CHANGE IN SOMATOSTATIN RECEPTOR EXPRESSION AFTER VASCULAR INJURY

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ABSTRACT

Somatostatin is an inhibitory neuroendocrine peptide that exerts its effects through 5 functionally distinct receptor subtypes (SSTR1-5). Somatostatin analogues have been shown to be effective in inhibiting intimal hyperplasia after balloon induced vascular injury. However, the exact SSTR subtype responsible for the inhibitory effect of somatostatin on intimal hyperplasia is unknown.

***Purpose*:** To define the presence and abundance of the SSTR-2 subtype in a rat iliac balloon injury model of intimal hyperplasia.

***Methods*:** Transaortic balloon injury of the rat iliac artery was carried out. Rats were sacrificed at 48 hours, 1 week and 1 month post injury, perfusion fixed and samples of iliac arteries were immunostained with antibodies against SSTR 1,2, and 3. Expression of SSTR-2 was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). The PCR product was sequenced to confirm its identity.

***Results*:** Immunocytochemical staining demonstrated the presence of SSTR2 on the intimal surfaces of normal and injured vessels. SSTR2-immunoreactivity was more prominent at 1 week and 1 month post injury compared with 48 hours post injury. There was no immunostaining with SSTR1 and SSTR3 antibodies. PCR results confirm elevated SSTR2 in injured arteries.

***Conclusion*:** SSTR2 is expressed on endothelial cells in normal and injured rat vessels. Its abundance in the injured vessel was increased up to 1 month post injury.

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I. INTRODUCTION

Vascular injury is an important subject in clinical medicine. Many of the current therapies for coronary and peripheral vascular disease (angioplasty, endovascular stenting, endarterectomy, bypass surgery) induce vascular injury and are plagued by its repercussive effects. These interventional procedures cause a cascade of cellular responses to injury leading to renarrowing or restenosis of the artery which often translate into recurrence of symptoms in patients. Clinical studies have shown that restenosis occurs in 30-50% of coronary angioplasties 6 months after treatment[1-5].

Recent evidence has indicated that a somatostatin analog, angiopeptin, is effective in inhibiting intimal hyperplasia in animal models. Intimal hyperplasia (IH) is a proliferative process that represents the major cause of restenosis after arterial injury[6].

Somatostatin (SS) is a neuroendocrine peptide originally isolated from the hypothalamus as a growth hormone inhibitory substance. Subsequent studies of SS demonstrated a general inhibitory action on a variety of physiologic functions in different organ systems. These multiple effects of SS are transduced by a family of specific G-protein linked membrane-spanning receptors, of which 5 subtypes (SSTR 1-5) have been cloned to-date. Subsequent studies demonstrated each receptor subtype possesses a different affinity for SS as well as chemically synthesized agonists such as SMS-201-995 (octreotide), MK 678 (seglitide) and BIM-23014 (angiopeptin) [7].

In the following sections, the physiological function of SS and its receptors, the pathophysiology of vascular injury, and the evidence for SS as a factor in vascular injury are reviewed.

1. SS and SS analogues:

SS is widely distributed in many organs including the central and peripheral nervous system, pancreas, gastrointestinal tract, thyroid, kidney, adrenals, prostate and placenta [8,9]. In the peripheral nervous system, studies using immunohistochemical techniques have shown that SS is present in the sympathetic and sensory nervous system[10] as well as in the cardiac vagal nerves [11,12]. It has been shown to be released from primary sensory neurons via voltage and calcium dependent mechanisms[13].

The physiologic functions of SS include modulation of cognitive processes, inhibition of endocrine and exocrine secretion, neurotransmission and modulation of other neurotransmitter systems, intestinal motor activity, absorption, and vascular smooth muscle contractility [8, 14,15]. SS also has antiproliferative effects and may be an important hormonal regulator of cell proliferation and differentiation [16,17]. In the splanchnic circulation, SS effectively reduces blood flow. When given as intravenous injections, SS reduces portal venous pressure and hepatic blood flow by 9-28% [18-20].

Specific to the cardiovascular system, SS is particularly abundant in the right atrium and atrioventricular node of canines where it may act as a modulator of cholinergic transmission[22].

There are two naturally occurring forms of SS, SS-14 and SS28 [23,24]. The former is the result of proteolytic cleavage of the C-terminal portion of SS-28. The smaller 14 amino acid form is produced by neurons, gastric and pancreatic endocrine cells; the larger form is produced by enteric endocrine cells. In the circulation, SS is only present in picogram amounts since it is rapidly metabolized with half-life of 2-3 minutes[25]. The primary mode of action for SS produced in the endocrine cells is via paracrine or autocrine transmission.

Paracrine refers to transmission by diffusion to receptors of surrounding cells while autocrine refers to transmission to receptors on the same cell.

On the basis of its widespread inhibitory effect, SS has the potential to treat many human diseases caused by hyper-functional states. However, the clinical use of SS has been hampered by several disadvantages. These include the very short half-life in circulation (3 minutes) that dictates continuous infusion as the only way to ensure adequate systemic drug concentration. In addition, its diverse action contributes to multiple side effects including gall stone formation, malabsorption, hypoglycemia and hypothyroidism. Research into development of stable structural analogs has led to synthesis of a number of SS analogs; thus far, four of them, octreotide (SMS201-995, sandostatin), BIM-23014 (Lanreotide, Angiopeptin) and RC-160 (Octastatin, Vapreotide), are either in use or being evaluated in clinical studies. Figure 1 shows the amino acid sequence of each of these analogs, in comparison with that of the native peptide. These analogs possess a longer half-life and substitution of amino acids at key positions enhances the specificity of these drugs for individual SSTR subtypes.

2. SS receptors

Some of the more important functions of SS are neuromodulation, control of glandular secretion, smooth muscle contractility and cell proliferation[26]. These are transduced by a family of high affinity membrane receptors which are present in brain, gut, pituitary, endocrine and exocrine pancreas, adrenals, thyroid, kidneys, and immune cells [9,27-31]. Rat heart smooth muscle cell (SMC) express receptors for SS14. Binding of these receptors by SS14 leads to inhibition of SMC growth[33].

Figure 1. Structure of somatostatin 14, Octreotide, BIM-23014, RC-160

Somatostatin-14 H-Ala-Gly-Cys-Lys Asn-Phe-Phe-Trp-Lys-Thr-Ser-Cys-OH

Octreotide H-DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr(ol)

BIM-23014 H-DNal-Cys-Tyr-DTrp-Lys-Val-Cys-Thr-NH_2

Angiopeptin (BIM-23014C) NH_2-DNal-Cys-Tyr-DTrp-Lys-Val-Cys-Thr-NH_2

RC-160 H-DPhe-Cys-Tyr-DTrp-Lys-Val-Cys-Trp-NH_2

Sequence analysis of the cloned SS receptors indicates that they belong to the super family of G protein-coupled receptors with seven alpha-helical transmembrane segments[34]. There are 5 receptor subtypes described to date. (Table 1)

The availability of the DNA sequences for these receptors has made possible studies of their relative distribution, although the precise receptors expressed on blood vessels is presently unknown [35,36].

The 5 subtypes have a 42 to 62 percent identity in their amino acid sequences. However, they are pharmacologically, and functionally distinct. The receptors 1-4 have a higher affinity for SS 14 than SS28 while SSTR5 has a higher affinity for SS28. Activation of SSTR1 and SSTR2 results in the activation of tyrosine phosphatase, which is related to the anti-mitotic effects in some types of cells [37,38] Inhibition of cell proliferation with stimulation of SSTR5 is mediated through a different mechanism, probably involving changes in intracellular calcium mobilization[39]. SSTR2 may be the receptor subtype responsible for regulating release of growth hormone from the pituitary but not SSTR1 or SSTR3 since RNA blotting and in situ hybridization studies have shown SSTR2 to be selectively expressed in rat anterior pituitary. In contrast, SSTR1 and 3 are expressed at low levels. All 5 subtypes are capable of regulating intracellular cAMP levels, in part, through activation of inhibitory G proteins [40,41,42].

3. Pathophysiology of vascular injury, intimal hyperplasia and restenosis

Vascular injury, whether via open surgery or endovascular procedure initiates a process of proliferation analogous to generalized wound healing. The classical model for vascular injury is the balloon injury model produced by placing a balloon inside an artery,

Table I. Characteristics of Human Somatostatin Receptors

	SSTR1	SSTR2a	SSTR2b	SSTR3	SSTR4	SSTR5
Amino Acids	931	369	356	418	388	360
MRNA(kB)	4.8	8.9	2.3	5.0	4.0	4.0
Agonist specificity	SS-14	SS-14	SS-14	SS-14	SS-14	SS-28
G-protein activation	+	+	+	+	+	+
Adenyl cyclase coupling	+	+	+	+	+	+
Chromosomal location	14	17	17	22	20	16

inflating it and dragging the balloon to injure the vessel wall by distension[44-47]. Histopathologically, the healing process can be categorized into 4 stages.

Stage i: Thrombosis

The earliest response to arterial injury is the formation of thrombus at the injury site. Dependent upon nature of the injury, dragging the balloon strips off the endothelial cells lining the vessel exposing the subendothelial matrix. Platelets from the bloodstream quickly attach to this denuded surface along with fibrin and erythrocytes. The resultant thrombus usually accumulates within minutes after the arterial injury. This process involves the attachment of the platelet membrane by means of glycoprotein receptors to the subendothelial matrix and is modulated by soluble factors such as Von Willebrand's factor, fibrin, thrombospondin, and fibronectin. By 24 hours, the thrombus becomes more dense and homogeneous as platelets lyse and agglutinate discharging granules that release many bioactive substances within the thrombus. The substances released contain potent growth factors for smooth muscle cells including platelet derived growth factor (PDGF), transforming growth factor (TGF), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF).

Stage ii: Cellular recruitment

Within 48 hours, fibroblasts from the adventitia, medial smooth muscle cells, and endothelial cells begin to proliferate and migrate into the injured site. Both adventitial fibroblasts and smooth muscle cells from the media are activated and undergo phenotypic transformation before migrating into the intima where they accumulate and cause intimal

thickening. Monocytes and lymphocytes are attracted from the flowing blood to the injured area at 4 to 8 days. These cells migrate into the degenerating thrombus where monocytes become macrophages. Both macrophages and lymphocytes release a variety of growth factors and cytokines that promote SMC proliferation. Endothelial cells from adjacent uninjured sources begin to repave the denuded surface by growing as a continuous sheet.

Stage iii: Cellular proliferation

The majority of the proliferative process occurs in the intimal layer of the vessel wall hence referred to as intimal hyperplasia. Traditionally the proliferative tissue was thought to be composed of SMC of the adjacent media where proliferation can be seen after 24 hours post injury. These SMC change their phenotype from contractile spindle cells to secretory cells and migrate to the intima where proliferation continues for several cycles. Proliferation and migration of the smooth muscle cells can be abruptly curtailed in regions of endothelial regeneration. Even in the absence of any overlying endothelial surface, rat SMC stops its proliferation 2 to 4 weeks post injury. More recently, study of porcine adventitial fibroblasts using bromodeoxyuridine (BrdU) labeling demonstrates that fibroblasts in the adventitia also contribute cells to the intima. These cells can be seen undergoing rapid proliferation within 48 hours of vessel injury, acquire α-actin to become myofibroblasts and migrate into the intima at day 18-35 after injury [48,49].

Stage iv : Matrix deposition

The development of thickened intima occurs in two steps: first there is the accumulation of smooth muscle cells as a result of migration and proliferation and second there is the subsequent and pronounced accumulation of matrix synthesized by the smooth muscle cells. The accumulation of extracellular matrix in the intima accounts for the majority of the intimal volume once proliferation has subsided. This increase in intimal volume results in luminal narrowing in some vessels and vascular occlusion in others. Interestingly, some vessels can compensate for this increase in intimal volume by increasing their diameter and thereby expanding the luminal area to maintain flow in a process referred to as vascular remodeling.

4. Vascular remodeling

The vascular system is made of living tubes that transport blood throughout the body. Each vessel is an active integrated organ capable of adapting to long term changes in haemodynamic conditions by altering its own composition and morphology in a process known as vascular remodeling. The remodeling process begins in the embryo during vascular development as first described by Thoma in 1893 and is commonly referred to as Thoma's law:

1. Caliber of the blood vessel is determined by the velocity of the blood flow.
2. Vessel length is determined by the pull on the vessel walls from surrounding organs and tissues.
3. Vessel thickness is determined by the blood pressure within it.
4. New capillaries will form in response to increased terminal blood pressure in the region, whereas capillary size is reduced in the presence of decreased pressure.

In the mature vessels some of these laws remain applicable as the vessel continues to adapt and remodel in response to acquired changes in hemodynamics such as vascular injury and disease. Glagov et al. were the first to describe the compensatory arterial dilatation early in the human coronary artery atherosclerotic disease process. This remodeling process enlarges the luminal diameter which delays the development of focal stenosis despite significant plaque accumulation [50,51]. Vascular remodeling has been documented in animal models of vascular injury as well as in patients with serial intravascular ultrasound [52-55].

The remodeling process involves every component of the blood vessel. The endothelium, smooth muscle, and fibroblast cells of the media and the adventitia are all coupled to each other in a complex interactive system. Cell processes such as migration, proliferation, hypertrophy, extracellular matrix metabolism, and cell death are regulated by a dynamic interaction with local cytokines, vasoactive substances and hemodynamic changes.[56] These interactions result in the observed structural changes in the vessel wall. Examples of remodeling include vasodilatation in response to high flow (e.g. arteriovenous fistula), arterial medial matrix loss resulting in aneurysm formation, and neointimal production in response to vessel wall injury.[56]

5. Endothelium as the Signal Transducer of vessel remodeling

The endothelium plays a key role in the homeostatic mechanisms underlying the normal function of the artery wall. In response to hemodynamic, hormonal and inflammatory stimuli, various of biologically active substances are secreted by endothelial cells. However, the exact factors produced by the endothelial cells that are responsible for the

functional and structural change in arteries are incompletely understood. An example of this can be seen in vessels with arteriovenous fistulas where, in response to long term flow increase, the vessel enlarges and the lumen of the vessel increases in diameter. Conversely, a long-term decrease in blood flow through an artery results in structural decrease in luminal diameter. Both of these adaptations are endothelium dependent processes[57]. The endothelium appears to be capable of sensing shear stress in the arterial wall and activating the vessel remodeling process to maintain a constant predetermined level of shear stress [56,58]. This phenomenon can be partially explained by the release of biologically active substances. In response to increased flow, mediators of vasodilatation such as prostacyclin and nitric oxide are released from the endothelium. Increased tension promotes release of mitogenic factors such as platelet-derived growth factor (PDGF) as well as matrix-promoters to produce vessel wall hypertrophy and an increase to vessel diameter.[56]

The endothelium actively participates in many aspects of remodeling releasing substances that influence vasomotor tone, cell growth, migration, death, and the composition of the extracellular matrix. Some of the factors produced by the endothelium are listed in Table II.

6. Role of adventitia and perivascular Nerves in vessel structural remodeling

Until recently, research on restenosis focused mostly on the morphology and mechanisms within the intima and media while the role the adventitia has been largely ignored. More current studies using porcine coronary arteries have shown that adventitial fibroblasts respond to endoluminal balloon injury by differentiating into myofibroblasts

Table II. Factors produced by the endothelium

Vasoactive mediators
Endothelin-1,2 and 3
Nitric oxide
Eicosanoids (PGE2, PGI2, PGF2α)
Angiotensin II
Endothelium derived hyperpolarzing factor

Adhesion molecules
Endothelial adhesion molecule (ELAM-1)
Intercellular adhesion Molecule 1 and 2 (ICAM-1, ICAM-2)
VCAM-1

Growth regulatory molecules
Platelet derived growth factor (PDGF)
Basic fibroblast growth factor (bFGF)
Transforming growth factor (TGFβ)
Macrophage colony stimulating factor (M-CSF/GM-CSF)
Tumor necrosis factor alpha (TNFα)
Heparan Sulfate

Chemotactic factors
Monocyte chemotactic protein-1 (MCP-1)
Oxidized low density lipoproteins (oxLDL)

Immune response mediators
Interleukin-1
Interleukin-6
Interleukin-8

[49,59]. These cells may contribute to the process of vascular lesion formation by proliferating, synthesizing growth factors and migrating into the neointima. The adventitial myofibroblasts with α-actin may also contribute to vessel narrowing (unfavorable geometric remodeling) and late lumen loss. This is analogous to the process of wound healing where presence of myofibroblasts is the pathological hallmark of tissue contraction.

Besides fibroblasts, the adventitia of the blood vessels also contains an intricate network of nerves that are important in modulating the vessels' physiologic function and morphology.

The nerve fibers supplying blood vessels are generally arranged in two plexuses: an outer plexus located in the middle of the adventitia and an inner plexus, or terminal ground plexus, which derives from the former and is typically restricted to the adventitial-medial border[60]. The terminal parts of the nerves innervating blood vessels generally consist of nonmyelinated axons surrounded by a Schwann cell sheath and exhibit periodic swellings or varicosities in the vicinity of the adventitial-medial border.

For many years, the only neurotransmitters considered in the perivascular nerves were norepinepherine and acetylcholine. Since the discovery of these two chemical messengers in the 1960s, many more neural transmitters have been identified. (Table III) The pathophysiological function of these vasoactive peptides in relation to restenosis is still unclear. It has been suggested that neuropeptides may be part of a rapidly responding system linked into central mechanisms [61]. However, there is also evidence to suggest a neural influence on the long-term structural dimension of the vessel [36,62,63].

Table III. Established and putative transmitters in the perivascular nerves

ESTABLISHED	PUTATIVE
5 hydroxytrptamine(5HT)	Cholecystokinin-gastrin(CCK-GAS)
Acetylcholine(ACh)	Neurotensin(NT)
Adenosine 5'-triphosphate(ATP)	Vasopressin like peptide(VP)
Calcitonin gene related peptide(CGRP)	Galanin(GAL)
Neuropeptide Y(NPY)	Gastrin releasing peptide(GRP)
Noradrenaline(NA)	Enkephalin-dynorphin(ENK-DYN)
Somatostatin(SS)	Dopamine(DA)
Substance P(SP)	Adrenocorticotropic hormone(ACH)??
Vasoactive intestinal peptide(VIP)	Angiotensin(ANG)??

Although vascular muscle unlike skeletal muscle does not depend on its innervation for survival during early development, surgical denervation of the saphenous artery of 10 days old rats results in a thinner tunica media at 3 weeks. Following denervation, the central rabbit ear artery had a thinner, stiffer wall [64]. Following sympathectomy in aortas of rats and rabbits, there was increased collagen synthesis and increased cholesterol content in the walls [65,66]. On the other hand, chronic stimulation of perivascular nerves using electrode implantation induced structural and immunocytochemical changes in the vascular endothelium of rabbit ear artery[67].

Interestingly, there is also evidence that perivascular nerves may adapt to changes in the blood vessel. When the endothelium of the dog coronary artery was injured mechanically without disruption of the elastic lamina, neuron-specific enolase-positive nerve fibers were increased in number at both 1 and 3 months[68]. An increased density of SP-containing nerve fibers was also observed in the dog coronary artery 3 months after mechanical injury to the endothelium.

The perivascular nerves are capable of synthesizing, storing and releasing more than one neurotransmitter[69]. These nerves are chemically coded, so that individual neurons contain a particular combination of transmitter substances to affect specific target sites. For example, sympathetic nerves in the distributing arteries of guinea pigs contain NPY and NA, in the smaller arteries they contain dynorphin and NPY and NA, while in precapillary arterials only dynorphin and NA are present[70]. The local differences in innervation may allow the modulation of general homeostatic activity to suit the requirements of the particular region.

Developmentally, the nerve density around rat blood vessels reaches maximal levels between 3 and 8 weeks postpartum [71]. After a relatively homogeneous period of postnatal nerve growth, plexuses show heterogeneous changes with both marked reductions and increases being recorded in different regions [72,73]. During postnatal development a process of diversification occurs that results in the development of the mature locally specific pattern of innervation. With aging, this pattern of innervation changes. In the aging rats, where as the expression of vasoconstrictors, and cerebrovascular neurotransmitters (NA and 5-HT) decreases, there is an increase in vasodilator neurotransmitters (VIP and CGRP) [35]. The effect these changes in innervation have on the remodeling characteristics of these aging vessels is presently unknown.

7. Possible role of SS in vascular injury

In the peripheral vasculature, SS is found in nerve fibers innervating blood vessels. In-situ administration of SS to rat saphenous vessel adventitia can cause arterial vasodilatation and venous constriction. These effects are mediated by nitric oxide since deendothelialization or local infusion of NG-nitro-L-arginine, (a nitric oxide synthesis inhibitor) completely abolish the effects of SS[74].

Of all the SS analogues, angiopeptin is the most extensively studied with respect to effects on vascular tissue. Angiopeptin is capable of inhibiting vessel wall proliferation (thymidine incorporation), and intimal hyperplasia as well as inducing beneficial vascular remodeling after vascular balloon injury in rabbits[75-80].

Angiopeptin is a stable synthetic SS analogue also known as BIM-23014C. There are several mechanisms of action proposed for the analogue:

i) *Interruption of intracellular signal transduction pathways.* SS activates a membrane bound phosphatase which dephosphorylates tyrosine kinase in the intracellular domain of many growth factor receptors.

ii) *Inhibition of growth factor secretion.* Native somatotatin inhibits the release of many hormones. There is evidence that SS curtails the release of various promoters of restenosis from cells such as endothelial cells, smooth muscle cells, and monocytes and macrophages.

iii) *Decrease leukocyte adhesion to endothelial cells.* Angiopeptin and SS have been shown to decrease the adhesiveness of activated endothelial cells for mononeuclear leukocytes; this is an important event in the mechanism of restenosis.

iv) *Inhibit SMC migration.* Angiopeptin inhibits SMC migration in vitro through a Gi protein mediated pathway[91].

v) *Promote endothelial cell regrowth.* Local delivery of angiopeptin in the rabbit aorta after balloon injury results in marked improvement in endothelial coverage of the injured area[79].

vi) *Induce nitric oxide mediated vasodilatation.* SS causes arterial vasodilatation via a nitric oxide dependent mechanism[74]. Nitric oxide inhibits intimal hyperplasia as well as promote favorable vessel remodeling [92-96].

8. Clinical trials

Three double blind randomized clinical trials have been conducted using angiopeptin for prevention of restenosis in coronary artery angioplasty. In the North American trial (n=1241), Kent et al. failed to show an effect on clinical events of angiographic stenosis.

However, the dosage used may have been too small [97]. In the Scandinavian trial using a higher dosage (n=112), significant reduction in angiogram defined restenosis was shown but, the clinical event rate was not significantly reduced [98]. In the European Angiopeptin Study (n=553), a significant reduction in clinical event was shown but, there was no significant reduction in angiographic stenosis [99]. The mixed results demonstrated in these clinical studies are puzzling. Clearly, a better understanding of the function and mechanism of action of SS and its analogues is necessary to resolve these conflicting findings.

Angiopeptin as well as the other two analogues mentioned above all interact with 3 of the 5 receptors, SSTR 2, 3 and 5 [100]. It is unclear at present whether all three receptor subtypes are involved in the action of SS on intimal hyperplasia or whether a single subtype predominates. The answer to this question may be potentially of great clinical significance. If a single receptor subtype is associated with the inhibitory effect on cell division then it should be possible to design a target specific drug to activate only that receptor whilst leaving other systems regulated by SS unaffected. The molecular cloning and sequencing of the neuropeptide receptors has made possible the generation of specific antibodies which will allow high resolution mapping of their distribution.

II. HYPOTHESIS

The inhibitory effect of SS analogues on vascular intimal hyperplasia is mediated through specific receptors on the injured vessel.

III. SPECIFIC AIMS

1) To determine the distribution and/or abundance of SS nerves and its receptors in normal and injured rat iliac arteries.

2) To quantitate the expression of mRNA for SSTR-2 using RT-PCR.

IV. MATERIALS AND METHODS

1. Surgical Procedures

Fifteen male Wistar rats weighing 400-500 gm were anaesthetized with intravenous pentobarbital and halothane inhalation. Through a midline abdominal incision in the abdomen, aorta and bilateral iliac arteries were isolated. A transverse opening was made in the infrarenal aorta. Balloon endothelial injury was created by introducing a No. 2 French Fogarty balloon catheter from the aorta into the left common iliac artery. The balloon were inflated with 0.2 ml of air and pulled proximally 6 times to injure the entire common iliac artery. The right iliac arteries were left uninjured as controls. After the balloon catheter was removed, the aorta was closed with interrupted 7-0 prolene sutures. At 48 hours, 1 week and 1 month post injury groups of 4 , 6, and 5 animals respectively were sacrificed. Two animals from each group were perfusion fixed with 500 ml of 4% paraformaldehyde (Fisher) at perfusion pressure of 100 mmHg. Bilateral iliac arteries were harvested, post fixed for 4 hours in 4% paraformaldehyde and processed for for light microscopy and immunohistochemistry.

In 2, 4, and 3 animals from the 48 hour, 1 week and 1 month post injury groups their iliac arteries were prepared for reverse transcription and polymerase chain reaction (RT-PCR). These animals were perfused with 500 mls of cold normal saline before bilateral iliac arteries were harvested from the aortic bifurcation to the common iliac bifurcation and immediately frozen in liquid nitrogen.

2. Light microscopy and immunohistochemistry:

Immunohistochemical staining for nerves, SS nerve fibers, endothelium, and SS receptors 1,2 and 3 were completed. Antibodies to PGP 9.5, and rat endothelium antigen-1 were used as markers for nerve fibers and endothelial cells, respectively.

For nerve fiber staining, whole mounts were prepared by gently teasing away the adherent fat tissue to expose the surface of the vessels. The vessels were cut open and placed in wells in a 12 well culture plate containing the relevant antisera diluted in phosphate buffer containing 0.3% Triton X100 (Sigma) to improve penetration into all layers of the tissue. After a 72 hour incubation at 4^0C, the whole mounts were washed in PBT for 30 min with 3 changes of buffer at room temperature. Finally, the tissues were incubated for 2 hours at room temperature in the secondary layers: goat anti-mouse IgG conjugated to Cy 3 for the SS10 antibody, donkey anti-rabbit IgG conjugated to FITC for the PgP 9.5. The whole mounts were washed in PBT for 1h with 3 changes of buffer prior to placing on a slide and applying coverslips in PB/Glycerine (1:9). The whole mounts were then examined using a Zeiss Axiophot microscope.

For staining endothelium and SSTR 1,2,and 3, six micron frozen cross sections of each iliac artery were mounted on coated slides and dryed at 37^0C overnight prior to staining. Hemotoxylin and eosin staining was done on representative sections to document the histology. Adjacent sections were immersed in the relevant antisera diluted in phosphate buffer containing 0.1% Triton X100. After a 48h incubation at 4^0C, the sections were washed in PBT for 30 min with 3 changes of buffer at room temperature. Finally, the sections were incubated for 2 hours at room temperature in the secondary layers: goat anti-mouse IgG conjugated to FITC for the anti-RECA-1 antibody, donkey anti-rabbit IgG

conjugated to Cy3 for the SSTRs. The sections were then examined using a Zeiss Axiophot microscope.

3. Reverse transcriptase-Polymerase chain reaction (RT-PCR)

RT-PCR was carried out to determine if SSTR-2 mRNA was expressed in both control and injured iliac arteries.

i) RNA extraction Total RNA was extracted from each iliac artery by the method of Czomczynski and Sacchi[101]. In brief, the artery was homogenized in guanidinium thiocynate solution (250g guanidinium thiocynate, 293 ml water, 17.6 ml 0.75 M sodium citrate, 26.4 ml 10 % sarcosyl, 2 ml 2-mercaptoethanol). RNA was extracted from this homogenate by the addition of phenol, and chloroform-iso-amyl alcohol mixture (49:1). The total RNA extract was then precipitated twice with 1 volume of isopropanol, washed with 75% ethanol and solublized in 10 µl of PCR buffer (67mM Tris pH9.01, 1.5mM $MgSO_4$, 166 mM $AmSO_4$ and 10 mM 2-mercaptoethanol) , containing 10 U Rnasin and 4 U DNase. The samples were incubated for 30 minutes at 37°, followed by 90° for 5 minutes to inactivate the DNase prior to RT-PCR.

ii) Primers Oligonucleotide primers were synthesized on Applied Biosystems Model 391 DNA synthesizer. Paired oligonucleotide primers were selected from a region of the rSSTR-2 gene that had minimal overlap with other members of the SSTR family. The sequence of the forward primer was 5'-GATGAAGACCATCACCAACA-3' which corresponds to nucleotides 301-320 of the human SSTR-2 gene; the sequence of the

reverse primer was 5'-TTCCACTTCAGGAGACCTTA-3' which corresponds to nucleotides 782-801 of the human SSTR-2 gene.

iii) First strand reaction. First-strand cDNA was prepared with 3μl of the total RNA extract. The RNA was first denatured at 90°C for 2 minutes and immediately chilled on ice. Reverse transcription was performed at 42°C for 1 hour in First-strand buffer (Pharmacia) containing 100ng of 0.1 mM DTT, random primers (200 ng), 10 U of RNAase inhibitor (Pharmacia), 1mM dNTPs (Pharmacia), and 100 U Superscript reverse transcriptase (Gibco). The reverse transcriptase was inactivated by heating the samples to 99°C for 4 minutes.

iv) PCR The PCR was carried out in PCR buffer using 2 ul of first strand cDNA. Forward and reverse primers (100ng of each) were added as well as $MgCl_2$ (2.5mM final concentration), 5% DMSO, 1mM dNTPs, and Taq polymerase (1.25U) (Gibco). Simultaneous reactions using primers for mRNA of beta-actin (an ubiquitous structural protein) were completed for each arterial sample as controls to reflect the amount of RNA isolated in each sample. The samples were overlaid with mineral oil to prevent evaporation and run for 30 cycles at annealing temperature of 55 °C. PCR products (4 ul aliquots) were separated by electrophoreses through a 1% agarose gel, stained with ethidium bromide, then examined under florescent light. The intensity of the bands was quantitated by computer assisted densitometric analysis (Eagle Eye™ 3.0 Image Capture and Analysis Software, Stratagene). The relative concentration of mRNA in each sample

was normalized to the amount of beta-actin by taking the ratio of the pixel density of SSTR2 over the pixel density of beta-actin band (SSTR2/actin).

To determine the absolute concentration of the PCR product, known concentrations of standard DNA were run out on 1% agarose gel and their pixel density measured. The band intensity was plotted against the concentration of the standard DNA and a standard curve generated. The concentrations of the unknown samples were determined by measuring the intensity of the band and obtaining its corresponding concentration using the standard curve.

v) Sequencing of PCR Product. To confirm its identity, the nucleotide sequence of the PCR product was determined as follows. The PCR product was purified and cloned using pGEM T vector system (Promega) into competent *E. coli* cells. The competent cells were grown and vector containing cells selected out using ampicillin plates and blue –white β-galactosidase selection. Single-stranded DNA was produced with the addition of M13K07 helper phage. Sequence analysis were carried out using the chain termination method with deoxynucleotides and radioactive labeling with alpha-[^{35}S]-thio dATP. Reaction products were electrophoresed on urea-polyacrylamide gel, autoradiographed and manually read.

Table IV. Reagents
Agarose (Gibco Life Technologies, Grand Island, New York)
Alpha-[^{35}S]-thio dATP (Amersham Canada Ltd., Ockville, Ontario)
Anti- PGP 9.5 antibodies (Ultra Clone Ltd. Isle of Wight, England)
Anti- rat endothelial cell antigen-1 antibodies (Serotec Ltd, Oxford, England)
Anti- somatostatin antibodies (Dr. A. Buchan in University of British Columbia, Vancouver, Canada)
Anti- somatostatin receptor 2A antibodies (Dr. John Walsh in the CURE/Gastroenteric Center, Antibody/RIA core)
Anti- somatostatin receptor 2B antibodies (Dr. John Walsh)
Anti- somatostatin receptor 3 antibodies (Dr. John Walsh)
Anti- somatostatin receptor 5 antibodies (Dr. John Walsh)
Chloroform (Sigma Chemical Co., St. Louis, MO)
DH5a Competent E coli cells (Gibco)
DMSO (Sigma)
dNTPs1mM (Pharmacia Biotech, Uppsala, Sweden)
Donkey anti-rabbit IgG Cy 3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.)
Donkey anti-rabbit IgG FITC (Jackson)
DTT (Sigma)
Eosin (Fisher Scientific Co., Fairlawn, N.J.)
Ethanol (BDH)
Ethidium bromide (Sigma)
First strand buffer (Gibco)
Goat anti-mouse IgG Cy3 (Jackson)
Goat anti-mouse IgG FITC (Jackson)
Guanidium thiocynate (Gibco)
Hemotoxylin (Fisher)
Iso-amyl alcohol (Fisher)
Isopropanol (BDH)
M13K07 helper phage (Promega Corp. Medison, WI)
Mercaptoethanol (BDH)
$MgCl_2$ (Fisher)
OCT compound (Fisher)
Paraformaldehyde (Fisher)
PCR buffer (Gibco)
PGEM T vector system (Promega)
Phenol (Gibco)

Polyacrylamide (Gibco)
RNAase inhibitor (Pharmacia)
Sarcosyl (N-Lauroylsacrosine, Sigma)
Sodium citrate (Fisher)
Superscript reverse transcriptase (Gibco)
Taq polymerase (Gibco)
Triton X100 (Sigma)
Urea (Gibco)

V. RESULTS

1. Light microscopy

In the uninjured iliac artery, the intima consisted of the lining endothelial cells and a thin layer of subendothelial connective tissue with very few smooth muscle cells.

In the injured artery, after 48 hours post balloon injury, the vessel cross sections revealed complete denution of the endothelial layer with the exception of small patches of cells within and adjacent to the ostia of minute side branches (Figure 2a). The artery surface was covered with adherent platelets, scattered erythrocytes and leukocytes. The elastic lamellae were fragmented in some places and some necrotic smooth muscle cells and intramural hematoma were observed. In the adventitia, clusters of inflammatory cells could be seen.

At 1 week after injury, the injured sites had been mostly re-populated by neo-endothelium. The new endothelium had a cuboidal and uneven appearance. There was an area in the middle of the injured segment which remained uncovered by the endothelium. Patchy areas of intimal hyperplasia could be seen underneath the endothelial layer. It was thicker in some areas reaching 3 to 4 cells thick while in other areas there was minimal intimal hyperplasia. The area of intimal hyperplasia did not narrow the luminal area and there was no stenosis detected. (Figure 2b). Inflammatory cells were observed in the adventitia as well as some areas of the media.

At 1 month after injury, vessel sections showed a well-formed continuous neo-endothelial surface covering the previously injured surface. The endothelial cells had a smoother oval appearance. Prominent areas of intimal hyperplasia were seen involving

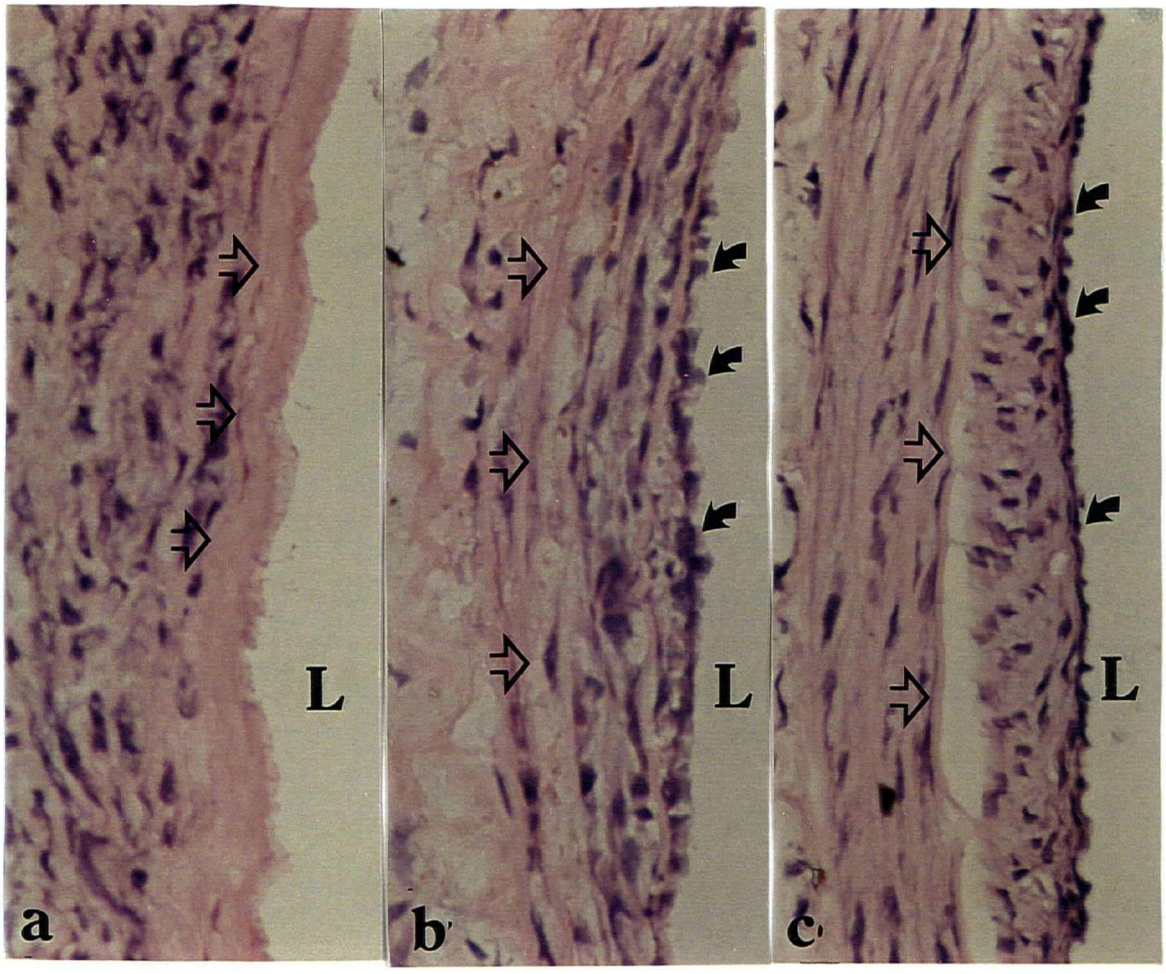

Figure 2. Results of light microscopy (a) Cross section of 48 h after balloon injury. Note absence of an endothelial layer. (b) One week after injury, the injured site had been repopulated by new endothelium. There were also areas of intimal hyperplasia. (c) One month after injury, a smooth endothelial surface covering the previously injured surface has occurred. Intimal hyperplasia is prominent. L, lumen of the artery; open arrow, the internal elastic lamina (separation between intimal and medial layer); solid arrow, endothelial cells. (hemotoxylin and eosin, 250X)

most of the circumference of the vessel. The inflammatory cells had largely disappeared from the adventitia (Figure 2c).

2. SSTR staining

Immunostaining with antisera against SSTR2 in the normal control arteries revealed very light membrane staining of the endothelium (Figure 3a).

In vessels stained 48 hours after balloon injury, no specific staining of the artery apart from occasional endothelial cells left over from the balloon stripping was seen (Figure 3b). Double staining with the RECA-1 antibodies confirmed these cells were endothelial cells.

SSTR2 staining of vessels at 1 week and 1 month after balloon injury revealed intense membrane staining of the neo-endothelium (Figure 4a, 4b). The specificity of the SSTR2 anti sera was confirmed by pre-absorption testing of the anti-sera with the parent antigen (synthetic SSTR2 peptide). This resulted in eliminating the endothelial staining.

Immunostaining with anti sera against SSTR1 and SSTR3 did not demonstrate specific staining in any of the vessels.

3. Nerve staining

Immunostaining with the rabbit antibody against PGP 9.5 (a marker for all nerve fibres) showed abundant peri-vascular nerve fibers within the adventitia layer of the iliac artery (Figure 5a). There were larger nerves that ran in a longitudinal fashion and smaller tortuous nerves with prominent varicosities that were organized in circular plexus. Double staining with the monoclonal antibody SS10 showed some of these fibers to contain SS (Figure 5b). The SS fibers were mostly the smaller tortuous fibers with periodic varicosities consistent with sensory afferent nerves.

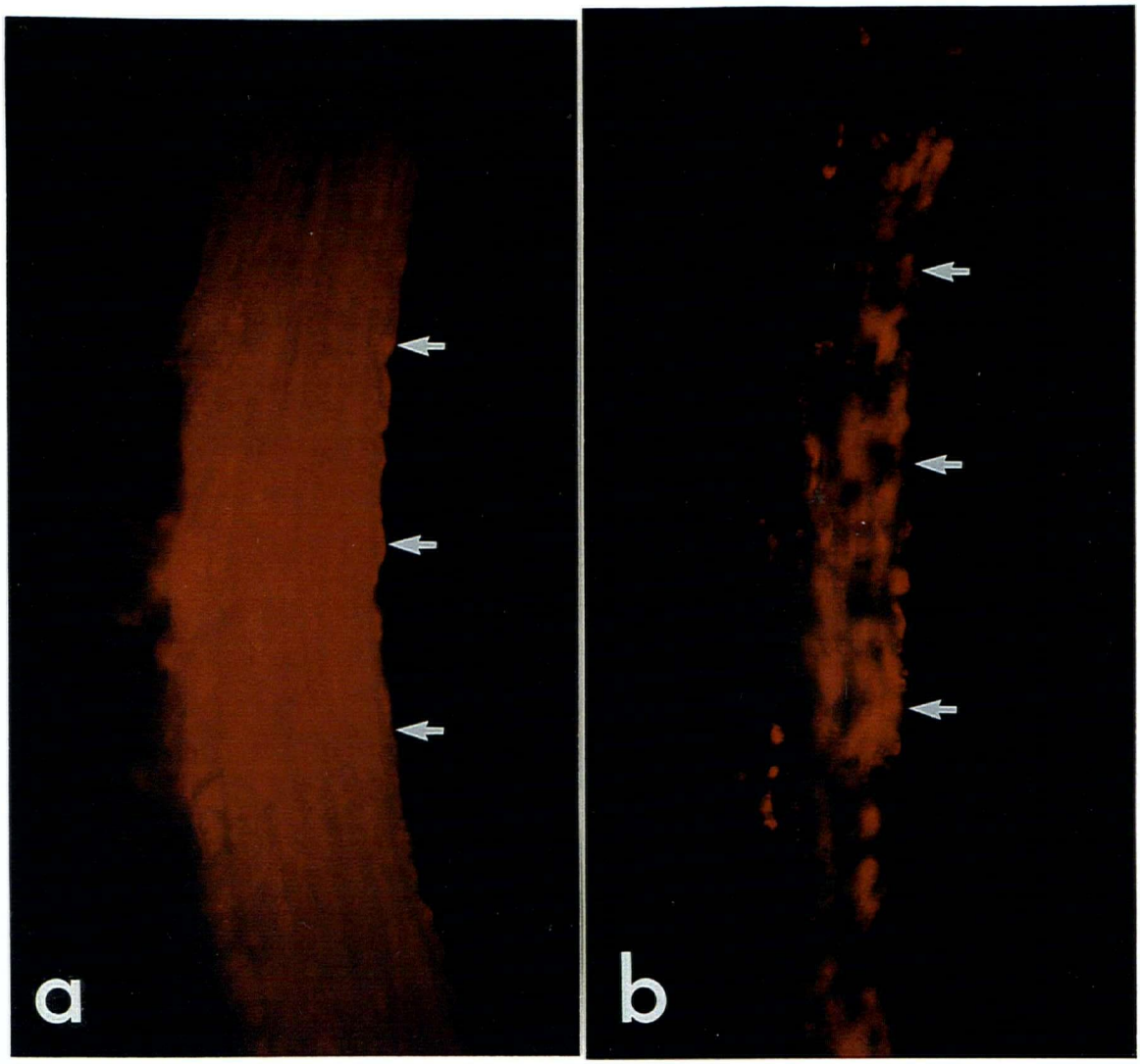

Figure 3. Results of SSTR2 staining of control and 48 hour artery cross sections. (a) normal rat iliac artery and, (b) iliac artery at 48 hours after injury. Arrows indicate luminal surface.

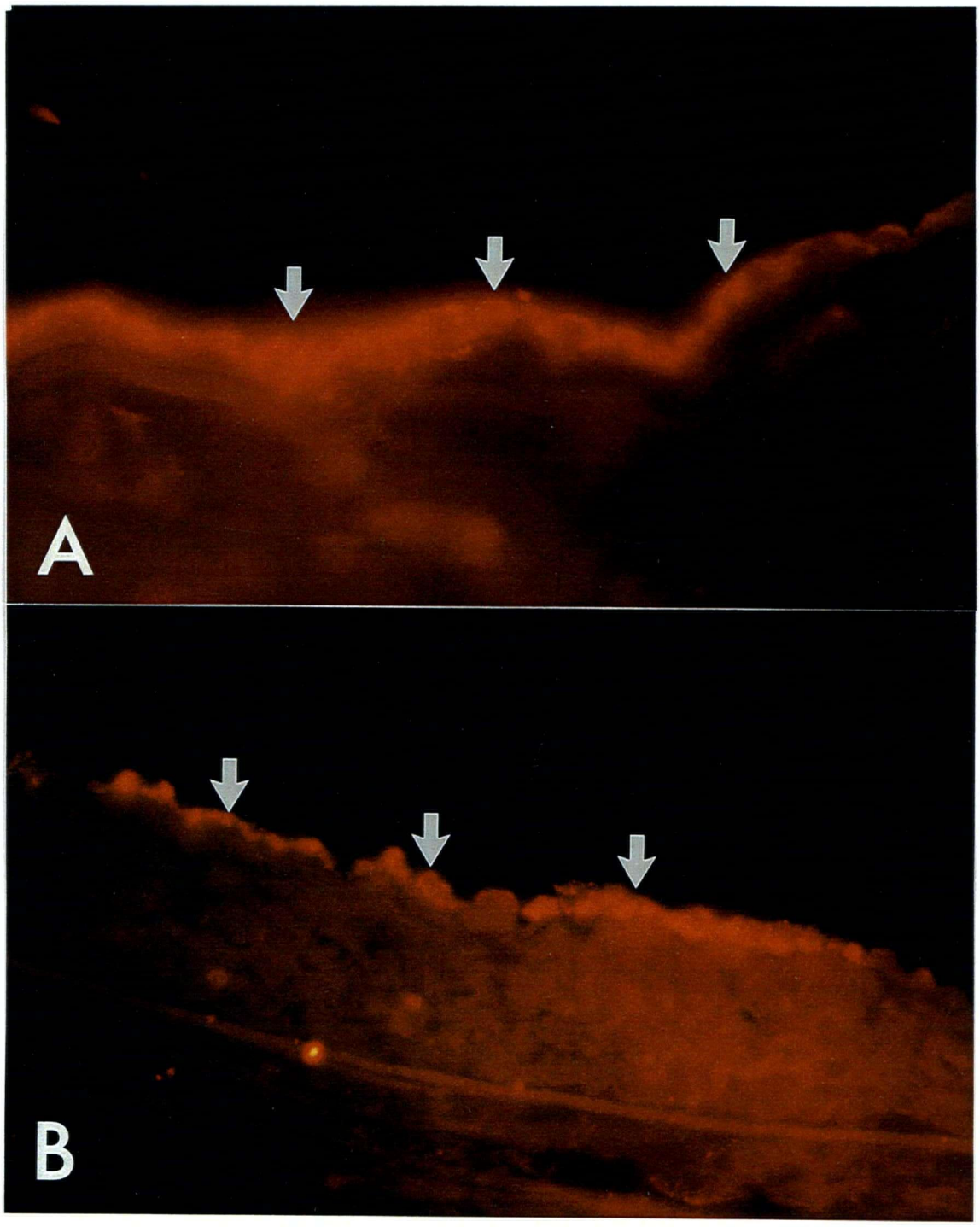

Figure 4. Results of SSTR2 staining of 2 weeks and 1 month artery cross sections (a) 1 week and (b) 1 month after injury. Arrows indicate luminal surface.

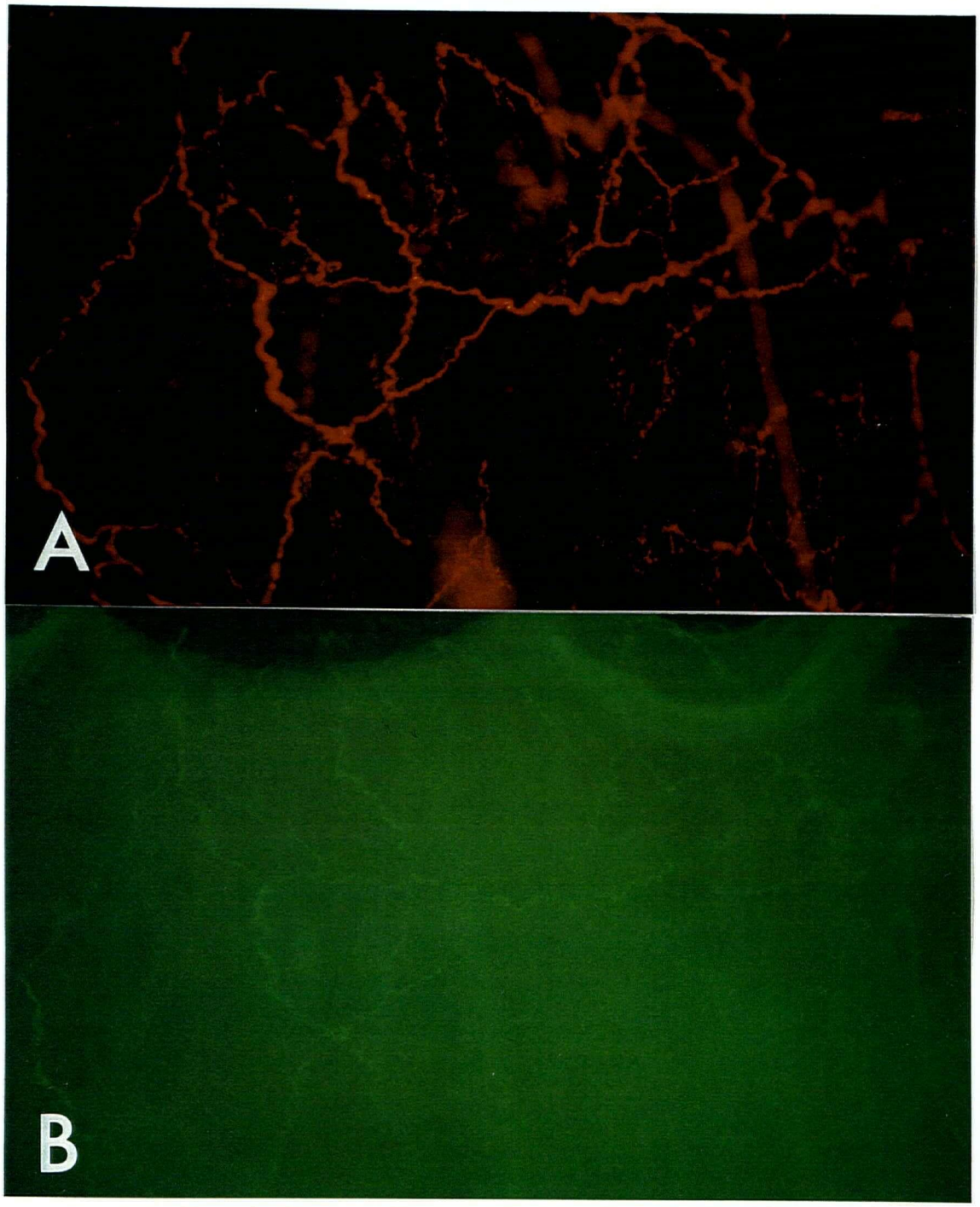

Figure 5. Results of nerve immunocytochemical staining (A) Immunostaining with antibody against PGP showing abundant perivascular nerve fibers within the adventitia. (B) Counterstaining with antibody against SST showing that some adventitial nerve fibers contain somatostatin.

4. RT-PCR

The RT-PCR yielded fragments of DNA of predicted size for SSTR2 mRNAs as well as actin mRNA in all samples. However the abundance of PCR product was different according to the time lapse after the vessel injury (Figure 6).

At 48 hours post injury, the injured vessel showed a deceased expression of mRNA for SSTR2 as its control counterpart. At one week post-injury, the amount of mRNA for SSTR2 was increased as indicated by brighter bands and higher pixel density ratio (SSTR/actin) compared to that of the contra lateral control vessel. In the 1 month post injury rats, the pixel density ratio was again higher in the injured artery compared to the controls. This indicates that the increase in SSTR2 mRNA expression persisted up to 1 month post injury.

In order to estimate the absolute quantity of the PCR product, Known amounts of double stranded DNA were run on 1% agarose gel and their pixel density measured to construct a pixel density/concentration scale. Absolute amount of PCR products were derived from this scale as illustrated on Figure 7. Results of these semi-quantitative measurements are tabulated in table V. The absolute concentration of PCR product were quite variable among rats in the same time group. When these concentrations were normalized to beta-actin and compared with the control vessels, the 1week as well as 1 month post injury (experimental) vessels showed consistent increase in SSTR expression. (Experiment/Control greater than 1)

To confirm the identity of the PCR product, the DNA was eluted from the gel and the nucleotide sequence determined (Figure 8). This sequence had 100% identity the

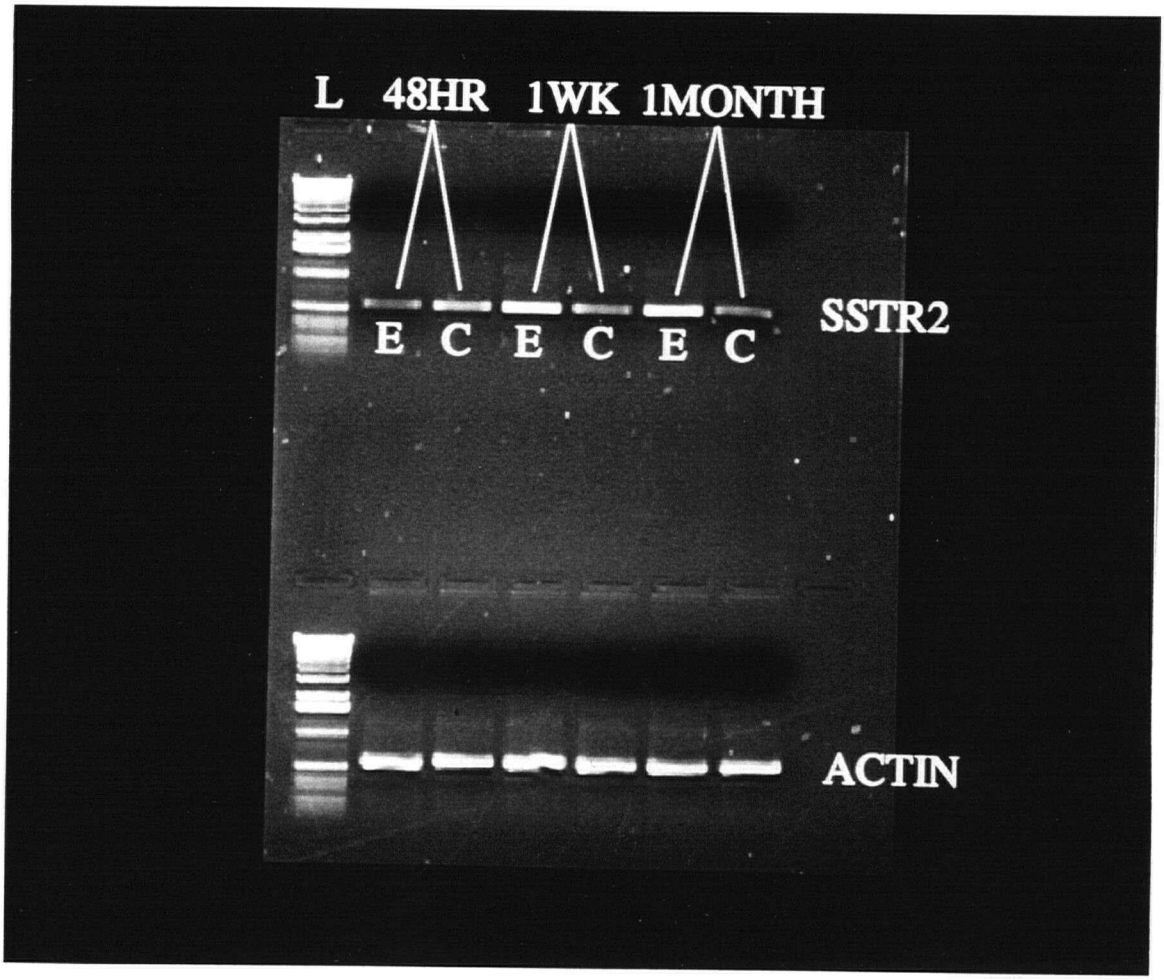

Figure 6. Results of RT-PCR using SSTR2 and actin primers on arteries 48 hours, 1 week, and 1 month after balloon injury. Injured arteries (E) at 1 week and 1 month showed brighter SSTR2 bands compared to the control vessels (C).

FIGURE 7. DERIVATION OF ABSOLUTE AMOUNT OF PCR PRODUCTS BY PIXEL DENSITY/CONCENTRATION SCALE

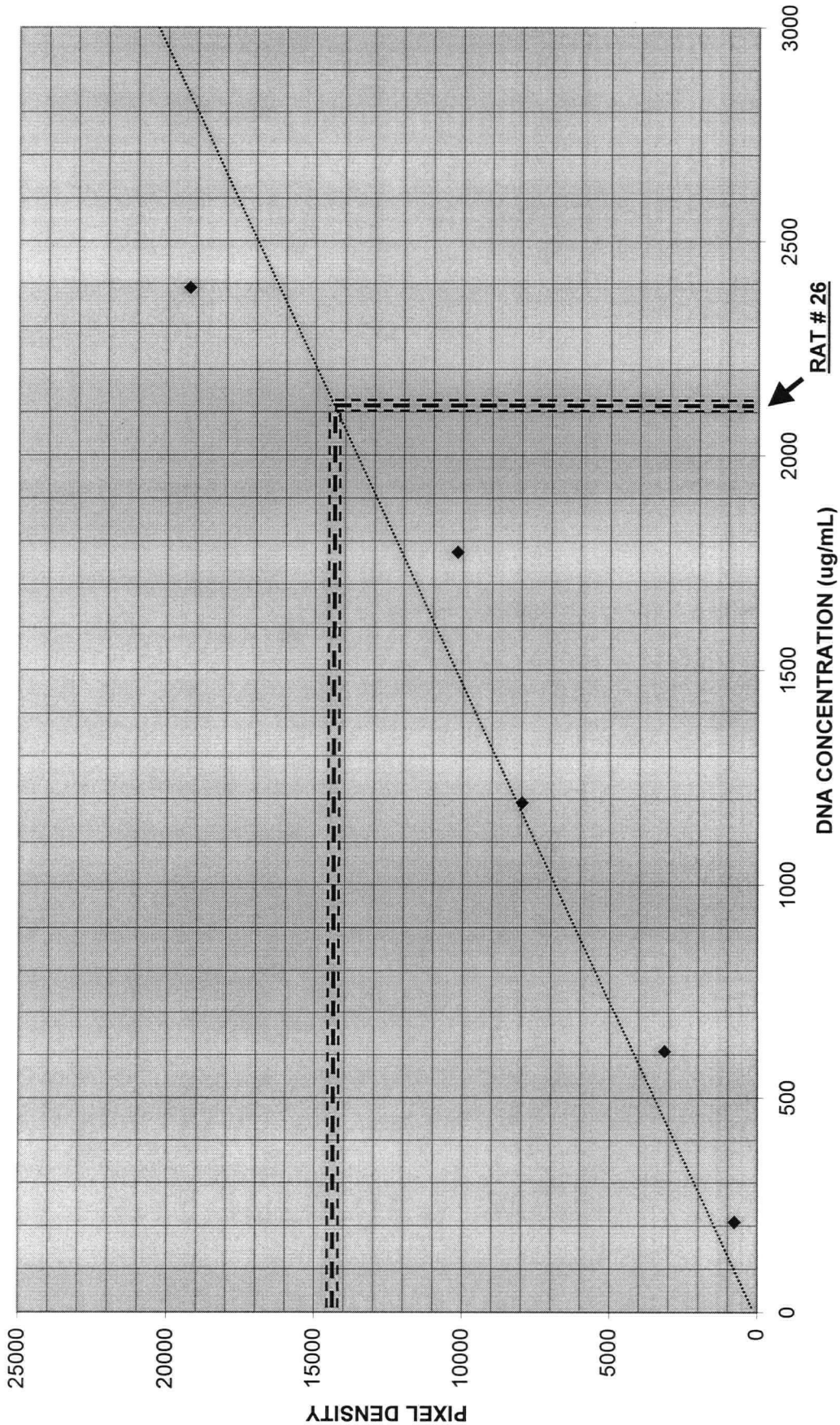

TABLE V. RESULTS OF SEMI-QUANTITATIVE MEASUREMENT OF PCR

RAT#	TIME FROM INJURY	PIXEL DENSITY	CONCENTRATION (UG/ML)	sstr/actin RATIO	EXPERIMENT/ CONTROL
24	48 HOURS	2994	410	0.19	0.49
25	48 HOURS	2793	390	0.21	0.48
11	1 WEEK	2110	300	0.17	2.13
20	1 WEEK	2196	310	0.68	6.18
22	1 WEEK	3874	590	0.13	1.4
26	1 WEEK	14499	2150	0.67	2.09
10	1 MONTH	1966	290	0.12	1.09
13	1 MONTH	12627	1850	0.61	5.08

Figure 8. Sequence of PCR product and its translated amino acid sequence

ATG AAG ACC ATC ACC AAC ATT TAC ATC CTC AAC CTG GCC ATC GCA
GAT GAA CTC TTC ATG CTG GGG CTG CCC TTC TTG GCC ATG CAG GTG
CTG GTC CAC TGG CCT TTT GGC AAG GCC ATC TGC CGG GTG GTC ATG
ACT GTG GAC GGT ATC AAC CAG TTC ACC AGT ATC TTC TGC TTG ACG
GTC ATG AGC ATC GAC CGT TAC CTG GCC GTG GTC CAC CC

Met Lys Thr Ile Thr Asn Ile Tyr Ile Leu Asn Leu Ala Ile Ala Asp Glu Leu Phe Met
Leu Gly Leu Pro Phe Leu Ala Met Gln Val Leu Val His Trp Pro Phe Gly Lys Ala Ile
Cys Arg Val Val Met Thr Val Asp Gly Ile Asn Gln Phe Thr Ser Ile Phe Cys Leu Thr
Val Met Ser Ile Asp Arg Tyr Leu Ala Val Val His

previously published sequence for rat SSTR.[81] When translated, the amino acid sequence of this PCR product had 100% identity to the human SSTR2 sequence (Gene Bank accession number: M81830) reflecting the highly conserved nature of this receptor between species.

VI. DISCUSSION

This study has shown the presence of SSTR2 receptor sub-type on the surface of normal and regenerated endothelium after balloon catheter injury. The abundance of SSTR2 as detected by immunocytochemistry was increased after injury and this was verified by demonstration of increased mRNA production.This observation suggests that SS and its receptors on endothelial cells may function as regulators in the setting of vascular injury and play a role in intimal hyperplasia.

SS via activation of subtypes 1 and 2 increase the activity of a membrane bound phosphatase which dephosphorylates phosphorylated tyrosine kinase in the intracellular domain of many growth factors receptors[81]. Many growth factors such as PDGFb, FGF, IGF and PDGF play important roles in the development of intimal hyperplasia and restenosis. Studies using inhibitors of these growth factors in various animal models have been shown to reduce the degree of restenosis. By interrupting the common intracellular pathway of these growth factors, SS may block the pathophysiologic process of restenosis at multiple sites, making it an effective inhibitor of restenosis. Since the effect of SS is potentiated by the up regulation of its receptors, increased expression in the post injury endothelial cells constitutes an augmentation of the inhibitory mechanism within endothelial cells to dampen the process of restenosis after injury.

Another mechanism through which SS may regulate the vascular injury response is by the inhibition of growth factor secretion. Native SS is a potent general inhibitor known to inhibit release of many hormones from different organs. There is evidence that SS curtails the release of various promotors of restenosis from cells such as endothelial cells, smooth muscle cells, and monocytes and macrophages. SS analogues, such as angiopeptin,

lanreotide and octreotide, are effective in inhibiting vascular expression of insulin like growth factor, an important mediator of intimal hyperplasia after arterial injury[82-86]. Compared with untreated rats, angiopeptin has been shown to suppress the level of PDGF, IGF-1, and EGF by 35-75% after aortic allograft transplantion[87]. Both angiopeptin and octreotide have been shown to stimulate the release of IGF-I binding protein which is known to inhibit IGF-I actions [84]. Rabbits treated with angiopeptin before aortic balloon denudation has demonstrated a lower expression of the oncogenes c-fos and c-jun which is the earliest events associated with growth factor stimulation [88]. By dampening the release of these growth factors, intimal hyperplasia is inhibited.

A third mechanism through which SS may regulate vascular injury response is by decreasing leukocyte adhesion to endothelial cells. Leukocytes are important players in the development of intimal hyperplasia. In the injured vessel, the leukocytes and endothelial cells are activated by cytokines and chemoattractant peptides which cause them to express adhesion molecules on their surface. These adhesion molecules cause the leukocytes to stick to the endothelial surface and migrate below the endothelial cell layer into the evolving lesion where they secrete more proliferation enhancing factors. Angiopeptin and SS have been shown to decrease adhesiveness of unstimulated and IL-1β activated endothelial cells for mononuclear cells in vitro. This seems to be mediated by a decrease in the cAMP pathway and possibly also involves PKA signal transduction downstream from cAMP [89,90] Whether this decrease in adhesiveness is mediated through down regulation of adhesion molecule expression is presently unknown.

Another mechanism through which SS may inhibit the vascular injury response is through inhibition of smooth muscle migration. Immediately after vascular injury the medial

smooth muscle cells are activated and enter a proliferative and migratory mode. Angiopeptin has been shown to inhibit SMC migration in-vitro through a Gi protein mediated pathway [91]. This seems to be a direct effect of the SS on the SMC. SS receptors have been demonstrated by Leszynski et al. on SMC of rat coronary artery using ^{125}I labeled SS [33]. Interestingly, the immunohistochemistry results in this study showed only staining of the endothelial layer without SMC staining. These results suggest the SSTR on SMC may not be SSTR2 but are made up of other subtypes of SSTR. Alternatively, SSTR expression may also be different in coronary as compared to iliac arteries used in this study.

A fifth mechanism by which SS acts to reduce the injury response may be by promoting endothelial cell regrowth. Lack of endothelial cells on the injured vessel has been shown to have a permissive, if not facilitatory, impact on SMC proliferation. [102-105]

This relationship between endothelial and SMC has been attributed to functions of the endothelium, including barrier regulation of permeability, regulation of thrombogenicity, and leukocyte adherence, as well as production of growth inhibitory molecules. [106]

Typically, within 48 hours after balloon catheter injury, endothelial cells begin to grow in from adjacent uninjured sources and repave the denuded surface by growing as a continous sheet [107]. Regrowth of the neo-endothelium over the denuded area has been associated with regression of intimal hyperplasia in the vessel wall [107,108]. Local delivery of angiopeptin in the rabbit aorta after balloon injury results in marked improvement in endothelial coverage of the injured area[79]. The mechanism of this is presently unknown. One possibility is that stimulation of SSTR on endothelial cells surrounding the injury may potentiate the actions of endothelial cell specific mitogens such as vascular

endothelial cell growth factor (VGEF) [109-111]. Gene transfer of 165-amino acid isoform of VGEF has been shown to accelerate restitution of endothelial integrity and endothelial dependent function. [112]

Another way that SS may act on injured vessels is through nitric oxide mediated mechanisms[75]. Nitric oxide is capable of not only vasodilatation but also has been shown to inhibit intimal hyperplasia as well as promote favorable vessel remodeling [92-96]. The neo-endothelium formed after balloon injury does not have the same ability to produce nitric oxide as normal endothelium[113]. Treatment with angiopeptin has been shown to have a beneficial effect on neo-endothelial function by enhancing nitric oxide dependent acetycholine-induced relaxation [77]. Perhaps it is through this same mechanism of nitric oxide enhencement that angiopeptin is able to prevent restenosis[80].

Finally, SS may decrease vascular injury response through its anti-inflammatory properties. SS has been shown to have anti-inflammatory effects both in vitro and in vivo and is capable of suppressing the expression of many inflammatory mediators. [114] Lymphocytes and mononuclear leukocytes express SSTR on their surfaces[115-116]. SS can suppress the proliferation of T lymphocytes[117-118]. Since these inflammatory cells play important roles in intimal hyperplasia by releasing growth promoting factors, the effects of SS on these cells constitute another way through which SS may inhibit intimal hyperplasia.

It is unknown to what extent each of the above mechanisms (or a combination of them) underlies the ability of SS to curtail the damaging effect of vascular injury. The up-regulation of SSTR2 receptors on endothelial cells demonstrated in this experiment

strongly suggests that the endothelial cells are a major target for SS and implicates SSTR2 as a mediating mechanism for the effect of angiopeptin on intimal hyperplasia.

The mechanism of SSTR2 up-regulation with injury is unknown but may be attributed to the release of growth factors in the injured vessel. When pancreatic tumor AR42J cells were treated with epidermal growth factor, SSTR2 density was increased in parallel with an increase in the antiproliferative effect of SS analogues [43]. SSTR2 has also been found to mediate inhibition of tumour cell proliferation [16]. In endothelial cells, SSTR2 receptors may be upregulated by a similar process and would serve to increase the ability of SS to regulate the proliferative process in vessels after injury.

If SS is to play a role in regulation of vascular response to injury, is it aneural or endocrine process that is responsible? Physiologically, SS is synthesized and released by a wide variety of cells throughout the body. Although, SS usually acts in a paracrine, neurocrine or autocrine fashion, and is rapidly metabolized with half life of 2-3 minutes[15], plasma SS concentrations in peripheral blood of normal subjects can be measured and have been reported at between 17 to 100 picograms per milliliter[119-120]. The finding of appreciable quantities of SS in peripheral blood supports the possibility that SS may act on the endothelial receptors in a hormonal fashion.

The peripheral nervous system constitutes other source of SS. In this study, SS was demonstrated in the adventitial peri-vascular nerves. SS produced at the dorsal root ganglia is transported to peripheral sensory nerve terminals, from which it is released in reponse to local injurious stimuli [8]. The idea of peri-vascular nerves sensing injury and responding promptly with secretion of suppressive neurotransmitters is appealing.

The other possible sources of SS include lymphocytes and the endothelium. SS had been reported to be synthesized in cells of the immune system [121,122]. SS has been found by immunostaining in B lymphocytes as well as macrophages inside granulomas[122, 123]. Although the endothelium has not been demonstrated to synthesize SS it has been shown to release neurohumoral substances such as acetylcholine, ATP, bradykinin, and substance P in response to stimulation with increased flow or hypoxia. These substances function in a autocrine/paracrine fashion on endothelial receptors to modify vascular tone.

The immunohistochemical staining for SSTR2 utilized both anti sera for SSTR2A and SSTR2B since both forms has been described in rat tissue[124]. Only SSTR2A produced positive staining. These two forms of SSTR2 represent alternative splicing with SSTR2A been the unspliced and SSTR2B the spliced version, encoding for 369 and 346 amino acids, respectively. The sequences of residues 1 to 331 are identical between the two forms but the length and sequence of the intracellular C-termini differ. The staining results indicate that only SSTR2A is expressed in the rat endothelium.

The amount of SSTR mRNA expression in this study was measured using mRNA for beta-actin as a control. Use of this internal control allowed the variations in the initial RNA extraction to be taken into account as well as controlling for RNA degradation. Using beta-actin as an internal control, however, adds another source of error since beta-actin is a structural protein that has been shown to change its expression with balloon injury. Fifteen days after balloon injury of rat aorta, there was a decrease in the proportion of alpha- actin synthesis and a moderate increase in beta-actin synthesis[125]. This increase in beta-actin production impacts on quantitation of PCR product when SSTR/actin density ratio is used and results in under estimation of the SSTR expression post injury. Hence the

increase in sstr2 expression in injured arteries is likely greater than was documented in this study.

Another source of error with the quantitation of mRNA in this study is the fact that PCR products were measured rather than the mRNA isolates. PCR was needed to amplify the amount of mRNA since our samples were very small. However, this amplification process also allowed measurement errors to be amplified. In addition, the amplification efficiency may be different between tubes due to difference in the amount of substrate between samples, and plateau effect caused by degradation of nucleotides or primers, inactivation of Taq polymerase, reassociation of single stranded DNA, and substrate excess after prolonged cycling. This accounts for the variability of PCR product concentrations within group of rats with the same injury age. Unfortunately the size of the actin control was the same as the SSTR product which precluded running both PCR in the same aliquot of PCR mixture. Future studies to further minimize this source of error may include using actin controls with different size of PCR products or carry out competitive PCR within each sample. A larger number of animals and larger animals to increase the yield of mRNA would also be helpful.

Taken together, the results of this study suggest that SS and its analogues exert their effect on the endothelium and play a role in mediating the development of intimal hyperplasia. Further knowledge regarding the impact and specific mechanism of SS/SSTR regulation of vascular injury and restenosis should help to improve therapy for this disease.

VII. CONCLUSION

SSTR2 was expressed on endothelial cells in normal and injured rat arteries. Its expression in the injured vessel was increased up to 1 month post injury.

VIII. FUTURE DIRECTIONS

The finding in this study supports the hypothesis that SS has a regulatory function in vascular injury. However, further studies in several areas are needed to better define the role of SS and its receptors in this setting.

The function of endothelium with respect to SS needs further investigation. Are the endothelial cells capable of stimulating its SSTR receptors in an autocrine fashion? What is the mechanism for accelerated endothelial cell regrowth with SS treatment and is it related to other endothlial cell regrowth promoters such as VEGF? Is the reduction in adhesiveness of endothleial cell to leukocytes related to adhesion molecule expression?

The role of SS nerves and other neuropeptide containing nerves in the perivascular nerve plexus needs further studies. Does stimulation or denervation of these nerves change the response of the vessel to injury? Alternatively, can chronic changes in blood flow due to injury or disease cause change in innervation?

With regard to the SSTR2 receptors further work is needed to define the triggers for increase SSTR2 expression. Which growth factors stimulate SSTR expression? Does treatment with SS increase or decrease the expression of its receptors?

This study looked at SSTR subtype 1,2, and 3 using immunohistochemistry. Are other SSTRs expressed in the blood vessel? What is the effect of stimulating these functionally distinct receptors with specific ligands?

Finally, the aim of biomedical research must be linked to practical applications. How can the information derived from this study be applied in the clinical setting? Are these animal model results transferable to human diseases? Can SS analogs be used to prevent restenosis after surgery? Another area of potential benefit is in the field of transplantation immunology where endothelial/leukocyte interaction plays a vital role in chronic graft rejections. Can an increase in SSTR expression be useful in predicting rejections? Can SS be an effective treatment in chronic rejection patients? Future studies to address the above questions can better define the role of SS in vessels and allow effective treatments to be designed.

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APPENDIX 1	
GLOSSARY	
5-HT	5 hydroxytryptamine or serotonin
Actin	Abundant protein that forms actin filaments in all eucaryotic cells. Alpha actin are found in various types of muscle cells, whereas beta and gamma actin are principle constituents of nonmuscular cells
Adhesion molecules	Transmembrane proteins involved in the adhesion between cells or to extracellular matrix
Adventitia	Outer most layer of a blood vessel containing perivascular nerves and fibroblasts
Allograft	Transplantation of tissue between adult individuals of same species
Amino acids	Organic molecule that serve as building blocks for proteins
Angiopeptin	A synthetic somatostatin analogue
Angioplasty	Surgical procedure involving direct repair of an arterial narrowing
Antibody	Protein produced by B lymphocytes in response to a foreign molecule
Antigen	Molecule that provokes an immune response
Antiserum	Serum which contain polyclonal antibodies against a protein
Apoptosis	Programmed cell death
Autocrine	Cell secretes signaling molecules that binds back to its own receptors
Autoradiography	Technique in which a radioactive object produces an image of it self on a photographic film
Bacteriophage	Any virus that can infect a bacterium
BrdU	Bromodeoxyuridine
Bypass surgery	Surgical procedure during which a conduit is connected to replace an obstructed channel
Cyclic AMP	A signaling molecule that is generated from

	ATP by stimulation of cell surface receptors
CDNA	Complementary DNA molecule made as a copy of mRNA
CGRP	Calcitonin gene related peptide
Clone	Population of cells formed by repeated division from a single common cell
Cytokines	Extra-cellular signaling protein that act as mediator in cell-cell communication
Denervation	Disconnecting neural connection from an organ or tissue
Dephosphorylation	Reaction in which phosphate group is removed from a molecule
DNA	Deoxyribonucleic acid
Domain	Region of a protein that folds to give a stable tertiary structure of its own
EGF	Epidermal growth factor
Endarterectomy	Surgical procedure which involves removal of diseased inner lining of a blood vessel
Endocrine	Secretion of signaling molecule which travel via blood stream to act on other cells
Endothelium	Single layer of endothelial cells that form the lining of all blood vessels.
Endovascular stenting	Surgical procedure involving placement of device inside blood vessel to keep it open
FGF	Fibroblast growth factor
Fibroblasts	Common cell type found in connective tissue secrets extra-cellular matrix rich in collagen
First layer	Specific antibody against the antigen to be detected in immunochemical staining
First strand reaction	Laboratory procedure converting RNA to DNA using reverse transcriptase. First step of the RT-PCR procedure
G protein	One of a large family of heterotrimeric GTP-binding proteins that are important intermediaries in cell-signaling pathways
Growth factors	Extra-cellular polypeptide signaling molecule that stimulate a cell to grow or proliferate
IL	Interleukin. Secreted peptide or protein that mainly mediates local interactions between white blood cells

Immunohistochemistry	Laboratory technique involving use of specific antibody to stain for a particular antigen
Intimal hyperplasia	Reactive proliferative process in the intima of a blood vessel in response to stress or injury
Lumen	Cavity enclosed by an endothelial sheet or a membrane
Leukocytes	White blood cells
Ligands	Any molecule that binds to a specific binding site on a protein or other molecule
Lymphocytes	A class of small mononuclear white blood cells mainly involved in immune response
Extra-cellular matrix	Connective tissue between cells that perform mechanical functions. They are composed of mostly water and a variety of macromolecules such as collagen, elastin, ground substance, and fibronectin.
Media	Middle layer of blood vessel wall defined by the internal elastic lamina internally and external elastic lamina externally. Smooth muscle cells constitute the dominant cell type
Monocytes	A class of large mononuclear white blood cells that when activated become macrophages
MRNA	Messenger ribonucleic acid
Myofibroblasts	Fibroblasts that contain myofibrils capable of contraction
NA	Nor-adrenaline
Neo-endothelium	Regrowth of endothelium after vessel denudation. These endothelial cells are functionally impaired
Neucleotides	Organic molecule that serves as building blocks for DNA and RNA
Neuropeptide	Peptides secreted by neurons as signaling molecules either at synapsis or elsewhere
Neurotransmitters	Small signaling molecule secreted by the presynaptic nerve cells at a chemical synapse to relay the signal to the postsynaptic cells
NG-nitro-L-arginin –	A nitric oxide synthesis inhibitor
Nitric oxide	An molecule that is the physiologic mediator of endothelium mediated vasodilation. It is made

	from L-arginine by nitric oxide synthase in a Ca^{++}-calmodulin dependent reaction
NO	Nitric oxide
NPY	Neuropeptide Y
Octreotide	An somatostatin analogue
Paracrine	Secretion of signaling molecules that binds to receptors of neighboring cells
PCR (polymerase chain reaction)	Technique for amplifying specific regions of DNA by multiple cycles of DNA polymerization, each with a brief heat treatment to separate complimentary pairs
PCR product	End product of the PCR consisting of multiple copies of the amplified region
PDGF	Platelet derived growth factor
Perivascular nerves	Nerve plexus surrounding blood vessels mostly contained within the adventitia
PGP 9.5	Protein gene product 9.5; a nerve specific protein
Phosphoprotein phosphatase	Enzyme that removes a phosphate group from a protein by hydrolysis
Platelet	Cell fragment, lacking a neucleus, that breaks off from a megakaryocyte in the bone marrow and is found in large numbers in the blood stream. It helps initiate blood clotting when blood vessel is injured
Protein kinase	Enzyme that transfers the terminal phosphate group of ATP to a specific amino acid of a target protein
Pre-absorption	A laboratory procedure before immunochemical staining where the specimen is bathed in nonspecific protein to decrease background staining
Primers	A pair of oligonucleotides with similar G+C content each complementary to one end of the DNA target sequence
Receptor	Protein that binda a specific extracellular signaling molecule and initiates a responds in the cell
Remodeling (of vessels)	Change in vessel structure in response to chronic change in hemodynamic stress

Restenosis	Recurrent narrowing of a vessel after treatment to widen it
Reverse transcription	Process of making complimentary DNA from RNA
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction. A process which amplifies minute quantity of RNA into detectable quantities of cDNA
Second layer	Second step in the protocol of immunochemical staining where antibodies against the primary antibodies are put on
Sequencing	Determination of order of nucleotides in a DNA molecule
SMC	Smooth muscle cells. Located mainly in the media of the blood vessel
Somatostatin	A peptic hormone as well as neurotransmitter with a diverse, mainly inhibitory function throughout the human body
Somatostatin analogues	Synthetic peptides that have similar structure and function as somatostatin
SS	Somatostatin
SSTR 1-5	Somatostatin receptors; 5 subtypes have been described.
Stenosis	Lumional narrowing in a hollow tubular viscus
Substance P	One of the neuropeptides that induces vasodilatation
Sympathectomy	Interruption of sympathetic nerve supply
Taq polymerase	A thermal stable DNA polymerase used in PCR
TGF	Transforming growth factor
Thrombus	Solid mass formed in living blood vessels or heart
Thymidine incorporation	
Translation	Process by which the sequence of nucleotides in a messenger RNA molecule directs the incorporation of amino acids into protein; occurs on a ribosome
Transmodulation	Cell surface receptor regulation by heterogeneous ligands
Tyrosine kinase	Enzyme that transfers the terminal phosphate of