, TH-positive fibers were equally abundant in operated femoral arteries and in control specimens. NF and GAP-43 immunoreactivity showed the beginning of degeneration at this point and the staining fibers had swollen (IV).

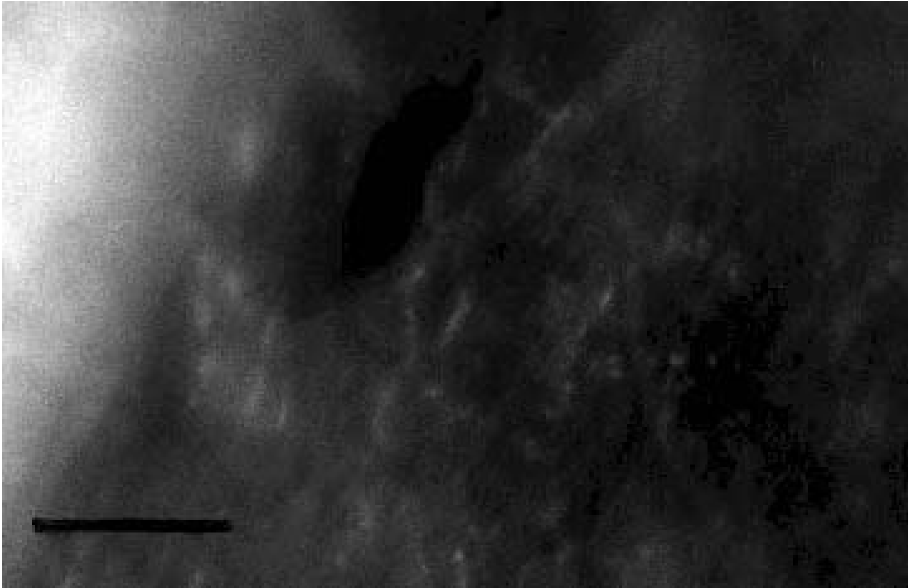

Fig. 8. A graft harvested 24 hours after operation showing scant fluorescence with no identifiable neuronal structures. Black bar, 100 μm.

48 hours (II)

In the 48-h group of grafts there were three specimens in which the concentration of noradrenaline was below the detection limit of high-pressure liquid chromatography, and they were marked 5 pg/mg (the lower detection limit of our method). The noradrenaline concentration had dropped to an almost nonexistent level.

72 hours (IV)

At 72 hours both NF and GAP-43 staining patterns were sparser but they had not disappeared completely. TH immunoreactivity had disappeared almost completely and so had that of the parasympathetic marker VIP, which tended to disappear more rapidly than the other parasympathetic marker nNOS. Some fibers positive for nNOS immunoreactivity were still seen three days after the operation.

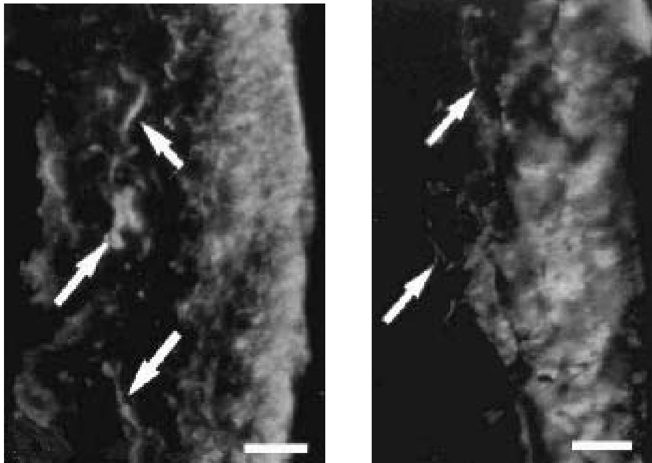

Fig. 9. Tyrosine hydroxylase immunoreactivity 1 (left) and 3 days (right) after the operation, showing a clear decrease in the amount of fluorescence (IV). Arrows indicate the white lines of tyrosine hydroxylase fluorescence

7 days (III and IV)

In HPLC, the noradrenaline concentration was about 1.5% of that of the controls at one week (III), a value significantly lower ($p<0.001$).

In immunohistochemistry some neurofilaments were still present at the ta-tm border. Only some very thin fibers had immunoreactivity for GAP-43. TH immunoreactivity was almost absent at this time. The parasympathetic markers VIP and nNOs had all but disappeared (IV). One week after the operation the denervation process had been completed.

4 weeks (III and IV)

At this point the mean noradrenaline level in the grafts was 19.8% of that in the controls, using HPLC (III).

TH-IR was seen mostly in the ta, but there were only a few fibers. Only a few GAP-43-positive fibers were encountered in immunohistochemistry. There was an abundance of NF-staining fibers, mostly in the ta and sometimes in the tm. Only a few nNOS- and VIP-staining fibers were seen and these were mostly in the ta (IV).

12 weeks (III and IV)

At 12 weeks the graft NA concentration measured by HPLC was significantly lower than in the controls (27%, p<0.01) and in the 5-month graft group, but it did not significantly differ from that in 1-week and 1-month grafts (III).

TH-IR had increased when compared with the 1-month group and there was even some immunoreactivity on the surface of the tunica media. GAP-43 immunoreactivity had profoundly increased in the tunica adventitia and NF immunoreactivity was abundant both in the tunica adventitia and in the tunica media. NF immunoreactivity was found in relatively thick bundles. Very dense nNOS-positive immunoreactivity was found in outer parts of the tunica adventitia and in occasional fibers on the surface of the tunica media. VIP showed a different pattern of distribution – it was found mostly in outer parts of the tunica media, with only some fibers in the tunica adventitia.

20 weeks (III and IV)

After 5 months noradrenaline levels were 44.9% of those in the controls, a value significantly lower than in the controls and significantly higher than in one-week grafts (p=0.004), but not significantly different from values in the 0-week or 12-week groups (III).

TH-IR was present both in nerve fibers and bundles. GAP-43 immunoreactivity had decreased to a level corresponding to that at one month, whereas NF-IR was observed to be increased but only in thick bundles, not in single fibers. There was an increase in the number of nNOS-positive fibers when compared with the three-month group (IV).

eNOS and iNOS findings

Immunohistochemistry for iNOS revealed no cellular profiles in the control specimens. A small number of iNOS-positive cells was observed in the tunica adventitia 1 day after grafting, whereas at 3 and 7 days after surgery, the number of these positively stained cells was somewhat increased. The iNOS-positive cells were equally distributed along the graft periphery and the graft-host junction, and they were round in morphology, resembling activated mast cells. High-intensity staining for eNOS was seen in all tissues with intact endothelium. There was no decrease in the staining for eNOS during the 7 days of follow up; however, the endothelium was partially detached in some of the grafts, probably because of minor inadvertent trauma to the vessel walls during the grafting procedure (IV).

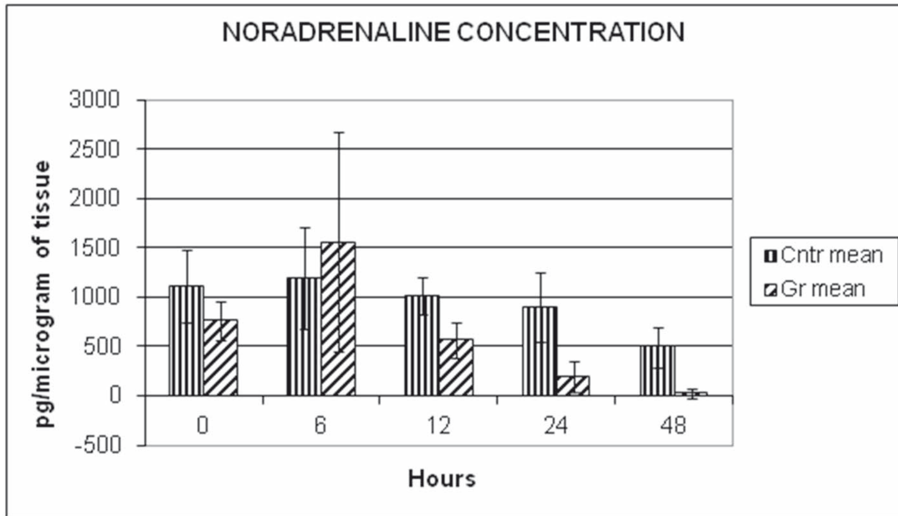

Fig. 10a. Noradrenaline concentrations (pg/µg tissue) for each group of rats. Bars show mean values for the graft (Gr) and control (Cntr) groups, with SDs. The rise in noradrenaline concentration in grafts at 6 h was statistically insignificant ($p>0.05$). After 6 h the concentration in grafts fell significantly ($p<0.05$). In 48-h control specimens the decrease in noradrenaline concentration was also statistically significant ($p=0.04$) compared with 0-h controls.

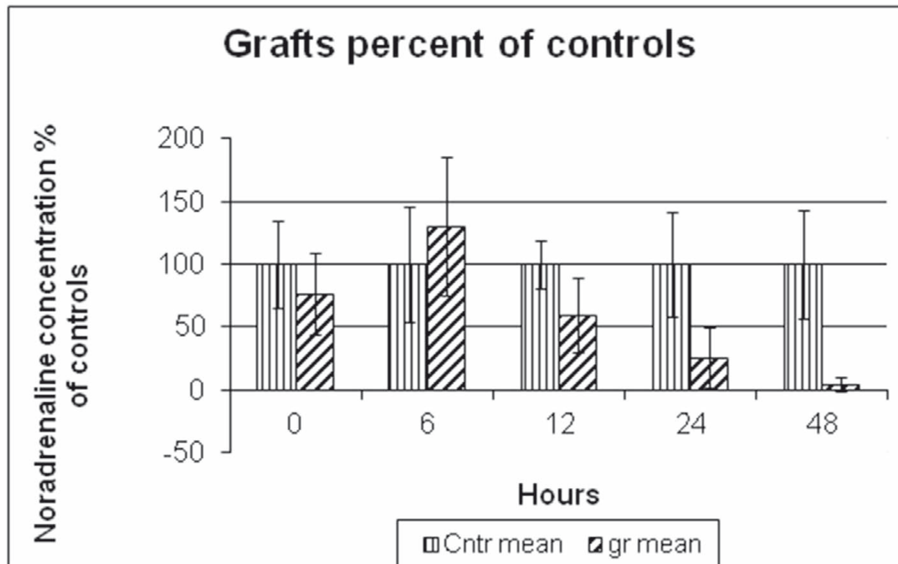

Fig. 10b shows the same results as in Fig. 10a, but as percentage of controls.

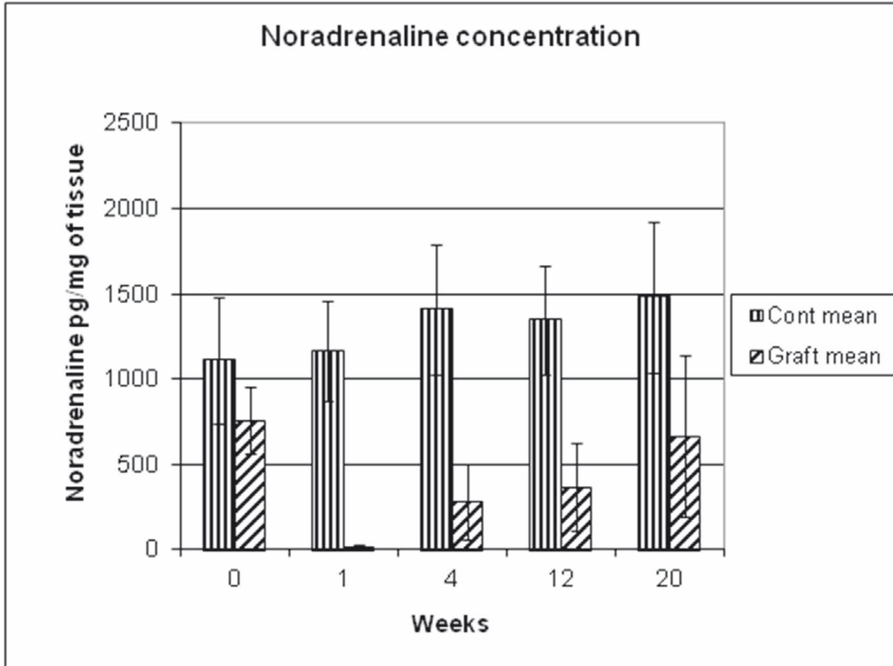

Fig. 11a. A chart showing noradrenaline concentrations (pg/mg tissue; mean ± SD) in graft and control arteries during the postoperative period (weeks). The difference between controls and grafts immediately after operation was statistically insignificant (p=0.053). One week postoperatively the difference was statistically significant (p<0.001). In the grafts, noradrenaline concentrations showed a rising trend from one to 20 weeks (statistically significant p=0.004). Each group consisted of six rats except for the 20-week group, which consisted of seven rats. The data was analyzed by means of Student's t-test (paired).

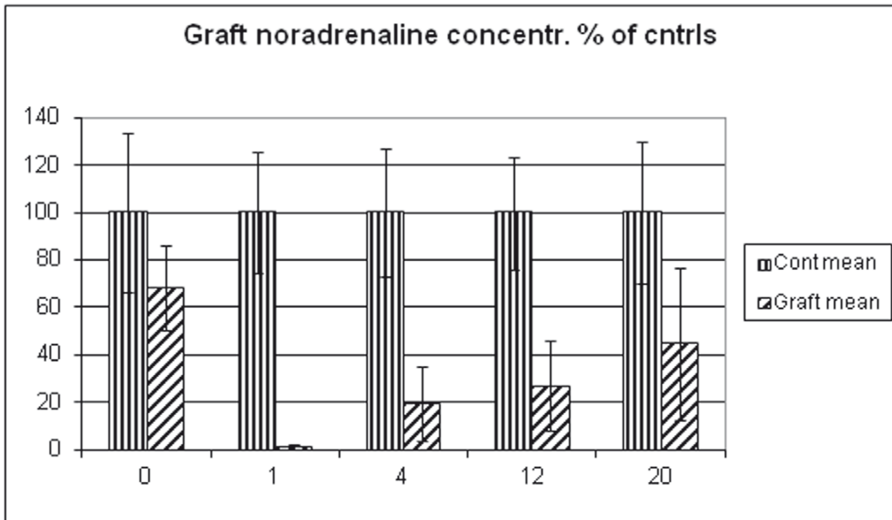

Fig. 11b shows the same results as Fig. 11a, but as percentage of controls.

Combinet results of the original article are shown on pages 48-49 (Table 7).

Table 7. Results of the original articles

Method ↓ Time →	0 h	6 h	12 h	1 d	2 d
Glyoxylic acid-induced fluorecence	almost intact	little diminished		none left	
HPLC	no significant difference between grafts and controls	significant rise compared with 0-h grafts	significant fall in NA concentration	significant fall in NA concentration	significant fall in NA concentration
Histochemistry					
Tyrosine hydroxylase	initially rich IR against TH				
nNOS	initially rich IR against nNOS			fewer fibers with IR against nNOS	
eNOS				intense IR against eNOS	
iNOS	few cells present in the ta				
VIP	rich IR against VIP			significantly less IR	
GAP-43	rich IR throughout ta			less IR than in 0-h group	
Neurofilament	rich IR throughout ta			less IR than in 0-h group	

3 d	1 w	1 m	3 m	5 m
	significant difference in NA concentration vs. 0-h group	significant rise in NA levels vs. 1-w group	significant rise in NA levels vs. 1-w group	No significant difference vs. 0-h group in NA concentration
IR becoming sparse	IR against TH disappeared	only occasional fibers with TH IR	more fibers with TH IR	still more IR against TH
IR becoming sparse	IR against nNOS disappeared	sparse IR against nNOS	more IR against nNOS	rich IR against nNOS
intense IR against eNos	intense IR against eNOS			constitutive expression of IR against eNOS
	marked increase in the number of cells with IR			only a few cells present
none present	completely absent	sparse IR against VIP	more than in 1-m group	rich IR against VIP
less IR in ta	few fibers to be seen	few nerve fibers with IR present	dense network with IR present	less IR than in the 3-m group
less IR in ta	fewer NF-staining fibers to be seen			

6. DISCUSSION

6.1. GENERAL METHODOLOGY

Several methods have been employed to assess adrenergic nerve plexuses quantitatively. These methods include visual and grid-counting techniques of nerve densities and estimates of visual intensity (Cowen and Burnstock 1980). Other methods, such as automatic quantitative image analysis have been introduced for the same purpose. High-performance liquid chromatography (HPLC) with electrochemical detection is a well-established method for measuring tissue catecholamines. It is a highly sensitive method and capable of revealing even small changes in catecholamine content (Taylor et al. 1983, Cheng et al. 1995). The sensitivity of the method is indicated by the changes in the noradrenaline content of the control samples during the postoperative period. Severed nerve axons are probably unable to maintain biochemical processes that replenish the stores of noradrenaline in nerve endings.

There is an initial breakdown of axonal elements in transected nerves (Waller 1850). Noradrenaline is expected to disappear from vascular grafts shortly after the operation due to Wallerian degeneration. The fall in noradrenaline content in the 0-h graft group can be attributed to handling of the grafts during the operation, compared with the controls. However, the noradrenaline concentration in control specimens was within the 95% confidence interval of the concentration in the grafts. This decrease in noradrenaline content due to perioperative handling is clinically significant and may be the explanation behind instances of vasospasm occurring during and immediately after operation.

Catecholamines have a half-life of a few minutes in tissues, but they do not disappear from grafts within the first few hours, as one might expect. In the 12-, 24- and 48-h groups there was a steady decrease in noradrenaline content. This is well in accordance with other findings reported in the literature (Cowen et al. 1982, Hukki et al.1989) and also as regards distal parts of denervated arteries (Kaarela 1991a, Kaarela et al. 1991c, Waris 1991). Our findings concerning glyoxylic acid-induced fluorescence suggest that noradrenaline disappears in a linear fashion. The relationship between fluorescence and noradrenaline content is known not to be completely linear (Van Orden et al. 1967). It is also very difficult to visually estimate the amount of non-neuronal tissue catecholamines. We propose that contrary to what is believed, severed axons might retain some ability to take up and concentrate catecholamines for some hours after transplantation of an arterial graft into a recipient vessel. This could be connected with the integrity of axonal

membranes and the presence of energy stores needed for biochemical processes that maintain noradrenaline stores. It may be of great clinical significance.

6.1.1. GLYOXYLIC ACID-INDUCED FLUORESCENCE

Histochemical demonstration of catecholamines by way of formaldehyde-induced fluorescence has long been the method of choice for demonstration of the transmitter substance itself. Glyoxylic acid fluorescence was originally described by Axelsson and others, and by Lindvall and Bjöklund (Axelsson et al. 1972, Lindvall et al. 1974), and it was a refinement of the formaldehyde-induced method originally described by Eränkö (Eränkö 1955, 1967, Falck 1962). Tyrosine hydroxylase, an enzyme that is rate-limiting in the formation of catecholamines, indicates only if catecholamine synthesis is present in tissues, or more precisely if such capability is present in tissues. Therefore, although glyoxylic acid fluorescence is an old method used for demonstration of catecholamines, it still holds its place in histochemistry. Stretch preparations have widely been used for demonstrating various neuronal structures, both by glyoxylic acid-induced fluorescence and by immunohistochemistry (Waris and Partanen 1975, Seiger et al. 1984). The resolution of catecholamine detection improved after glyoxylic acid was introduced as a condensation agent (Lindvall and Björklund 1974, Waris and Partanen 1975), and there have been many modifications of the glyoxylic acid method (Korkala and Waris 1977, Waris et al. 1978a, Waris et al. 1978c, Seiger et al. 1984). The glyoxylic acid-induced fluorescence method has been shown to be both specific and sensitive (Waris 1978a, Waris 1978c) and it has been found to be useful in histochemical studies on the degeneration and regeneration of adrenergic nerves following sympathectomy (Kaarela et al. 1991a, b, c, 1994).

The use of glyoxylic acid-induced fluorescence allowed the transmitter substance to be demonstrated, not the enzymes or other protein components of the nerve fibers, as in immunohistochemistry (Waris 1977, Waris et al. 1978a, c). It explains the difference between the results in Study I, in which catecholamines from the adrenergic nerves of arterial grafts disappeared within 24 hours, and those of Seiger et al. (1984), who immunohistochemically demonstrated the disappearance of reactive nerve fibers in transplanted irises after one week. This finding, on the other hand, is in concordance with the findings in Study IV, where NF-IR persisted for a week after grafts had been inserted in the carotid artery. The disappearance of catecholamine fluorescence from arterial grafts within one day corresponds well with earlier observations on the disappearance of catecholamines from surgical flaps (Waris et al. 1978 b.) and from vein grafts (Hukki et al. 1989). Our results correspond well to earlier observations in other organs; adrenergic denervation occurred 16–28 hours after axotomy in cat irises (Malmfors and Sachs 1965) and a similar observation has been observed in rat irises (Olson and Malmfors1970).

6.1.2. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography is a method that is used for assay of numerous compounds, including biogenic amines. It was developed in the 1970s to improve the existing techniques of open-column chromatography, thin-layer chromatography and paper chromatography, which were inadequate as regards quantification and differentiation of related compounds. In precise measurements of the amount of catecholamines in tissues it has proven to be an invaluable tool (Taylor et al.1983, Cheng et al.1995). The system comprises a solvent reservoir from which the solvent is drawn. There is an injection port through which the sample is injected into the system. The sample is carried by the solvent through the column, which is a glass tube of 3–5 mm internal diameter, or a microcolumn of 3–200 µm diameter. The glass column is filled/surface-coated with a substance that separates the different compounds as they pass through it. Identification of the compound is accomplished by way of detectors. These can be of different types, namely refractive index, ultraviolet, fluorescent, radiochemical, electrochemical, near-infra-red, mass spectroscopic, nuclear magnetic resonance and light scattering. Each one of these involves use of a different kind of physical or chemical phenomenon to detect changes in the content of the solvent phase and a signal is emitted when such a change is detected. Electrochemical detectors, as used in our studies, detect compounds that undergo oxidization and reduction reactions on the detector surface. The detector is placed just behind the column in the array. Quantification is an important part of the process. The signal from the unknown concentration in the sample is compared with a series of peaks in a chromatogram created by a series of known concentrations of sample solutions. By using a triangular equation the areas of these peaks are calculated to create a calibration curve and the measurement obtained from the sample is then compared with this calibration curve.

6.1.3. IMMUNOHISTOCHEMISTRY

Immunohistochemistry was first used by Marrack to demonstrate typhoid and cholera organisms (Marrack 1934). Coons et al. (1941) demonstrated pneumococcus type III organisms, using β-anthryl isocyanate-conjugated antibodies against them. He was able to demonstrate the difference between types II and III pneumococci. This was a direct reaction and it had poor sensitivity. Later, an indirect method was developed with an unlabeled primary antibody against the tissue antigen and a labeled secondary antibody against the primary one. This enhanced the color reaction because several secondary antibodies were bound to the primary antibody. The method also allows several different kinds of secondary antibodies to be used with the primary one. Fluorescent markers are unstable and later in development the

antibodies were linked with enzymes such as horseradish peroxidase. This made it possible to demonstrate the antibody with a permanent stain in the specimen brought about by a deposit from a diaminobenzidine reaction with hydrogen peroxide as substrate. Later still the sensitivity of immunohistochemistry was increased with the introduction of the peroxidase-antiperoxidase (PAP) complex and the avidin-biotin complex (ABC). Normal serum is used in the first incubation to block out nonspecific binding of primary antibodies. (Burnett et al. 1997)

6.1.4. EXPERIMENTAL MODEL

Because of the availability of rats in our laboratory, all rats were in the age range of 2–5 months, except for those in the 0-h group (n=3), which were aged 10 months. Since the control values in this group were no different from those in the other control groups, with the exception of the 48-h group, we found that age should have no effect on the outcome of the experiment. Also, since we were studying the disappearance of noradrenaline, which results from the breakdown of the molecule in a nonenzymatic manner, our assumption was that the age of the animals was not critical. It would be a completely different matter if we had been studying the regeneration of nerves or the synthesis of noradrenaline. However, age homogeneity should be considered in future studies.

We used six animals per group. In this kind of study, five animals per group is the minimum needed. A larger group of animals would reveal smaller differences, but that would mean a greatly increased labor input and since there is a recommendation that as few laboratory animals as possible should be used, we opted for this group size.

In this work we demonstrated the rate of disappearance of noradrenaline in arterial grafts. This rate is comparable with other findings (Cowen 1982), but it is definitely faster than the disappearance of neuronal structures from transplanted arteries.

At 6 h there was a significant rise in noradrenaline content of the grafts compared with the 0-h grafts. The mean control value fell within the 95% confidence interval of noradrenaline concentrations in the grafts at six hours. All concentrations in other grafts were below control values, even in the 0-h group. The 6-h group differed significantly from the 12-, 24- and 48-h groups ($p<0.005$) but did not significantly differ from its own control group. No corresponding reports in the literature have been found. This may be due to the fact that the nerve fibers do not receive stimuli that would make them release noradrenaline to the synaptic void. At the same time the active, energy-dependent, re-uptake of noradrenaline occurs in the terminal ends of the fibers. Only when the nerve fibers lose their energy stores and start to degenerate do they liberate these accumulated stores of noradrenaline.

There was no significant difference between the 0-h, 6-h, 12-h and 24-h control groups. In the 48-hour control group there was a statistically significant fall in noradrenaline content compared with the other control groups. This may be because there was a longer period of time between the operation and sample collection and, hence, the rats were no longer undergoing a metabolic response to surgery. This could be reflected in the noradrenaline levels seen in the control specimens. As such, this is a clinically important finding, since it shows that during and immediately after the operation the rats were under stress and exposed to increased adrenergic stimuli.

The present results are clinically significant in microvascular- and coronary by-pass surgery in which arterial grafts are used. Catecholamines liberated from degenerating adrenergic nerves provide a strong vasoconstrictive stimulus to arterial wall smooth muscle cells and cause vasoconstriction in the graft. It is important to note that there is a critical period when there is a significant rise in catecholamine concentrations in arterial grafts during the first 6 postoperative hours. This may very well raise the risk of vasospasm and measures to minimize this risk should planned well in advance. The other important observation that may deserve special attention is the fact that the disappearance of noradrenaline from the grafts was slower than has been presumed (2 days instead of a few hours), which prolongs the postoperative period associated with a risk of vasospasm. We planned our experiments to cover 20 weeks of follow-up, since reinnervation reaches its final level at 18 weeks in vein grafts (Hukki 1989).

The effects of perivascular sympathectomy have been studied in animal models. It is known that perivascular sympathectomy does not remove distal vascular adrenergic innervation (Kaarela et al. 1991a, Kaarela et al. 1991c, Waris et al. 1991). Only the segment from which the adventitia is removed loses its adrenergic innervation. However, a graft significantly differs from a sympathectomized vessel, since it has been completely removed from its surroundings and all the afferent axons have been severed.

Neuronal regeneration is a subject of wide debate in the current medical literature, especially in transplantation surgery (Ferretti 2002). There are contradictory reports about its occurrence and clinical significance in different fields of surgery (Leverenz 2001). In microvascular surgery, reinnervation, and arterial spasm during the immediate postoperative period are important aspects, whereas, for example, the metabolic needs of a free TRAM flap (Transverse Rectus Abdominis Muscle) in breast reconstruction or a free latissimus dorsi muscle flap in the repair of open tibial fracture are, perhaps, not so great.

In conclusion, noradrenaline disappeared almost completely from arterial grafts in rats within one week postoperatively. Thereafter, noradrenaline levels increased over the follow-up period of 20 weeks, most probably as a result of reinnervation of the grafts. In rat femoral arterial grafts transplanted into carotid arteries, NA concentrations rose to levels that did not significantly differ from those in the controls

or the day 0 group. This can be interpreted as a sign of reinnervation of the grafts. There was an increase in the standard deviations along with the increase in mean values after immediate measurement in the grafts, but not in the controls. This might reflect different regenerative and growth capacities of individual rats as regards the grafts but not the non-operated (control) arterial segments where such processes are not expected to take place.

6.2. DEGENERATION

In the present study we elucidated the postoperative sequence of degeneration and regeneration of neuronal cytoskeletal structures, NFs and GAP-43, and changes in sympathetic and parasympathetic innervation. While our findings are in good agreement with those of previous studies on axonal degeneration (Waller 1850, Schlaepfer and Hasler 1979, Glass and Griffin 1991, Miki et al. 1992) and on nerve fiber growth into host grafts (Kawaguchi et al. 1998, Uberfuhr et al. 2000a, b, Ulrich-Lai and England 2000), we have extended the existing data by studying these phenomena in arterial grafts.

Immunoreactivity against the main axonal cytoskeletal protein, NF, almost completely disappeared during the first week after grafting. This indicates destruction of the axonal elements in degenerating fibers (Schlaepfer and Hasler 1979, Glass and Grifffin 1991, Miki et al. 1992). The few NF-positive axon profiles seen at seven days after the operation probably represent the remains of degenerating axonal fragments, and not necessarily functional fibers, since all markers of the different transmitters had disappeared at this time point.

In the present study there was still some TH-IR left in the enlarged, degenerating axonal endings one day after the operation, while all TH-IR had disappeared at later time points. Thus, enzymatic capacity for the synthesis of catecholamines may remain for a longer time than the catecholamines themselves. Lack of catecholamines in the terminals one day postoperatively may be a result of rapid release, or lack of other mechanisms required for synthesis (e.g. lack of precursor uptake or energy stores).

Similarly to the markers of sympathetic innervation, both markers of parasympathetic innervation, VIP-IR and nNOS-IR, were also lost during the first postoperative week. Immunoreactivity to VIP was seen only on day one after operation, while some nNOS-IR was still seen at the third postoperative day. VIP is a peptide transmitter substance *per se*, and its disappearance is probably explained by cessation of axonal transport of newly synthesized peptides from neuronal somata. Neuronal NOS is an enzyme of nitric oxide synthesis, and the time course of its disappearance better parallels that of TH in the sympathetic fibers. The results of

numerous studies have suggested that *de novo* production of nitric oxide is often induced in injured neurons (Lumme et al. 1996, Gonzalez-Hernandez and Rustioni 1999a, b). Since we studied degenerating axonal fragments with no connection to their somata, we can safely preclude any *de novo* expression of nNOS in our grafts. Rather, we suggest that nNOS-IR represents only the remains of the enzyme in the degenerating axonal fragments.

6.3. REGENERATION

We observed a robust increase in fine nerve fibers showing GAP-43 immunoreactivity at one month after the operation. In a recent study, Harding and coworkers (1999) demonstrated that the expression of GAP-43 is correlated with rapid axonal regeneration, while slow axonal elongation may also occur, independently of GAP-43 protein. Our observations support such a concept: we saw the highest number of GAP-43-positive fibers at one month after the operation, while the number of neural elements, as revealed by NF-IR, increased throughout the observation period. Interestingly, we found that the growth of nerve fibers proceeded over the host-graft junction, which indicates that the origin of reinnervation of the grafted femoral artery is the same as that of the host carotid artery. However, visual assessment of innervation densities suggested that the nerve plexus in the femoral artery graft was denser than in the host artery. This suggests that there might be some as yet unknown intrinsic properties in the femoral and carotid arteries that differentially determine their innervation patterns.

Immunoreactivity against VIP and nNOS appeared slightly earlier than that against TH, which may reflect different rates of axonal regrowth in different neuronal systems. Cervical sympathetic neurons may show *de novo* expression of nNOS (Vanhatalo et al. 1995b, c, Lumme et al. 1996) and neuropeptides (e.g. VIP) (Schotzinger et al. 1994) in response to axonal injuries, and it is possible that some of the first nNOS-positive nerve fibers represent a subpopulation of regenerating sympathetic neurons. While such transient changes in transmitter phenotypes cannot be excluded, our findings show that reinnervation of an arterial graft is associated with marked changes in its neurochemical composition. It seems logical to assume that this is also reflected in a changing role of nerves in control of the vascular tone in the graft, which deserves further study.

Innervation patterns are known to differ between different arteries, as seen in the current study as well as in other studies (Dhall et al.1986, Morris et al.1998). We observed that innervation of the grafts resembled that of a normal femoral artery more than that of the host (carotid) artery. Recent studies in developmental neurobiology (Rao and Landis 1993, Schotzinger et al. 1994, Kentroti and Vernadakis 1995) and on various models of tissue transplantation (Kiyochi et al. 1995, Tan and

Harvey 1999, Asmus et al. 2000) have repeatedly demonstrated that the target tissue is of crucial importance for determination of both the intensity and neurochemical identity of innervation. Our observations suggest that vascular grafts are also able to influence their own reinnervation. This may have far-reaching implications in the selection of appropriate vascular grafts. Donor sites for vascular grafts are usually selected on the basis of their availability, while the present results suggest that one may need to attempt to find vascular grafts that have innervation patterns causing as few complications as possible during both the immediate postoperative period and longer follow-up periods.

Immunostaining for eNOS demonstrated that this enzyme continues its constitutive expression in grafted arteries. Recent studies have provided evidence that intravascular operations may increase or induce the expression of eNOS (Bosmans 1996, Poppa 1998), which may be important for proper relaxation, and hence for prevention of vasospasm in recently operated arteries. In some of our samples we observed partial detachment of the endothelium, which probably impairs nitric oxide-dependent vasorelaxation in the graft, predisposing the graft to vasospasm. A third source of nitric oxide in vascular structures is via iNOS. Our study (IV) shows that it is only seen in some cells in the tunica media (probably macrophages), and hence our results do not support a role for iNOS as an important factor in controlling vascular tone.

6.4. CLINICAL CONSIDERATIONS

With more and more patients undergoing reoperations because of coronary artery disease, and simultaneously suffering from intermittent claudication and critical ischemia of the lower extremities, coronary surgeons sometimes find themselves in a situation where they have no veins to harvest for grafts. In such situations, certain arteries, mostly radial arteries from the upper extremities are used (Chen et al. 1996). The internal mammary artery from either side is used routinely and the right gastroepiploic artery sometimes (Grandjean et al. 1996) in coronary bypass operations. Radial and right gastroepiploic arteries reportedly become spastic and susceptible to hypercontractibility thought to be caused by catecholamines (Cate et al. 1996) and for this reason topical use of spasmolytic agents is recommended (He and Yang 1998) during preparation of the artery. Our finding that catecholamines disappear soon after grafting gives a clue to the underlying mechanism. Remes and coworkers (Remes et al.1985, Remes et al. 1990) have demonstrated that instrumentation causes crush injury to the vessel wall with loss of metabolic activity and inflammatory cell infiltration. In our work a crush zone was seen in segmental dorsal arteries after handling with forceps. In the central portion of this

zone, fluorescence had almost completely disappeared, suggesting injury to the adrenergic nerves and rapid disappearance of transmitting substances.

Clinical and histochemical studies have indicated that when open heart surgery is performed catecholamines freed from heart tissue can be a risk factor. If the heart tissue contains large adrenergic nerves with “droplet fibers“ or “adrenaline bombs“ containing huge local intra-axonal concentrations of catecholamines, mostly noradrenaline, there is an increased risk of arrhythmia and even death (Kyösola et al. 1988). It has even been proposed that β-blocking agents or calcium antagonists should be used for those patients who are at a greater than normal risk (Kyösola et al. 1985, Kyösola et al. 1988, He and Yang 1998). In microsurgery, medical treatment has been used to prevent aggregation of blood platelets (Gateley et al. 1996, Renaud et al. 1996) and papaverine and guanidine are in clinical use to prevent vasospasm. In addition, the local anesthetic agent lidocaine can be used to prevent vasopasm, as can warm saline compresses.

Noradrenaline concentrations in rats arterial grafts are elevated significantly six hours postoperatively, raising the risk of vasospasm. They also take longer than expected to become reduced (drop to 4%) by 48 h postoperatively. Proper attention in this early postoperative period after arterial grafting and should be considered to improve the outcome of vascular grafting. This is a novel finding with no preceding similar reports. The re-uptake of noradrenaline is a substrate-dependent process that derives its driving force from the Na^+/K^+-ATPase-generated Na^+ concentration gradient (Bönich and Brüss 1994, Inazu et al. 2003). Hence, as long as there are energy sources to run this process, the axons will accumulate noradrenaline. At the same time there are no axonal excitation signals arriving to cause the release of adrenaline and this does not happen until axonal degeneration with lysis of the storage vesicles takes place. Noradrenaline is then released to the surrounding tissue from the graft, with its adverse effect of vasospasm. After this period, with the axons dead and no adrenergic innervation, the graft is subjected only to circulating catecholamines until reinnervation takes place.

Neuronal regeneration is a subject of wide debate in the current medical literature, especially in transplantation surgery (Odaka et al. 2001, Ferretti et al. 2002). There are contradictory reports about its occurrence and clinical significance in different fields of surgery (Leverenz et al. 2001, Mondelli et al. 2001). In microvascular surgery, reinnervation, and arterial spasm during the immediate postoperative period are important aspects, whereas, for example, the metabolic needs of a free TRAM flap (Transverse Rectus Abdominis Muscle) in breast reconstruction or a free latissimus dorsi muscle flap in the repair of open tibial fracture are, perhaps, not so great. This may be because the flow rates in microvascular flaps are small, 2.6 to 18.5 ml/min/100 g of tissue (Lorenzetti et al. 2001).

Reinnervation patterns have been shown to be graft-dominated, not host-dominated (IV, Schotzinger et al. 1994, Kiyochi et al. 1995, Cowen et al. 1996,

Asmus et al. 2000,), and differentiation of the growing nerves has been shown to be affected by their surroundings (Chong et al. 1996, Cowen et al. 1996).

From our earlier studies we know that glyoxylic acid-induced fluorescence and noradrenaline itself disappear from arterial grafts within 24 to 48 hours (I). After perivascular sympathectomy, adrenergic nerves do not disappear totally from arteries (Ferretti et al. 2002), but the situation is different with arterial grafts. A graft is by definition completely cut off from any source of intact nerve fibers, both from axial innervation along the artery and from those fibers entering from surrounding areas.

In the present work noradrenaline levels were so high at the end of the follow-up period that in our view it is clinically significant. It is important to understand that a free flap is not a separate piece of tissue but will be incorporated to its surroundings closely and will respond to nervous stimuli after the reinnervation process has been completed. Antispasmodic medication should be administered immediately after the operation for as long as it can be supposed that noradrenaline is liberated from the nerves.

In conclusion, noradrenaline disappeared almost completely from arterial grafts in rats within one week postoperatively. Thereafter, noradrenaline levels increased over the follow-up period of 20 weeks, most probably as a result of reinnervation of the grafts. In rat femoral arterial grafts transplanted into carotid arteries, NA concentrations rose to levels that did not significantly differ from those in the controls or the day 0 group. This can be interpreted as a sign of reinnervation of the grafts. There was an increase in the standard deviations along with the increase in mean values after immediate measurement in the grafts, but not in the controls. This might reflect different regenerative and growth capacities of individual rats as regards the grafts but not the non-operated (control) arterial segments where such processes are not expected to take place.

Our findings show that degeneration of innervation of arterial grafts in rats occurs within the first seven days. Regeneration is already evident one month after operation, and it continues to proceed for at least four more months. It seems that parasympathetic nerves may grow somewhat faster than sympathetic nerves. This might cause transiently altered neuronal control of vascular tone during the first few postoperative months. Finally, our findings suggest that graft tissue determines the pattern of innervation, not the surrounding tissue from which the nerves sprout.

7. CONCLUSIONS

ONE:
It was established that catecholamine-induced fluorescence had disappeared from the grafts within 24 hours after the operation.

TWO:
Using HPLC we established that noradrenaline levels are significantly elevated in rat arterial grafts at 6 hours and take 48 hours to come down. This is longer than one would expect from the fluorescence study (I). This was an unexpected finding.

THREE:
Using HPLC we established that catecholamine levels increased over the follow-up period of 20 weeks, which can be interpreted to be a sign of reinnervation of rat femoral arterial grafts transplanted into carotid arteries. Concentrations of NA rose to levels that did not significantly differ from those in the controls or the day 0 group.

FOUR:
Our histological findings showed that degeneration of innervation of arterial grafts in rats occurs within 48 hours. The dissolution of nerve fibers is complete within one week and there is growth of nerve fibers into the graft after one month. It seems that parasympathetic nerves regenerate sooner than sympathetic nerves. The reinnervation pattern of grafted femoral arteries resembles that of a femoral artery more than that of the host carotid artery.

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