New observations on the role of VEGF/VPF and nitric oxide in the vessel wall

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New observations on the role of VEGF/VPF and nitric oxide in the vessel wall

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
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volgens het besluit van het College van Decanen,
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Abbreviations

ACh	acetylcholine
AEC	3-amino-9-ethylcarbazole
ANOVA	analysis of variance
Ao	thoracic aorta
BSA	bovine serum albumin
CaM	calmodulin
cDNA	Complementary DNA
cGMP	cyclic GMP
Da	Dalton
DAB	3,3' diaminobenzide
EC	endothelial cell
EDRF	endothelium derived relaxing factor
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FMN	flavin mononucleotide
GAPDH	glyceraldehyde phosphate dehydrogenase
GMP	guanosine 3'-monophosphate
GTP	guanoside 5'-triphosphate
HC	high cholestoral diet
HIF	hypoxia-induced soluble factor
HUVEC	human umbilical vein endothelial cell
i-	inducible-
IEL	internal elastic lamina
IGF-1	insulin-like growth factor-1
IMA	internal mammary artery
IP ₃	inositol-tri-phosphate
IVC	inferior vena cava
LDL	low density lipoprotein
L-NAME	N ^ω -nitro-L-arginine
L-NMMA	N ^G -monomethyl-L-arginine
MAPK	mitogen-activated protein kinases
MCP-1	monocyte chemoattractant protein-1

NADP	nicotinamide-adenine-dinucleotide phosphate
n-LDL	native LDL
n-NOS	neuronal NOS
NE	norepinephrin
NF- κ B	nuclear factor- κ B
NGF	nerve growth factor
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
NZWR	New Zealand white rabbit
O_2^-	superoxide anion
ONOO^-	peroxynitrite
ONOOH	peroxynitrous acid
ox	oxidized
PA	pulmonary artery
PBS	phosphate-buffered solution
PDGF	platelet derived growth factor
PI-3	phosphatidylinostol-3'
RAM	rabbit antibody macrophages
rh-	recombinant human-
RTK	receptor tyrosine kinases
RT-PCR	reverse transcriptase-polymerase chain reaction
SA	surface area
SNP	sodium nitroprusside
SMC	smooth muscle cell
SV	sapheneus vein
TdT	terminal deoxynucleotidyl transferase
TNF- α	tumor necrosis- α
TUNEL	terminal deoxynucleotidyl transferase fluorescein nick end labeling
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VPF	vascular permeability factor
WHHL	Watanabe heritable hyperlipidemic

Chapter 1

Introduction

The preservation of normal vascular anatomy and function over time has been shown to be crucial in maintaining tissue perfusion. It has been demonstrated that the endothelial cells play an important role in conserving vascular integrity. In this thesis the hypothesis will be tested whether a growth factor specific for endothelial cells can exert a maintenance/repair role on vascular endothelium by augmenting the release of nitric oxide. Furthermore, additional studies on the aspects of this growth factor in a rabbit model of hypercholesterolemia will be presented, especially with regard to the biochemistry of nitrogen oxides.

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF)

VEGF/VPF-history

In 1989 Gospodarowicz¹ and Ferrara² reported on a protein secreted by bovine pituitary folliculo-stellate cells, that caused mitogenic activity in vascular endothelial cells, but not in other cell types tested, including epithelial, mesenchymal, or neuroectodermal cells or a variety of tumor cell lines.³ Because of the unique aspect of its narrow target cell specificity, this mitogen has been named vascular endothelial growth factor (VEGF).

Also in 1989 Connolly⁴ and Senger⁵ identified a multifunctional protein secreted by tumor cells. Since it functioned as a potent and fast acting mediator of vascular permeability,⁶ it was named vascular permeability factor (VPF). VPF also has been documented as an endothelial cell specific growth factor^{1,2,7} and as an angiogenic agent *in vivo*.⁸ Analysis of receptor binding studies revealed two distinct classes of binding sites for this protein typically present on endothelial cells.⁹ Structural characterization and cDNA cloning of the involved factors revealed a single protein that hereafter will be referred to as VEGF/VPF.^{1,10}

VEGF/VPF-structure

VEGF/VPF is composed of two disulfide-bridged subunits each with a molecular weight of approximately 23 kDa. Analysis of cDNA clones predicts the existence of 206-, 189-, 165-, and 121-amino acid forms of the mature human VEGF/VPF subunit.^{11,12,13} The mRNAs for these isoforms arise by alternative splicing from a gene composed of 8 exons.¹¹ (figure 1) The alternative splicing involves exons 6 and 7; if neither exon is removed, VEGF/VPF₁₈₉ is generated; if only exon 6 is removed VEGF/VPF₁₆₅ is generated; and if both exons 6 and 7 are removed, VEGF/VPF₁₂₁ arises. Alternative splicing has profound effects on the behaviour of the translated proteins following secretion from the cell. The transcript of VEGF/VPF₁₆₅, the most common form, contains an additional 44 codons relative to VEGF/VPF₁₂₁, resulting in a homodimeric protein of approximately 46 kDa. These additional 44 amino acids convert it to a basic polypeptide with 7 cysteines not found in the shortest form. These 7 cysteines gives the protein heparin binding capability. VEGF/VPF₁₆₅ was secreted in a stably transfected embryonic kidney cell line, and 70% appeared to bind to unidentified components on the cell surface or in the extracellular matrix.¹² In contrast, the shortest form, VEGF/VPF₁₂₁, is secreted and freely soluble. Little or no VEGF/VPF₁₈₉ is found in a freely soluble form. The additional 24-amino acid segment of this protein encoded by exon 6 is even more basic in nature, containing 12 lysine and arginine residues. VEGF/VPF₂₀₆ has an additional insertion of 17 amino acids. There is no intron between exon 7 and the coding sequence for the additional 17 amino acid insertion found in VEGF/VPF₂₀₆. It is a very rare form so far identified only in a human fetal liver cDNA library.¹³

It has been suggested that the unknown VEGF/VPF-binding sites in cell cultures involve a heparin containing proteoglycan.¹⁴ Interestingly, a variety of studies demonstrated that heparin-containing proteoglycans are the constituents of the extracellular matrix that are responsible for binding and concentrating growth factors with angiogenic capacity.^{15,16,17}

The intron-exon pattern of the gene and the conceptual translation of the exon sequences indicate a relation with the genes encoding the platelet-derived growth factor (PDGF) (Figure 1).^{18,19} Data base searches with the amino acid sequences reveal approximately 21% and 24% homology respectively with both the mature forms of the A and B chains of human PDGF.^{20,21} Especially the locations of the seven cysteine residues involved in intra- and inter-molecular disulfide bridging are completely conserved among the sequences.^{10,22}

Despite the structural similarities between VEGF/VPF and PDGF, the two proteins have very different biological activities. PDGF is an effective mitogen on cells of mesenchymal origin such as fibroblasts and vascular smooth muscle cells, whereas VEGF/VPF is active primarily on cells of vascular endothelial origin.^{1,2,23} The different target cell specificity of VEGF/VPF implies that its action is mediated through distinct cell surface receptors.

Figure 1. Comparison of the intron-exon structure of the VEGF/VPF gene with those of the PDGF A and B chain genes. The number of each exon is given above each gene structure; coding regions are marked by diagonal lines and untranslated regions by open boxes. The precise lengths of the 3'-untranslated regions of PDGF A and VEGF/VPF are not known. The numbers between the exons refer to the size of each intron in kilobases. Both the normal and the alternative exon splicing patterns are shown. The scale refers to the exon sequences only. Figure from Tischer *et al* (ref. 11, with permission).

VEGF/VPF-receptors

In 1991 Terman and colleagues identified a new endothelial cell receptor tyrosine kinase gene, named *KDR*.²⁴ *KDR* was identified from a human endothelial cell cDNA library using primers homologous to the kinase domain of known receptor tyrosine kinases (RTK). The complete coding portion of *KDR* contains 4068 nucleotides and yields a 1356-amino acid residue protein. Matthews and colleagues reported the complete amino acid sequence of *Flk-1*, of which the strong sequence homology with the *KDR*-protein suggests that the two proteins are the human and mouse versions of the same gene.^{25,26,27} Seven immunoglobulin-like domains are present in the extracellular portion of the *KDR* protein, matching those of *Flk-1*. The mRNA for both receptors is selectively expressed in endothelial cells, but not in other cell types in adult²⁸ or in fetal²⁹ rat tissues.

It has been reported that the *fms*-like tyrosine kinase *Flt-1* is a VEGF-receptor also.^{30,31} Interestingly, *KDR/Flk-1* and *Flt-1* share many structural similarities. Each is a RTK-unit with kinase inserts domains of similar length. Each contains seven immunoglobulin-like domains in the extracellular portion, and there is a degree of structural similarity between the two receptors in each domain.³² The mRNA for both receptors is selectively expressed in endothelial cells and quantitative autoradiographic analysis of whole-body tissue sections from the adult rat demonstrates that ¹²⁵I-rhVEGF/VPF binds to these cells with high affinity in the 10-50 pM range and low capacity.²⁸ This binding is reversible and can be replaced by rhVEGF/VPF, but not by other growth factors. It is likely that high affinity binding identified in tissue sections reflects contribution of both *Flk-1* and *Flt-1* proteins.^{27,33} Because VEGF/VPF₁₆₅ is a heparin-binding protein, non-specific binding

to proteoglycans was evaluated by adding an excess heparin, which did not significantly influence specific (rhVEGF/VPF-displaceable) binding. Furthermore, excess of PDGF, which binds to heparin as well, did not result in displacement either.²⁸

Together with *Flk-1/KDR* and *Flt-1*, *Flt-4* constitutes another member of a new subfamily of RTKs.^{34,35} This receptor also contains seven immunoglobulin-like repeats and is structurally closely related to *Flk-1* and *Flt-1*. Interestingly, these similarities are also present between the A and B chains of the PDGF receptor.³⁶

More recently, new isoforms of VEGF/VPF, named VEGF/VPF-B and VEGF/VPF-C, have been isolated and cloned from a cDNA library prepared from the human prostatic adenocarcinoma cell line PC-3.^{37,38,39} For VEGF-C, it has been demonstrated that the protein is proteolytically processed, secreted to the cell culture medium, and bound to the extracellular domain of *Flt-4* where it induces tyrosine autophosphorylation of *Flt-4* and *KDR*. In addition, it has been shown that VEGF-C also stimulates migration of endothelial cells in collagen gels. Additional functional assays still are mandatory to assess the biological similarity of these highly related growth factors.

Since the earliest VEGF/VPF-binding sites were identified in the blood islands in the yolk sac, the expression of VEGF/VPF receptors may be one of the earliest events occurring in endothelial cell differentiation.^{29,40} Consequently, VEGF/VPF may play an important role in the development of the vasculature of mammalian embryos. The tight temporal and spatial regulation of mRNA expression, documented by Breier⁴¹ and Jakeman,²⁹ together with the ubiquitous nature of VEGF/VPF binding to endothelial and preendothelial cells are likely to serve the highly regulated development of the embryonic vascular system. Further support for this notion is the abnormal blood vessel development in transgenic mice embryos missing a single VEGF/VPF allele.⁴² VEGF/VPF deficiency impairs most steps of early vascular development, including *in situ* differentiation of blood islands (vasculogenesis), sprouting from preexisting vessels (angiogenesis), lumen formation, the formation of large vessels, the establishment of interconnections, and the spatial organization of intra- and extraembryonic vessels. Similar results were obtained in *Flk-1*⁴³ and *Flt-1*⁴⁴ deficient mice embryos.

Postnatally, expression of VEGF/VPF receptors by quiescent endothelium is reduced,^{33,45,46} or absent.⁴⁷ Both the *Flt-1* and the *KDR/Flk-1* receptors have been shown to be upregulated only at sites of recurrent angiogenesis, such as in the corpora lutea of the ovary,^{28,46} or in pathologic conditions as in psoriasis and tumor formation.^{45,47,48}

VEGF/VPF itself is not capable of upregulating the expression of its receptors.⁴⁹ Interestingly, hypoxia has been shown to be a potent stimulus for augmented transcription of the VEGF/VPF gene in vascular endothelial cells in culture^{50,51,52} and *in vivo*.⁵³ In addition, it has been demonstrated both *in vitro*^{49,54} and *in vivo*,⁵⁵ that ischemic tissues produce hypoxia-induced soluble factors (HIFs), capable of upregulating VEGF/VPF receptor expression on endothelial cells. Erythropoietin is another example of a protein that is upregulated under hypoxic conditions. An intriguing observation is

the homology between a sequence in the VEGF/VPF promotor and a nucleotide sequence in the erythropoietin promotor, identified as a binding site for an HIF.^{11,56}

VEGF/VPF-function

The increased VEGF/VPF expression and the upregulation of its receptors under hypoxic conditions constitute a paracrine loop that stimulates endothelial cell mitogenesis, which is the initiating feature in angiogenesis.⁵⁷ For new blood vessel growth the activation of endothelial cells is followed by disruption of the basement membrane and subsequent migration of endothelial cells into the interstitial space in the direction of an ischemic stimulus. Intracellular vacuolar lumen formation, pericyte capping, and production of a new basement membrane complete the developmental sequence.⁵⁷ This process is facilitated by VEGF/VPF-induced endothelial cell secretion of serine proteases such as urokinase-type and tissue-type plasminogen activators.⁵⁸ In addition, VEGF/VPF increases the expression of matrix metalloproteinases, thus promoting a prodegradative environment that enables endothelial cells to migrate.⁵⁹ It has been shown that VEGF/VPF stimulates tumor growth by enhancing tumor angiogenesis in renal cell carcinoma, colon carcinoma and several intracranial tumors.^{60,61,62,63} A strong correlation has been reported between the degree of vascularization of the malignancy and VEGF mRNA expression.⁶⁴ Consequently, monoclonal antibodies capable of inhibiting VEGF/VPF-induced angiogenesis in vivo exert a dramatic inhibitory effect on the growth of human sarcomas.^{63,65} Whether anti-VEGF/VPF therapy has therapeutical applications in highly vascularized tumors remains to be investigated.

VEGF/VPF mediated angiogenesis has been reported not only in hypoxic areas of tumors, but also in nonneoplastic ischemic conditions such as coronary artery disease,^{52,54} peripheral artery disease,⁶⁶ and diabetes mellitus.⁵⁴ VEGF/VPF therapy has been used to promote angiogenesis in conditions of insufficient tissue perfusion in a model of rabbit ischemic hindlimb,^{67,68,69} rat and rabbit cornea,^{7,70} the chorioallantoic membrane,² and dog ischemic myocardium.⁷¹ Currently, studies are performed to establish therapeutic angiogenesis in human peripheral artery disease following arterial gene transfer with a plasmid encoding the 165 amino-acid isoform of VEGF/VPF.^{72,73}

Interestingly, perfusion through collateral circulation produces endothelial dysfunction in the recipient downstream microvessels as opposed to microvessels perfused by normal arterial circulation.⁷⁴ It has been suggested that the collateral circulation fails to develop at a rate sufficiently rapid to prevent ischemic damage to endothelial cells of the downstream microvasculature. Expedited development of collaterals in response to administration of an angiogenic growth factor such as VEGF/VPF has been shown to markedly improve endothelium-dependent flow.⁷⁵

A further potential therapeutical application of VEGF/VPF therapy is the prevention of restenosis following angioplasty. It has been proposed that damage to the endothelium is the crucial event triggering fibrocellular intimal thickening.⁷⁶ Asahara and colleagues

showed that local delivery of VEGF/VPF accelerated endothelial cell mitogenesis and reendothelialization and as a result attenuated intimal hyperplasia in balloon-injured rat carotid arteries.⁷⁷ The specificity of VEGF/VPF for endothelial cells may be especially useful to avoid concomitant mitogenesis of vascular smooth muscle cells.

Nitric oxide (NO)

NO-history

In 1980 Furchgott and Zawadzki observed that vascular endothelium is mandatory in acetylcholine-induced relaxation of smooth muscle cells.⁷⁸ The involved endothelium-dependent relaxing factor (EDRF) has been shown to be the free radical nitric oxide (NO).^{79,80,81} NO is a gas that is poorly soluble in water. It has a short half-life of approximately 10 to 60 seconds in tissue, because it readily reacts with oxygen, superoxide radicals, and transition metals such as iron and copper.^{82,83} Consequently, NO does not function as a hormone since oxyhemoglobin rapidly combines with NO to yield methemoglobin and nitrate (NO₃⁻).⁸⁴ However, NO is particularly useful as an intracellular messenger because it can easily diffuse through most cells and tissues.⁸⁵

NO-chemistry

NO is generated from the terminal guanidino-nitrogen of L-arginine by an N^ω-hydroxyl-L-arginine intermediate yielding citrulline. This reaction is catalyzed by a nitric oxide synthase (NOS):

Three different isoforms of NOS have been documented so far, based on three different genes that have been cloned and sequenced: two isoforms of constitutive NOS present in vascular endothelial cells (ec-NOS) or in brain tissue (neuronal NOS, n-NOS), and one isoform of inducible NOS (i-NOS). These isoforms differ in cell and organ localization and in sensitivity to calcium stimulation.⁸⁶ NOS proteins share approximately 50% amino acid sequence identity each. Within each isoform group (ec-NOS, n-NOS, i-NOS), there is a high degree of amino acid identity across species (80-94%).⁸⁷ The human ec-NOS contains 1,203 amino acids. A schematic representation of the amino acid sequences is shown in figure 2. Comparison of the deduced amino acid sequences from the three isoforms of NOS demonstrates that the greatest similarity in structure is found in the last two-thirds of the molecule, the carboxyl terminal. The amino terminal is unique for each isoform and contains sites for potential posttranslational modification.⁸⁸

All isoforms require four co-factors: flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), iron-protoporphyrin IX (heme), and tetrahydrobiopterin.

Neuronal NOS **Cytokine-inducible NOS** **Endothelial NOS**

Figure 2. Schematic representation of the three different isoforms of nitric oxide synthase (NOS). Neuronal NOS contains an additional amino acid sequence with unknown function. The amino terminal of ec-NOS has been myristoylated, which is mandatory for membrane attachment. CaM: calmodulin; other abbreviations: see text.

Furthermore, nicotinamide-adenine-dinucleotide phosphate (NADPH) is mandatory for NOS activity and therefore has been used to determine NOS capacity.

The two isoforms of constitutive NOS (ec-NOS, n-NOS) are calcium and calmodulin dependent and continuously generate small quantities of NO. A variety of agents capable of increasing intracellular free calcium such as acetylcholine and bradykinin have been shown to cause activation of ec-NOS, which subsequently leads to endothelium-dependent vasorelaxation.⁸⁹ NO synthesis in vascular endothelial cells is regulated by two known mechanisms. Activation of NOS is dependent on the intracellular free calcium concentration and the rate of L-arginine transport into endothelial cells.⁹⁰ Although ec-NOS activity is inhibited by high doses of exogenous NO (NO donors), it has been shown not to be sensitive to feedback inhibition by endogenous NO production.⁹¹

The N-terminal region of the ec-NOS protein contains a unique sequence for attachment of myristic acid. This N-myristoylation allows this enzyme to attach to a specific membrane site.⁹² Consequently, only membrane-bound ec-NOS has been shown to be activated by modulations of flow conditions: shear stress-induced endothelial NO release represents a highly effective and sensitive system for local control of vascular tone.^{93,94}

The NO synthesizing enzyme in macrophages is referred to as i-NOS and is capable of producing nanomolar quantities NO throughout the life of the enzyme, which is approximately 1000-fold more than the constitutive production by ec-NOS. Calmodulin

is bound tightly to i-NOS, but the enzyme is not sensitive to fluctuations in intracellular free calcium concentrations.^{95,96} In quiescent conditions i-NOS is not expressed in leukocytes, but it can be induced in several different cell types by cytokines such as interleukin-1 β , tumor necrosis factor- α (TNF- α), and interferon- τ as well as by bacterial lipopolysaccharides.⁹⁷ The induction of i-NOS is responsible for the NO-mediated cytotoxic properties of macrophages.⁹⁸ In rabbits, 30 minutes after administration of lipopolysaccharides an NO-related decrease in blood pressure has been observed, reflecting the time necessary for i-NOS to become expressed.⁹⁹ Its induction is inhibited by glucocorticoids, which explains the beneficial action of these type of agents in the treatment of septic shock.¹⁰⁰ It has been observed that a combination of cytokines and endotoxins exerts a synergistic action in cytotoxicity, which possibly reflects a primordial, but effective mechanism for microcidal activity of macrophages.¹⁰¹

NO-function

Of the known 92 naturally occurring elements in the universe 6 are present in all living creatures and are indispensable to life as we know it on this planet, including nitrogen and oxygen.¹⁰² The simple molecule NO has been incorporated from the early occurrence of life, which might be an explanation for the great number of phenomena that are mediated by this free radical gas, including endothelium-dependent vascular relaxation,⁸⁰ but potentially also cardiac and skeletal muscle relaxation,¹⁰³ endothelial permeability, macrophage-mediated cytotoxicity,¹⁰⁴ inhibition of platelet adhesion and aggregation,^{105,106} inhibition of leukocyte adhesion,¹⁰⁷ DNA modifications and repair, transcriptional regulation,¹⁰⁸ tissue injury and inflammation, hormone production and secretion, relaxation of the human penile corpus cavernosum,¹⁰⁹ regulation of basal blood pressure,¹¹⁰ long-term potentiation of synaptic transmission,¹¹¹ glomerular and medullary microcirculation,¹¹² ventilation-perfusion matching,¹¹³ intestinal secretion and ion transport, including prevention of pylorospasm in infantile hypertrophic pyloric stenosis.¹¹⁴

NO reacts with a variety of low and high molecular weight molecules, such as guanylyl cyclase, cyclic nucleotide protein kinases and phosphodiesterases, cyclooxygenase, heme proteins, and phospholipase C.¹⁰³ The NO generated in endothelial cells binds to the heme component of soluble guanylyl cyclase-guanosine 5'-triphosphate (GTP)-complex located in adjacent smooth muscle cells. Nitrosation by NO dislocates the heme-iron out of the plane of the porphyrin ring, which activates the catalytic site of guanylyl cyclase, resulting in an increase of cyclic guanosine 3'-monophosphate (cGMP) production. The intracellular concentration of cGMP is determined by the rate of formation and hydrolysis of cGMP by the type V isoenzyme of cyclic 3',5'-nucleotide phosphodiesterases.¹¹⁵ The mechanism by which cGMP produces vasorelaxation is not entirely clear. It has been suggested that vasorelaxation is mediated by a cGMP-dependent protein kinase causing

activation of Ca^{2+} -dependent K^+ channels.^{116,117} Other groups proposed that cGMP impairs angiotensin II activity in mesangial cells, thus promoting vasorelaxation.¹¹⁸

Recently, it has been reported that cGMP-elevating agents suppress proliferation of vascular smooth muscle cells.¹¹⁹ Experiments conducted by several groups have elucidated growth factor-related signaling pathways to mitogenesis of vascular smooth muscle cells. Cytokines and several growth factors such as fibroblast growth factor (FGF), platelet derived growth factor-AB (PDGF-AB), and epithelial growth factor (EGF) have been described to contribute to growth of vascular smooth muscle cells in injured blood vessels. These growth factors stimulate the synthesis of DNA and cell proliferation by activating the phosphorylation cascade of mitogen-activated protein kinases (MAPK).¹²⁰ This activation initiates the cell cycle transition via phosphorylation of nuclear transcription factors of genes such as *c-fos*, *c-jun*, and *c-myc*.¹²¹ It has been reported that interleukin-1 β acts synergistically with these growth factors to promote smooth muscle cell mitogenesis.¹²² Inhibitors of NOS did not block this co-mitogenic effect, suggesting that this synergism is independent of the NO/cGMP pathway.

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) has been shown to act exclusively on endothelial cells and to be capable of augmenting endothelial cell NO release (this thesis). So far, no evidence has been provided that VEGF/VPF promotes vascular smooth muscle cell mitogenesis via the NO/cGMP pathway. Support for this notion is found in the observation that during differentiation of neuronal cells nitric oxide triggers a switch to growth arrest rather than to cell proliferation.¹²³ In addition, it has been postulated that NO is involved in the control of vascular smooth muscle cell apoptosis.¹²⁴ NO is capable of inducing smooth muscle cell apoptosis, thus contributing to tissue homeostasis in the media of the vessel. Some controversy exists whether programmed cell death is mediated directly by NO¹²⁵ or via the NO/cGMP pathway.¹²⁶

NO-oxidative stress

The production of free radicals has been associated with oxidative stress-related tissue injury. A major mechanism in the generation of oxidative stress in atherosclerosis is associated with the production of NO *in vivo* and its reaction with the superoxide anion (O_2^-) to form peroxynitrite:

The direct toxicity of NO is relatively low, but greatly enhanced by the formation of peroxynitrite.¹²⁷ Peroxynitrite is a strong oxidant that can react with a number of biological molecules. Nitration of structural proteins, for example, mostly has major pathological consequences.¹²⁸ It has been observed that myocyte depression associated with NO production is due to nitration of the contractile proteins.¹²⁹ Normally, the concentration of superoxide is kept low by natural scavengers such as superoxide

dismutase, although a large flux of superoxide is produced by aerobic metabolism.¹³⁰ However, the reaction rate for the formation of peroxynitrite has been determined to be $6.7 \pm 0.9 \cdot 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is approximately six times faster than the scavenging of superoxide with copper or zinc superoxide dismutase at physiological conditions.¹³¹ Consequently, NO is the only known biological molecule produced in high enough concentrations under pathological conditions to outcompete endogenous superoxide dismutase for superoxide. In atherosclerotic lesions the production of both O_2^{-132} and NO (this thesis) is significantly increased, culminating in a sharp increase in oxidative stress employed by the production of peroxynitrite.

One of the most important sources of a pathological increase in NO are lipid-laden foam cells in the subendothelial space. The recruitment of monocytes into the arterial wall has been shown to be one of the earliest events in the pathogenesis of atherosclerosis.¹³³ The mechanism whereby lipoproteins are initially oxidized has never been proven, but Graham¹³⁴ and colleagues have shown that peroxynitrite modifies low-density lipoproteins into a form recognized by the macrophage scavenger receptor. Experimental studies in animal models of atherosclerosis have demonstrated that these cells accumulate subendothelially after attachment to the arterial endothelium.¹³⁵ Cytokine-induced oxidative stress is an important regulatory signal in endothelial cells that mediates the expression of vascular cell adhesion molecule-1, which is a crucial initiating event in the development of atherosclerosis.¹³⁶ Subsequent penetration of the endothelial layer presumably occurs in response to chemotactic factors such as monocyte chemoattractant protein 1 (MCP-1) released from cells in the vascular wall.¹³⁷ Once accumulated in the subendothelial space, macrophages start to ingest oxidized lipoproteins and become foam cells. In time, these nests of foam cells lead to proliferation of smooth muscle cells, the deposition of increased extracellular matrix, the formation of extracellular pools of lipid, and the development of necrotic foci to form intermediate lesions. Eventually, the development of fibrous plaques occurs, which ultimately produces the clinical manifestations of atherosclerosis.¹³⁸

NO-measurements

A variety of techniques have been previously employed to measure NO. NO production has been studied using a chemiluminescence assay;¹³⁹ this approach measures the intensity of fluorescent radiation emitted after oxidation of NO by ozone. The use of this method for measurement of NO production in an aqueous solution first requires reduction of NO_2^- back to NO, and second transfer of NO from solution to gas. A limitation of this technique is that removal of NO from solution may introduce significant error at lower concentrations.

Kiechle and Malinski reported an electrochemical method, based on registration of current, secondary to oxidation of NO.¹³⁹ This technique permits measurements to be made within short time intervals on the surface of the cell membrane, using

electrochemical sensors with a diameter between 0.2 and 1.0 μm . The size of the sensor, however, restricts its use to measurements of NO release from cells, and is not applicable for NO registration from intact tissue specimens.

In the current studies, we therefore employed an alternative method, UV-visible spectrophotometry, which is relatively easy to perform, yields reproducible results, and allows measurements of significant differences of NO concentrations in the micromolar range from intact vascular segments. This approach will be discussed in detail in the *Methods*-section of chapter 2.

Hypothesis

The function of VEGF/VPF on quiescent endothelial cells remains enigmatic. It has been thought that VEGF/VPF receptors are importantly downregulated in mature endothelium.^{33,48} However, Peters and colleagues observed expression of *Flt-1* mRNA by quiescent endothelium of the adult mouse in multiple organs, including brain,¹⁴⁰ corresponding to a similar pattern of ^{125}I -VEGF binding described earlier by Jakeman and colleagues;²⁹ these studies thus suggested that VEGF/VPF, produced by vascular smooth muscle cells, macrophages, fibroblasts or other cell types in the vessel wall, might have a function in mature vessels other than mediating vascular growth, such as conserving the integrity of the endothelial layer in mature blood vessels.

NO has been implicated as a key molecule involved in the regulating function of vascular endothelium and, consequently, the prevention of intimal thickening responsible for compromised arterial patency.^{141,142} VEGF/VPF, too, has been shown to inhibit intimal thickening in rat and rabbit models of balloon-injured arteries.^{77,143} The results of these animal studies suggest that NO is at least in part responsible for mediating this inhibitory effect of VEGF/VPF on intimal thickening.

Defective endothelial function has been shown to have a permissive impact on intimal thickening. This inverse relation is attributed to the loss of certain NO-mediated putative endothelial functions, including barrier regulation of permeability, thrombogenicity, leukocyte adherence, and production of growth-inhibitory molecules. Interestingly, it has been observed that aging, an important determinant of vascular disease, specifically is associated with reduced concentrations of endothelial NO release.¹⁴⁴ Also, in the human coronary circulation, it was observed that despite the absence of angiographic evidence of atherosclerosis, exposure to risk factors was associated with reduced bioavailability of NO.¹⁴⁵

We thus hypothesized that

- a. functional VEGF/VPF-receptors are present on quiescent endothelial cells of mature blood vessels harvested from different regions and from different adult species;
- b. VEGF/VPF has a maintenance/repair role serving the integrity of the endothelial layer.
- c. that VEGF/VPF increases the bioavailability of NO;

- d. the formation of peroxynitrite is associated with reduced bioavailability of endothelial NO and the occurrence of apoptosis; and
- e. VEGF/VPF protects endothelial cells, but not other cell types, from oxidative stress-induced apoptosis.

This thesis

Data supporting these hypotheses will be provided in chapter 2 and 3, where functional VEGF/VPF receptors on quiescent endothelium of rabbit and human blood vessels are shown to be involved in augmenting endothelial cell nitric oxide release. This observation supports the theory that some of the known effects of VEGF/VPF may reflect restored or enhanced NO production.

In chapter 4 data are presented supporting the hypothesis that VEGF/VPF exerts a maintenance role on vascular endothelial cells in a rabbit model of hypercholesterolemia. VEGF/VPF preserves endothelial function as evidenced by the induction of normal NO-mediated vasomotor responses. However, after the occurrence of neointima formation, this beneficial effect of VEGF/VPF on endothelium is shown to be lost. Oxidative stress implemented by the generation of peroxynitrite in the neointima results in reduced bioavailability of NO.

Although the conserving effect of VEGF/VPF on endothelial function will be lost in hypercholesterolemia-induced intimal thickening, in chapter 5 it will be demonstrated that VEGF/VPF does protect endothelial cells from peroxynitrite-induced apoptosis. Because of the narrow target specificity of VEGF/VPF, apoptosis of other intimal cells is not affected. The result is a reduced cellularity of the neointima, which may serve increased vascular patency.

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Chapter 2

Vascular Endothelial Growth Factor/Vascular Permeability Factor Augments Nitric Oxide Release From Quiescent Rabbit and Human Vascular Endothelium

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Abstract

Background

Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) is an endothelial cell (EC) mitogen. This feature is considered central to the documented role of VEGF/VPF in promoting angiogenesis. More recent evidence suggests that VEGF/VPF may also serve a "maintenance" function, modulating various aspects of EC biology. In the present study we sought to determine the extent to which VEGF/VPF may stimulate the release of nitric oxide (NO) from normal ECs.

Methods and results

Vascular segments from the thoracic aorta, pulmonary artery, and inferior vena cava of New Zealand white rabbits were placed in an organ chamber and exposed to VEGF/VPF; aliquots of organ chamber solution were then reacted with Griess solution to determine the production of NO. We observed a dose-dependent rise in NO concentration ([NO]) upon exposure to VEGF/VPF. In comparison to stimulation with acetylcholine, the onset of increased [NO] following administration of VEGF/VPF was slower, reaching a maximum value after 8 min. Preincubation of the aortic segments with L-arginine raised by 2-fold both baseline [NO] and [NO] stimulated by addition of 2.5 $\mu\text{g/ml}$ VEGF/VPF. Removal of CaCl_2 from the Krebs solution, disruption of the endothelium, and administration of L-NMMA abrogated the stimulatory effect of 10 $\mu\text{g/ml}$ VEGF/VPF. Similar findings were documented using an NO-specific polarographic electrode to measure NO release from cultured human umbilical vein ECs.

Conclusion

VEGF/VPF stimulates production of NO from rabbit and human ECs. This finding: 1) constitutes inferential evidence for the presence of functional VEGF/VPF receptors on quiescent endothelium of the adult rabbit, as well as human ECs; and 2) supports the notion that putative maintenance functions of VEGF/VPF may include regulation of baseline synthesis and/or release of EC NO.

The factors which govern the production of nitric oxide (NO) by endothelial cells (ECs) of the arterial wall have been shown to include physical factors such as shear stress^{1,2} and cyclic stretching^{3,4}, and neurohumoral factors, most notably acetylcholine (ACh) and bradykinin.⁵ The extent to which cytokines elaborated by the arterial wall may further modulate EC production of NO has been less well studied.

Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) constitutes a possible candidate for this role. VEGF/VPF is a 45-kD heparin-binding dimeric glycoprotein that is mitogenic for ECs.^{6,7,8} The possibility that the effects of VEGF/VPF on EC biology extend beyond mitogenesis and/or migration to involve a "maintenance role" has been previously suggested by Peters et al⁹ and others.^{10,11,12} Consistent with this notion, VEGF/VPF has been previously shown by Brock et al¹³ to increase cytosolic Ca^{2+} in human endothelial cells, and by Ku et al¹⁴ to cause dose-dependent relaxation in isolated canine coronary arteries that could be abolished by prior endothelium disruption and/or N^G -monomethyl-L-arginine (L-NMMA) pretreatment. Recent studies in our own laboratory have demonstrated that VEGF/VPF promotes recovery of disturbed endothelium-dependent flow in the rabbit ischemic hindlimb,¹⁵ causes NO-dependent hypotension in two different animal species,¹⁶ and inhibits intimal thickening in two different animal models of arterial injury.^{17,18}

Accordingly, in the present study we sought to determine more directly the extent to which VEGF/VPF may stimulate the release of NO from the normal arterial wall. We first used a spectrophotometric assay based on the reaction of nitrite with Griess reagent to demonstrate a dose-dependent rise in NO concentration in vascular segments freshly harvested from the thoracic aorta, pulmonary artery, and inferior vena cava of New Zealand white rabbits; this response was abrogated by endothelial denudation, L-NMMA pretreatment, and/or removal of CaCl_2 from the organ bath solution. These findings were then reproduced using an NO-specific polarographic electrode to measure NO released from cultured human umbilical vein ECs. The results of these experiments thus suggest a potential role for VEGF/VPF in the regulation of NO release from ECs in the vasculature, and parenthetically confirm the presence of VEGF/VPF receptors in quiescent adult endothelium.

Material and methods

Vessel preparations

Experiments were performed on vessels isolated from male New Zealand white rabbits of 3000-3500 g. Only male rabbits were used to avoid inconsistent outcomes attributable to gender alone.¹⁹ The experimental protocol described was conducted according to protocols approved by the St. Elizabeth's Institutional Animal Care and Use Committee.

Following premedication with xylazine, 2 mg/kg, rabbits were anesthetized with a mixture of intramuscular ketamine (Fort Dodge Laboratories, IA), 50 mg/kg, and acepromazine, 0.8 mg/kg. A small incision was made to expose the carotid artery, following which the upper-most level of the exposed artery was ligated. A 20-gauge catheter introduced into the carotid artery was used to infuse 500 ml of 0.9% saline, mixed with 3 cc heparin (Elkins-Sinn, NJ) and 0.6 ml ketamin at 100 mmHg. A second 20-gauge catheter introduced in one femoral artery was connected to a small plastic tube for exsanguination. The rabbits died after the larger part of blood was replaced by saline. Immediately after cessation of respiration, the chest was opened and the thoracic aorta (Ao) was excised from the aortic valve to the diaphragmatic hiatus. The inferior vena cava (IVC) was harvested from right atrium to the diaphragm. The pulmonary artery (PA) was excised from the pulmonary valve to its secondary branching. Care was taken during this procedure not to damage or stretch the vessels. After the vessels were taken out, they were bathed in Krebs solution containing (in mmol/l) NaCl 118, KCl 4.6, NaHCO₃ 27.2, MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, and glucose 11.1 (pH 7.4), aerated with a 95% O₂-5% CO₂ gas mixture, and maintained at a constant temperature of 37 °C. Connective and other adhesive tissue was completely removed because it contained blood. Any remaining blood was removed by placing the excised vascular segments in Krebs solution for 5 min. The segments were then cut at 5-mm intervals to allow each segment optimal time for exposure to oxygen and administered drugs. One segment of Ao from each rabbit was used for analysis of vasomotor reactivity following administration of VEGF/VPF (*vide infra*). For certain experiments, the endothelium was removed by gently rubbing the interior of the vessel with a cotton swab prewetted with Krebs solution.

Measurements of NO from vessel segments

Vessel segments were allowed to float for 10 min in the organ chamber, before its content was replaced with 10 ml of fresh Krebs solution, to remove any remaining hemoglobin. Fifteen minutes later ($t=0$), reagents to be tested were administered. In our experience 15 min was the minimal time necessary for the vascular segments to equilibrate with the Krebs solution in the organ chamber. Concentrated solutions were employed to avoid any significant increase in volume administered immediately after replacement of the Krebs solution (i.e. $t=-15$).

To measure nitric oxide concentration ([NO]) at different points in time, 0.7 ml aliquots of organ chamber solution were removed and added to a 1 ml cuvette with an optical path of 1 cm, containing 0.07 ml of Griess solution (1% sulfanilic acid, 0.1% naphthalene-ethylene diamine in 5% H₃PO₄). Naphthalene-ethylene diamine reacts with NO as well as with nitrite (NO₂⁻) to yield a product that renders the solution pink; the resulting product yields a spectrophotometric peak at 548 nm.²⁰ We found that a 1:10 ratio of Griess solution to sample volume yielded results which were equal to the

more commonly used ratio of 1:1; reducing the volume of Griess solution avoided diluting the [NO] of the sample volume in the cuvette, and increasing the sensitivity of the measurement. Immediately after removing the last sample from the organ chamber, the absorbance at 548 nm was measured using a diode array photospectrometer (8452A, Hewlett Packard, Andover, MA). Absorbance was proportional to [NO], according to a standard curve that, in our hands, was linear from 0.01 to 0.25 $\mu\text{mol/l}$ NO_2^- (figure 1). The Griess reaction has been typically reported to yield a sensitivity between 1 and 5 $\mu\text{mol/l}$. By diluting a 1 $\mu\text{mol/l}$ NO_2^- sample by a factor 2 until concentrations of 0.015625 $\mu\text{mol/l}$ were obtained, we registered absorbances in the different solutions as displayed in the Table. The resulting standard curve, based on four independent procedures, was virtually linear, including the range from 1 to 0.01 $\mu\text{mol/l}$ (Figure 1).

Table 1. The repetitive measurements for construction of the standard curve.

[NO ₂ ⁻] ($\mu\text{mol/l}$)	blank	0.015625	0.03125	0.0625	0.125	0.25	0.5
Absorbance							
I	-0.00003	0.00299	0.00624	0.01685	0.03285	0.07053	0.14297
II	0.00003	0.00290	0.00659	0.01707	0.03285	0.07066	0.14299
III	0.00003	0.00313	0.00661	0.01697	0.03264	0.07057	0.14323
IV	0.00005	0.00371	0.00681	0.01706	0.03305	0.07068	0.14316

Figure 1. Standard curve constructed with the Griess assay is virtually linear for nitrite [NO₂⁻] concentrations over range of 0.01 to 0.5 $\mu\text{mol/l}$.

The same cuvette and volumes were used for experiments involving vascular segments. For a blank, 0.7 ml of Krebs solution, in which no vessel segment had been placed, was added to 0.07 ml of Griess solution. The deviation obtained in the construction of the standard curve was much smaller than the variation in values obtained from processing the solutions derived from the organ bath experiments. Repetitive measurements per sample to ensure that the variability among measurement per sample did not exceed 10% and the reproducibility of the documented results suggest that the sensitivity of the Griess reaction for these type of experiments is sufficiently accurate at least to concentrations as small as 0.03 $\mu\text{mol/l}$. It should be noted that the values for NO reported here represent measured NO divided by the intimal surface area of the vessel segment (typically 250-700 mm^2 , *vide infra*); the product of NO reported here multiplied by 250-700 (mm^2) yields values which consistently fall within the calibration range of the linear relationship shown in Figure 1.

Calculations

Four measurements per sample were taken; these were used to compute the average absorbance for each time point. [NO] was determined in the manner described above. The increase in [NO] caused by sampling-induced decrease in volume of the organ chamber solution in the presence of an NO-producing source was corrected for according to the formula:

$$C_t = C_{x-2} + (C_t - C_{x-2}) \cdot ([V - \pi \cdot V_s] / V),$$

in which C_t is [NO] at time = t min; $x=15$ min; V is the initial volume in the organ chamber (i.e. 10 ml); π is number of sampling times; and V_s is the sample volume (i.e. 0.7 ml). The [NO] 2 min prior to drug administration was taken as the baseline value.

After completion of the above procedure, the surface area (SA) of the vascular segments were carefully measured. The [NO] per SA in $\mu\text{mol/l} \cdot \text{mm}^2$ at given points in time was described, assuming that production of NO was equal across the endothelium of the excised vascular segments.

Analysis of vasomotor reactivity

Aortic rings, 5 mm in length, were mounted using two L-shaped 30-gauge stainless steel hooks, one of which was immobile and the other of which was connected by a silk suture to a force displacement transducer (model 7D polygraph, Grass Instruments Company, Quincy, MA) for recording isometric tension development. The assay was performed with rings placed in Krebs buffer as described above. Vessels were passively stretched to 2.0 g isometric force. After 45 min of equilibration, the aortic rings were exposed to 70 mmol/l KCl solution to assess the effect of maximal depolarization. When the contractile response reached a plateau phase, the solution in the organ chamber was replaced by fresh Krebs buffer and again was allowed to equilibrate for 45 min in the presence of 5 $\mu\text{mol/l}$

indomethacin for complete inhibition of the cyclooxygenase and consequent production of vasoactive prostanoids. Norepinephrin (NE) was used to achieve submaximal tone, defined as 30-50% of maximal tone induced by KCl, following which VEGF/VPF was added in a cumulative fashion to the organ bath solution. The effect of VEGF/VPF was measured as the percentage change in NE-induced vascular tone.

Measurement of NO from HUVECs

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords by collagenase dissociation and grown onto 1% gelatin-coated plates in medium 199 (Life Technologies, Gaithersburg, MD) with 20% heat-inactivated fetal bovine serum (Life Technologies), 100 mg/ml endothelial cell growth supplement and 50 units/ml heparin (HUVEC medium).²¹ Cells were passaged at confluence following dissociation with 0.05% trypsin (Life Technologies). Cultured HUVECs were used between passages 5 and 6.

NO release from a cultured HUVEC monolayer was measured with an NO-specific polarographic electrode connected to an NO meter (Iso-NO, World Precision Instruments, Sarasota, FL).²² Calibration of the NO electrode was performed daily before beginning the experimental protocol according to the following equation:

The standard calibration curve was obtained by adding graded concentrations of KNO_2 (0, 5, 10, 25, 50, 100, 250, and 500 nmol/l) into the calibration solution containing KI and H_2SO_4 . The specificity of the Iso-NO electrode for NO was previously determined by measurement of NO from authentic NO gas.²³ The culture medium was removed and the HUVECs were washed twice with Dulbecco's phosphate buffered saline. The cells were then bathed in 5 ml of filtered Krebs solution in 6-well plates. Cell plates were kept on a slide warmer to maintain a temperature between 35 and 37 °C. For NO measurements, the sensor probe was inserted vertically into the wells; the tip of the electrode remained 2 mm under the surface of the solution. The wells containing the confluent HUVECs were randomly divided into five experimental groups: (a) VEGF/VPF (1 ng/ml), (b) VEGF/VPF (10 ng/ml), (c) VEGF/VPF (100 ng/ml), (d) the NO synthase inhibitor L-NAME (100 $\mu\text{mol/l}$) + VEGF/VPF (100 ng/ml), and (e) sodium nitroprusside (SNP, 100 $\mu\text{mol/l}$). NO release was expressed as picomoles per 1×10^6 endothelial cells. Measurements of NO released from HUVECs represent mean values of five to eight measurements in each group (number of cell culture wells).

Reagents

All reagents, except for VEGF/VPF, were purchased from Sigma Chemical (St. Louis, MO).

Heterodimeric recombinant VEGF/VPF, purified from *Escherichia Coli*, was the generous gift of Napoleone Ferrara, Bruce Keyt, and Stuart Bunting, Genentech Inc., South San Francisco, CA. All VEGF/VPF concentrations are reported in g/l. Previous analyses²⁴ disclosed that *E. Coli*-derived VEGF/VPF appeared as a single protein band on SDS-gels with a molecular weight of 39.8 kDa; the concentrations of VEGF/VPF used in the current experiments (2.5 and 10 µg/ml) thus correspond to 62.8 and 251.0 nmol/l, respectively.

Krebs solution and Griess reagent were prepared daily. Stock solutions of VEGF/VPF and ACh, dissolved in distilled water, were refrigerated until they were used. Pellets of L-arginine and L-NMMA were added to the organ chamber at established concentrations of 0.2 mmol/l.

Statistical analysis

Values are given as means $\pm$ standard error ($m \pm \text{SEM}$). If $n=2$, bars indicate the range of obtained values. The data were evaluated using a 2-factorial (significance over time, significance of drug effects) analysis of variance (ANOVA) for repeated measurements. Statistical significance was inferred when $p < 0.05$. In all experiments, n equals the number of rabbits from which the vessels were taken.

Results

Thoracic aorta

Stimulation of vascular segments of thoracic aorta (Ao) with ACh resulted in a nearly immediate and significant increase in [NO] (Figure 2). Stimulation with 10^{-5} mol/l ACh yielded a somewhat higher [NO] than observed with 10^{-6} mol/l ACh, but this difference was not statistically significant. No increase in [NO] was observed when 0.2 mmol/l L-NMMA was administered at time $t=0$ min.

In comparison with ACh, the onset of increased [NO] following administration of VEGF/VPF was slower, reaching a maximum value after 8 min (Figure 3). Both tested concentrations (2.5 µg/ml and 10 µg/ml) caused a significant rise in a dose-dependent fashion. Preincubation of the aortic segments with L-arginine raised by twofold both baseline [NO] and [NO] stimulated by addition of 2.5 µg/ml VEGF/VPF (Figure 4). Removal of CaCl_2 from the Krebs solution abrogated the stimulatory effect of 10 µg/ml VEGF/VPF, resulting in [NO] that did not differ significantly from baseline conditions or from unstimulated aortic rings (Figure 5). Similar results were observed following mechanical removal of the endothelium. Administration of 0.2 mmol/l L-NMMA at time $t=0$ min also prevented any rise in [NO] after stimulation with 10 µg/ml VEGF/VPF. Moreover, incubating with L-NMMA produced a tendency to diminished [NO], compared to the values obtained for the unstimulated aortic rings.

Figure 2. Acetylcholine (ACh), in concentrations of both 10^{-5} mol/l and 10^{-6} mol/l, augments nitric oxide (NO) production by thoracic aorta. No increase in [NO] was observed when 0.2 mmol/l N^G -monomethyl-L-arginine (L-NMMA) was administered at $t=0$ min. SA: surface area; ●, ■: $p<0.05$ versus ACh 10^{-5} mol/l/L-NMMA. The difference between ACh 10^{-6} mol/l and ACh 10^{-5} mol/l was not statistically significant.

Figure 3. Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) augments nitric oxide (NO) production by thoracic aorta. I, n: $p<0.05$ versus no VEGF/VPF. SA: surface area.

Figure 4. L-arginine augments production of nitric oxide (NO) by thoracic aorta. ●: $p < 0.05$ versus no vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF). ■: $p < 0.05$ versus VEGF/VPF 2.5 $\mu\text{g/ml}$.

Figure 5. Inhibition of nitric oxide (NO) production during application of vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) to thoracic aorta. No Ca^{2+} : CaCl_2 removed from Krebs solution in the organ chamber. I: $p < 0.05$ versus all other curves. The differences between curves demonstrating the inhibition of NO production were not statistically significant. L-NMMA: NG-monomethyl-L-arginine. SA: surface area.

Inferior vena cava

After stimulation with VEGF/VPF, vascular segments retrieved from the inferior vena cava (IVC) yielded results which were similar to those obtained for Ao segments (data not shown). [NO] for unstimulated IVC was nearly identical to that obtained for Ao; stimulation of IVC with 2.5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ VEGF/VPF, however, yielded a lower [NO] than recorded for the Ao.

In contrast to results obtained in the Ao with VEGF/VPF, ACh applied to the IVC failed to produce a significant increase in [NO] (Figure 6).

Pulmonary artery

For vascular segments retrieved from the pulmonary artery (PA), 2.5 $\mu\text{g/ml}$ VEGF/VPF did not lead to an increase in [NO] compared to results obtained for the unstimulated segments (data not shown). Stimulation with 10 $\mu\text{g/ml}$ VEGF/VPF, however, did yield a significant rise in [NO], though peak levels in the Ao and the IVC segments were higher. Preincubation with 0.2 mmol/l L-arginine increased [NO] similar to findings recorded for Ao and IVC.

Again, as in the case of the IVC, ACh did not result in a rise in [NO] (Figure 7).

Vasomotor reactivity

Figure 8A shows the representative vasomotor response obtained from an endothelium-intact ring of rabbit aorta following administration of VEGF/VPF. Norepinephrin was used to induce the initial vasomotor tone. VEGF/VPF produced slowly developing relaxation, reversible with L-NMMA. This effect was also abolished after mechanical removal of the endothelium and by removal of CaCl_2 from the organ bath solution (Figure 8B). The time course observed for VEGF/VPF-induced vasomotor reactivity, similar to that originally described by Ku et al¹⁴ in isolated canine coronary arteries, is consistent with the time course of VEGF/VPF-induced release of NO from isolated rings of rabbit aorta described above.

HUVECs

The Iso-NO electrode was calibrated by a chemical titration method as described in the Materials and Methods (Figure 9A). Stimulation of HUVECs with VEGF/VPF (1, 10, and 100 ng/ml) resulted in a concentration-dependent increase in NO release (Figure 9B). Peak NO release was observed between 5 to 8 min after addition of VEGF. Treatment of HUVECs with the NO synthase inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME) (100 $\mu\text{mol/l}$) attenuated the VEGF/VPF 100 ng/ml-stimulated release of NO under the same experimental conditions. Sodium nitroprusside 100 $\mu\text{mol/l}$ significantly increased [NO].

Figure 6. Acetylcholine (ACh), in concentrations of 10^{-5} mol/l and 10^{-6} mol/l, fails to augment production of nitric oxide (NO) by inferior vena cava. The differences between curves were not statistically significant. SA: surface area.

Figure 7. Acetylcholine (ACh), in concentrations of 10^{-5} mol/l and 10^{-6} mol/l, fails to augment production of nitric oxide (NO) by the pulmonary artery. The differences between curves were not statistically significant. SA: surface area.

Figure 8. A. Representative example of vasorelaxation in ring segment of rabbit aorta induced by vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF). Administration of N^G-monomethyl-L-arginine (L-NMMA) resulted in an immediate reversion of this effect. Both the response to L-NMMA and the timecourse of relaxation are consistent with previous observations regarding VEGF-/VPF-induced NO release from the vascular wall.

B. Summated results of VEGF/VPF-induced vasomotor reactivity. In absence of intact endothelium and/or Ca^{2+} , vasorelaxation is severely attenuated. NOR: norepinephrine.

Discussion

These experiments establish that VEGF/VPF causes a dose-dependent increase of [NO] in vascular endothelium. This increase in [NO] can be demonstrated in vascular segments harvested from different regions, including aorta, vena cava, and pulmonary artery, as well as from cultured human endothelial cells. Parenthetically, these experiments also establish that ACh fails to enhance production of NO in both IVC and PA.

A variety of techniques have been previously employed to measure NO. NO production has been studied using a chemiluminescence assay;²⁵ this approach measures the intensity of fluorescent radiation emitted after oxidation of NO by ozone. The use of this method for measurement of NO production by vascular segments in an organ chamber first requires reduction of NO_2^- back to NO, and second transfer of NO from solution to gas. A limitation of this technique is that removal of NO from solution may introduce significant error at lower concentrations.

Kiechle et al reported an electrochemical method, based on registration of current, secondary to oxidation of NO.²⁵ This technique permits measurements to be made within short time intervals on the surface of the cell membrane, using electrochemical sensors with a diameter between 0.2 and 1.0 μm . A modification of this method was employed

Figure 9. *A.* Standard calibration curve constructed with polarographic electrode employed for measurement of nitric oxide (NO) in cultured endothelial cells. *B.* Vascular endothelial growth factor (VEGF)/ vascular permeability factor (VPF) augments release of NO from human umbilical vein endothelial cells (HUVECs) in a dose-dependent fashion. This effect is abrogated by administration of N⁰-nitro-L-arginine methyl ester (L-NAME). Administration of an NO-donor (sodium nitroprusside, SNP) confirms the specificity of the registered NO values. ΔpA: differences expressed in pico-Ampères.

in the present study to measure NO release from cultured HUVECs treated with VEGF/VPF. The size of the sensor, however, restricts its use to measurements of NO release from cells, and is not applicable for NO registration from intact vascular segments.

In the current study, we therefore employed an alternative method, UV-visible spectrophotometry, which is relatively easy to perform, yields reproducible results, and allows measurements of significant differences of [NO] in the micromolar range from intact vascular segments. This approach is based on the premise that in common solutions [NO] is dependent upon the content of nitrogen oxides and nitrite:

The relationship between nitrous acid and nitrite is defined by:

$$\text{pH} - \text{pK} = \log\left(\frac{[\text{NO}_2^-]}{[\text{HNO}_2]}\right).$$

In dilute solutions, the $\text{pK} \approx 3.4$ at 37 °C. The experiments took place at $\text{pH} = 7.4$, implicating a 10,000-fold greater concentration of nitrite than nitrous acid. Assuming there is no NO or NO₂ present in the Krebs solution before starting the experiments, an equation can be obtained to relate NO and NO₂⁻:

$$\log[\text{NO}] = \text{constant} + \log[\text{NO}_2^-].$$

Thus, the nitrite concentration we measured in samples taken from the organ chamber runs parallel to the concentration of NO:

$$[\text{NO}] = \text{constant}' \cdot [\text{NO}_2^-].$$

Since the fluid in the organ chamber is exposed to atmospheric oxygen, there is some loss of NO and NO_2^- to air and also via the glass walls of the organ chamber. Samples taken after removal of the NO-producing vessels of the organ chamber, demonstrated a decrease in nitrite concentration of about 10% after 15 min (data not shown). This explains both the decrease in [NO] after NO production was blocked by L-NMMA, as well as the horizontal curve produced by the vascular segments exposed to neither VEGF/VPF nor ACh.

NO is a colorless, free radical, simple gas with a half-life time of 10 to 30 sec. The heme-group of hemoglobin readily combines with NO, yielding iron-nitrosyl adducts and/or nitrate (NO_3^-).²⁶ Misko and colleagues found, using a fluorometric assay, that as little as 10 $\mu\text{mol/l}$ hemoglobin completely abolished any signal representing nitrite.²⁷ The methodology employed in the current study likewise required removal of any blood which remained after harvesting the vessels. This, and handling of the excised vascular segments in a manner that avoids damaging the endothelium appear to be the two factors most responsible for minimizing variability in these types of experiments.

The onset for [NO] increase (4 min), and the time required for VEGF/VPF to induce maximum release of NO (8 min) were considerably longer than were observed for ACh. It is interesting to note that a similar time frame (5 to 8 min) was required for maximal relaxation of rings of thoracic aorta in the current study and isolated canine coronaries described previously by Ku et al.¹⁴ Furthermore, in vivo experiments recently performed in our own laboratory¹⁶ disclosed that the nadir of endothelium-dependent hypotension occurred approximately 8 min after VEGF/VPF administration. The apparent reduction in NO production after 8 min may be a function of the brief half-life of VEGF/VPF, reported to be approximately 3 min (N. Ferrara, oral communication, August 1995).

The rapid onset of increased NO production after stimulation with ACh is inferred to represent the expedited consequence of a G-protein which is coupled to the ACh receptor.²⁸

In contrast, VEGF/VPF binds to a non-G-protein coupled tyrosine kinase receptor *Flt-1* and/or *Flk-1/KDR*,^{29,30} which must dimerize in order to activate downstream signaling. Tyrosine phosphate-mediated phosphorylation of phospholipase $\text{C}_{\gamma-1}$, release of inositol 1,4,5-triphosphate from phosphoinositide, and a consequent increase in intracellular $[\text{Ca}^{2+}]$, are presumed to constitute the basis for increased EC production of NO in response to VEGF/VPF.¹⁴ These different signaling pathways may account for the temporal differences observed for the onset of increased [NO] in response to ACh versus VEGF/VPF.

VEGF/VPF caused an increase in [NO] in vascular segments from thoracic aorta, IVC, and pulmonary artery, implicating the presence of *Flt-1* and/or *Flk-1/KDR* on endothelial cells of all three vessel types. ACh, however, failed to augment [NO] in either the IVC or PA, a finding which to our knowledge has not been reported previously. This observation might represent the explanation for findings recently reported by Gao et al,³¹ regarding failure of ACh to induce endothelium-dependent relaxation in isolated pulmonary arteries of full-term fetal lambs; while ACh did induce relaxation in lambs 5 to 6 weeks in age, this effect could not be abolished by an inhibitor of NO synthase.^{31,32} Similar observations were reported by Miller in isolated canine femoral veins.³³ These differences in response to ACh may reflect a difference in muscarinic receptor density among different types of vessels. Moreover, activation of different muscarinic receptor subtypes are known to cause the release of different endothelial vasoactive factors.³⁴

VEGF/VPF receptor expression is widespread during vasculogenesis and angiogenesis in the developing embryo.³⁰ Postnatally, both the *Flt-1* and *Flk-1/KDR* receptors have been shown to be upregulated at sites of recurrent neovessel proliferation, such as the corpus lutea of the ovary,¹⁰ or in pathologic tissues,^{35,36,37} particularly in conjunction with hypoxia.^{38,39} In contrast, expression of VEGF/VPF receptors by quiescent endothelium in the adult has been considered to be typically reduced,^{30,35,36} and in some organs, such as the human adult brain, has been reported to be altogether absent.³⁷ Peters et al,⁹ however, observed expression of *Flt-1* mRNA by quiescent endothelium of the adult mouse among multiple organs, including brain, corresponding to a similar pattern of ¹²⁵I-rhVEGF binding described earlier by Jakeman et al;¹⁰ these studies thus suggested that VEGF/VPF might have a function in mature vessels other than mediating vascular growth.

The observation in the current study that VEGF/VPF stimulates production of NO when applied to endothelium-intact segments retrieved from three different vascular districts constitutes inferential evidence for the presence of functional VEGF/VPF receptors on quiescent endothelium of the adult rabbit. The fact that this response is blocked by a competitive inhibitor of NO synthase suggests further that putative maintenance functions of VEGF/VPF may include regulation of baseline synthesis and/or release of EC NO, and supports the notion of a “survival” or “maintenance/repair” role for VEGF/VPF.^{9,10,11,12} VEGF/VPF-induced recovery of disturbed endothelium-dependent flow in the rabbit ischemic hindlimb,¹⁵ for example, may reflect restored NO production by endothelial cells initially damaged by protracted ischemia in the collateral-dependent limb.

NO has been implicated as an inhibitor of intimal thickening responsible for compromised arterial patency.^{40,41,42,43,44} De Meyer et al, for example, showed that the NO donor SPM-5185 could inhibit neointima formation and restore vascular reactivity in collared carotid arteries of rabbits,⁴² Von der Leyen et al⁴³ likewise inhibited neointima formation in balloon-injured rat carotid arteries, and vascular reactivity in these vessels improved as well.⁴⁴ More recently, Marks et al used a long-lived NO adduct to inhibit

neointimal formation in injured rabbit arteries.⁴² VEGF/VPF, too, administered as the recombinant protein to the balloon-injured rat carotid artery,¹⁷ or as the cDNA to the balloon-injured rabbit femoral artery¹⁸ has been shown to inhibit intimal thickening. The results of these live animal studies in combination with the *in vitro* findings reported here, may be interpreted to suggest that NO is at least in part responsible for mediating this inhibitory effect of VEGF/VPF on intimal thickening.

The extent to which NO release may contribute to the proliferative and migratory roles of VEGF/VPF believed to be responsible for stimulating angiogenesis^{45,46} remains speculative. Leibovich et al⁴⁷ found that the angiogenic activity of monocytes stimulated with lipopolysaccharide was both L-arginine-dependent, and inhibited by inhibitors of NO synthase. Recent work from our own laboratory has established that dietary supplements of L-arginine enhance angiogenesis in the rabbit model of hindlimb ischemia,⁴⁸ consistent with a role for NO in promoting angiogenesis *in vivo*.

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Chapter 3

The human internal mammary artery releases more nitric oxide in response to vascular endothelial growth factor/vascular permeability factor than the human saphenous vein.

Evidence for VEGF/VPF-receptors in quiescent human endothelium.

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Abstract

Intimal thickening is an important cause of late coronary vein graft occlusion. The internal mammary artery (IMA) graft has been shown to have a better long-term patency than the vein graft, which might reflect a higher release of endothelial cell (EC) nitric oxide (NO). We therefore sought to determine to which extent vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) augments NO release in vascular segments of the human IMA and saphenous vein (SV), freshly harvested from patients undergoing coronary bypass surgery.

The vessel segments were placed in an organ chamber. NO was determined using photospectrometry on small samples taken from the organ chamber solution that subsequently were reacted with Greiss solution. For some experiments the vascular segments were homogenized for Western blotting.

We found that the NO production in the IMA was 2-fold higher than in the SV. In addition, VEGF/VPF augmented NO production in both types of vessels by approximately 50%. This effect was abrogated by co-administration of an inhibitor of nitric oxide synthase (NOS), by mechanical disruption of the endothelium, and by removal of calcium from the organ bath solution, thus providing evidence for the involvement of the calcium-sensitive EC constitutive NOS.

These results were supported by Western blotting of the harvested vascular segments, showing, to our knowledge for the first time, the expression of the VEGF/VPF-receptors KDR(Flk-1) and Flt-1 on quiescent mature human endothelium. The receptor density of the IMA was approximately 2-fold higher than in the SV.

In conclusion, the VEGF/VPF-receptors KDR(Flk-1) and Flt-1 are expressed on quiescent endothelium of human coronary bypass grafts, and VEGF/VPF augments EC NO release in the human IMA and SV.

Intimal thickening is an important cause of late coronary vein graft occlusion.^{1,2} The internal mammary artery (IMA), however, has been shown to have a much better long-term patency when this vessel is used as a conduit for coronary artery bypass.³ Lüscher and colleagues have suggested that the difference between endothelium-dependent relaxation in arterial and venous bypass grafts is due to a higher release of nitric oxide (NO) in the IMA.⁴

The NO produced in the endothelium exerts many putative features. Endothelium-dependent vasorelaxation is mediated by NO, a potent activator of soluble guanylate cyclase, which causes increased intracellular levels of cyclic GMP and subsequently smooth muscle relaxation.^{5,6} Furthermore, NO has an inhibitory effect on cell proliferation and adhesion, effects that are also mediated by increased levels of cyclic GMP.⁷ It has been reported, that NO reduces vascular smooth muscle cell mitogenesis.^{8,9,10} Platelet and leukocyte adhesion to the vascular endothelium and subsequent chemotaxis are also negatively regulated by NO.^{11,12,13} More support for the protective actions of NO was provided by Cooke and colleagues who documented the antiatherogenic effects of L-arginine, the precursor of NO, in hypercholesterolemic rabbits^{14,15} and humans.¹⁶

Recently, experiments in our own laboratory showed that the production of NO in quiescent rabbit vascular endothelium from arteries was significantly higher than in veins.¹⁷ In addition, vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) augmented NO release in arteries more than in veins.

VEGF/VPF is a 45 kD heparin-binding dimeric glycoprotein that has been reported to induce mitogenesis of endothelial cells, but not in other cell types.^{18,19,20} Because of the unique aspect of its narrow target cell specificity, VEGF/VPF has a crucial role in endothelial cell differentiation and consequently is obligatory for normal development of the vasculature in mammalian embryos.^{21,22}

The expression of VEGF/VPF receptors by quiescent endothelium in the adult, however, has been considered to be reduced or absent.^{23,24} Therefore, in the present study we sought to determine to which extent VEGF/VPF may stimulate NO production in freshly isolated segments of human IMA and the saphenous vein (SV), since a ligand-receptor interaction can be inferred from an augmented NO release. In addition, to further address the issue of *Flk-1/KDR* and *Flt-1* receptors on adult quiescent endothelium, the expression of mRNA of VEGF/VPF and its receptors in both types of human vessels is assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting.

Materials and methods

Vessel preparations

Experiments were performed on vessels isolated from 15 patients undergoing coronary artery bypass grafting for the first time after informed consent was obtained. The preparation of the vessels was conducted as described previously in rabbits.¹⁵ In short, immediately after excision of the SV and IMA segments, the vessels were washed with a phosphate-buffered solution (PBS). Connective and other adhesive tissue was completely removed. Then, the vascular segments were placed in an organ chamber and washed again in Krebs solution (in mMol/L: 118 NaCl, 4.6 KCL, 27.2 NaHCO₃, 1.2 MgSO₄, 2.5 CaCl₂, 1.2 KH₂PO₄, 11.1 glucose, pH 7.4), aerated with a 95%O₂-5%CO₂ gas mixture, and maintained at a constant temperature of 37 °C. When necessary, the segments were cut at 5 mm intervals to ensure optimal exposure of the endothelium to oxygen and reagents added to the organ chamber. For some experiments, the endothelium was mechanically removed by rubbing the interior of the vessel rings with a wetted cotton swab. Fifteen minutes before administration of the reagents (t=-15 min) the fluid in the organ chamber was replaced to remove any remaining hemoglobin. The reagents were administered at t=0 min in a concentrated fashion to avoid significant dilution of the NO or nitrite (NO₂⁻) concentration in the organ bath solution. PBS was added for negative control experiments. For a positive control acetylcholine was used.

Measurement of NO

NO was determined at different points in time before and after administration of the reagents to be tested. For each timepoint a 0.7 mL aliquot was taken out of the organ chamber and added to a 1 mL cuvette with an optical path of 1 cm, prefilled with 0.07 mL of Griess solution (1% sulfanilic acid, 0.1% naphthalene-ethylene diamine in 5% H₃PO₄). Naphthalene-ethylene diamine reacts with both NO and NO₂⁻; the products renders the solution in the cuvette pink. This color displays a spectrophotometric peak at 548 nm. The absorbance at 548 nm was determined with a diode array photospectrometer (8452A, Hewlet Packard, Andover, MA) and was translated into NO concentration using a standard curve, which was linear from 0.03125 to 0.25 μmol/L NO₂⁻. As a blank, Krebs solution, which had not been exposed to the vascular segments, mixed with Griess solution in a similar fashion, was used.

Drugs

Heterodimeric recombinant human VEGF/VPF, purified from *Escherichia Coli*, was a gift of Napoleon Ferrara and Stuart Bunting, Genentech, South San Fransisco, CA. All chemical components were obtained from Sigma Chemical (St. Louis, MO). Primary antibodies for Western blot analysis were monoclonal goat anti-human *Flk-1* and *Flt-1*

antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal goat anti-human VEGF/VPF antibody (R&D System, Minneapolis, MN). All used antibodies have been shown to be specific for the tested receptors.

RT-PCR procedures

Total RNA was extracted from homogenized vascular segments by phenol/ chloroform extraction,²⁵ and RNA concentration was calculated from the absorbance at 260 nm. Control experiments were performed on human umbilical vein endothelial cells (HUVECs) for *KDR/Flk-1*, *Flt-1*, and glyceraldehyde phosphate dehydrogenase (GAPDH). Umbilical vein smooth muscle cells (HVSMEs) were used as a control for VEGF/VPF.

Approximately 1 µg of RNA was reverse transcribed using the Promega reverse transcription system (Promega, WI) for 1 hour at 42 °C. PCR was performed using primers to *Flt-1*, *KDR/Flk-1*, VEGF/VPF, and GAPDH, which functioned as a control. PCR was performed with 2 µl cDNA in the presence of 500 ng of each primer and 0.2 mMol/L dNTPs in a total volume of 50 µl. Following a hot start (96 °C, 8 min) 1 U of DNA polymerase (Clontech, Palo Alto, CA) was added at 56 °C and amplified for 30 cycles under the following conditions: 94 °C, 1 min; 56 °C, 1 min; 72 °C, 2 min. Products were resolved on 1 % agarose gels and visualized by UV illumination following ethidium bromide staining. The paired primer sequences used were as follows (sense/antisense): for *KDR/Flk-1*, 5'-GGA AAT CAT TAT TCT AGT AGG CAC GAC G/5'-CCT GTG GAT ACA CTT TCG CGA TG (819 bp PCR product); for *Flt-1*, 5'-GCA CCT TGG TTG TGG CTG AC/5'-CGT GCT GCT TCC TGG TCC (735 bp); for VEGF/VPF, 5'-CCT GGT GGA CAT CTT CCA GGA GTA CC/5'-CTC ACC GCC TCG GCT TGT CA (438, 570, and 642 bp); for GAPDH, 5'-TGA AGG TCG GAG TCA ACG GAT TTG/5'-CAT GTG GGC CAT GAG GTC CAC CAC (983 bp).

Western blot procedures

The vessel segments were cut into small pieces and treated with lysis buffer containing protease inhibitors (PMSF, Pepstatin A, Aprotinin, and Leupeptin). For positive controls umbilical veins were used, for negative controls umbilical veins after removal of endothelial layer (data not shown). The tissue was homogenized, centrifuged (15,000g, 20 min, 4 °C), boiled, and centrifuged again. The supernatants were collected and protein concentrations were calculated using bovine serum albumin (BSA) as a standard. After loading of equal amounts per lane, proteins were separated by gel-electrophoresis and transferred onto 0.2 µMol/L PVDF-membranes (Bio-Rad Laboratories, Hercules, CA) in 25 mMol/L Tris, 200 mMol/L glycine, 1.3 mMol/L SDS, and 20% methanol at 30 mA for 3 hours. The membranes for detection of other proteins were incubated with 10% dry milk for 1 hour. Each membrane was incubated with a primary *Flk-1* or *KDR/Flt-1* antibody solution (1:100 dilution) at 4 °C overnight. For a negative control non-immune

goat IgG₁ was used. Then, biotinylated horse anti-goat immunoglobulin was applied, followed by incubation with streptavidin-horseradish peroxidase. Images were obtained on X-ray film using the ECL-kit (Amersham Life Science, Arlington Heights, IL).

Calculations and statistical analysis

The increase in NO in the organ bath solution caused by sampling-induced decrease in volume of the organ chamber in the presence of a NO-producing source was corrected for according to the formula:

$$C_t = C_{x-2} + (C_t - C_{x-2}) \cdot (\{V - \pi \cdot V_s\} / V),$$

in which C_t is the NO concentration at time $t=t$ min; $x=15$ min; V is the initial volume in the organ chamber (i.e. 10 mL); i is the number of sampling times; and V_s is the sampling volume (i.e. 0.7 mL).

To correct for the differences in size of the vascular segments, the NO production was standardized for surface area (SA). The NO concentration ([NO]) per SA in $\mu\text{Mol/L}\cdot\text{m}^2$ was documented, assuming that the production of NO was equal across the entire endothelium.

Values are given as means $\pm$ standard error ($m \pm \text{SEM}$). If $n=2$, bars indicate the range of obtained values. The data were evaluated using a 2-factorial (significance over time, significance of drug effect) analysis of variance (ANOVA) for repeated measurements. Statistical significance was inferred when $p < 0.05$. In all experiments, n equals the number of vessels that were used.

Results

NO measurements

The baseline production of NO in the arterial segments was observed to be significantly higher than in the venous segments (Figure 1). In addition, after administration of VEGF/VPF a significant increase in NO release in both the IMA and SV was documented. Interestingly, both vessels displayed a maximal value after 8 min, reflecting the time necessary for downstream signaling after ligation of the VEGF/VPF receptors.^{26,15}

Addition of L-arginine, the substrate for nitric oxide synthase (NOS), to the organ bath solution resulted in an increase of NO production by approximately 2-fold (Figure 2). Likewise, co-administration of N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS, totally abrogated the response to VEGF/VPF and decreased the baseline NO production.

Mechanical disruption of the endothelium also prevented a rise in NO production after administration of VEGF/VPF, implicating that intact endothelium is mandatory

Figure 1. NO production in the human internal mammary artery and the human saphenous vein. IMA: internal mammary artery; SV: saphenous vein; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; SA: surface area; *: $p < 0.05$ versus 'IMA; no VEGF/VPF'; #: $p < 0.05$ versus 'SV; no VEGF/VPF'.

Figure 2. NO production in the human internal mammary artery. VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; L-NMMA: N^G-monomethyl-L-arginine; SA: surface area; *: $p < 0.05$ versus 'no VEGF/VPF'; #: $p < 0.05$ versus 'no VEGF/VPF' at $t = 12$ min.

Figure 3. NO production in the human internal mammary artery. VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; SA: surface area; *: $p < 0.05$ versus all other curves.

for this growth factor to exert its action (Figure 3). In addition, removal of Ca^{2+} from the organ bath solution also abolished an increase in NO production. This observation is supportive for the notion that the calcium sensitive endothelial constitutive NOS (ec-NOS) is involved in the documented NO release, as opposed to the calcium insensitive inducible NOS (i-NOS), which can be expressed in other cell types of the vascular wall.

In the SV similar results were registered (Figure 4). Interestingly, after administration of L-arginine the baseline NO production increased to levels observed in the artery.

RT-PCR/Western blot

The expression of the receptor tyrosine kinase gene *KDR* (*Flk-1*) and the *fms*-like tyrosine kinase *Flt-1* in human adult vessel segments of both artery and vein is demonstrated (Figure 5a), an observation which, to our knowledge, has not been reported before. Furthermore, the expression of VEGF/VPF is recorded in both types of vessels, thus providing the constituents for a paracrine loop. The documented expression of both receptors and their ligand is supportive for the concept that VEGF/VPF augments NO release by mature quiescent endothelium.

Both *KDR* and *Flt-1* proteins were present in the vascular wall of both vessel types (Figure 5b). However, Western blotting revealed a higher receptor density in the IMA

Figure 4. NO production in the human saphenous vein. VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; SA: surface area; L-NMMA: NG^G-monomethyl-L-arginine; *: p<0.05 versus all other curves.

Figure 5. A) RT-PCR demonstrates KDR(Flk-1), Flt-1, and VEGF/VPF mRNA in isolated segments of human internal mammary artery (A) and human saphenous vein (V); n=3. cDNA for positive control is shown in lane 4. VEGF: vascular endothelial growth factor; lane 1: weight marker channel; GAPDH: glyceraldehyde phosphate dehydrogenase.

Figure 5. B) Western blot shows expression of VEGF/VPF receptors KDR(Flk-1) and Flt-1 in normal human saphenous vein (V) and human internal mammary artery (A) vessel segments; n=3.

for both VEGF/VPF receptors compared to the SV. This is in concordance with the observed higher baseline NO production by the artery.

Discussion

From these experiments it has been concluded that VEGF/VPF is capable of inducing a significant increase in NO release by both human IMA and SV. Furthermore, additional evidence is provided for the expression of the tyrosine kinase receptors *KDR (Flk-1)* and *Flt-1* as well as for the ligand VEGF/VPF by identification of the involved m-RNA and protein expression. The receptor density in the mammary arteries is higher than in the saphenous veins.

Constitutive release of NO from vascular endothelial cells has been shown to be crucial in preserving the integrity of a blood vessel. The simple molecule NO mediates a great number of phenomena in vascular biology, including endothelium-dependent vascular relaxation,^{5,6} endothelial permeability,^{27,28} inhibition of platelet aggregation,^{9,10} and inhibition of leukocyte adhesion by modulating the gene expression in endothelial cells of vascular cell adhesion molecule-1.^{13,29} The registered baseline NO release in arteries exceeds the venous NO production by approximately 50%, which might be an explanation for the superior long-term patency of IMA grafts. This observation warrants additional investigations as to the NO production of grafts that have been used as a conduit for a longer period of time.

From this study it can be concluded that VEGF/VPF augments NO production when ligated to its receptors *Flk-1/KDR* and *Flt-1*, uniquely present on endothelial cells. Accordingly, mechanical disruption of the vascular endothelium totally abrogated the response to VEGF/VPF. Endothelial cells contain nitric oxide synthase (ec-NOS), which constitutes a membrane-bound complex with calmodulin, thus becoming sensitive to cytosolic calcium fluctuations.³⁰ This complex is involved in the conversion of L-arginine to citrullin, yielding NO.³¹ VEGF/VPF binds to its tyrosine kinase receptors, which must dimerize to activate downstream signaling:³² phosphorylation of phospholipase $C_{\gamma-1}$, causing the release of inositol-tri-phosphate (IP_3) resulting in an increase in cytosolic Ca^{2+} , and a subsequent increase of ec-NOS activity and NO production.²⁶ Consistent with this mechanism is the observation that removal of Ca^{2+} from the organ bath solution abolishes the increase in NO production after VEGF/VPF administration.

The role of VEGF/VPF in adult human blood vessels still needs to be clarified. VEGF/VPF was originally discovered as a result of its ability to increase permeability of vascular endothelium³³ and to induce endothelial cell mitogenesis.³⁴ VEGF/VPF has been reported to be mandatory in the normal development of the vasculature of mammalian embryos.^{21,22} The tight temporal and spatial regulation of VEGF/VPF mRNA expression, together with the ubiquitous nature of receptor binding to endothelial and preendothelial cells is documented by Jakeman³⁵ and Breier.³⁶ Postnatally, however,

both *Flt-1* and *Flk-1/KDR* receptors have been shown to be upregulated only at sites of recurrent neovessel proliferation, such as the corpora lutea of the ovary,³⁷ or in pathologic conditions like psoriasis and tumor formation,^{38,39} especially in conjunction with hypoxia.^{40,41,42} In contrast, expression of VEGF/VPF receptors by quiescent endothelium is reduced in the adult,²³ or in some organs, such as the human adult brain, altogether absent.²⁴

In this study, the expression of both VEGF/VPF and its receptors in combination with an increase in NO production after VEGF/VPF administration in both types of adult human vessels is documented. This observation suggests that in healthy, mature vessels the biological function of this growth factor is not limited to angiogenesis. Preservation of an intact barrier between circulating blood and the vessel wall constitutes a critical condition for normal vessel behavior. Throughout life, blood vessels are exposed to local expression and/or infiltration by cytokines, proteases, oxidative stress and other factors that may damage or impair endothelial function.^{4,43} Under these circumstances VEGF/VPF-augmented NO production may be required for recurrent repair of damaged or dysfunctional endothelium, thus protecting the vessel from thrombotic events,^{11,12} leukocyte adhesion,¹³ and/or vasospasm.^{5,6} In addition, intact functional endothelium is mandatory to inhibit medial smooth muscle cell proliferation.^{8,9,44} This feature is relevant to the preservation of vascular patency as well, since migration of proliferating medial smooth muscle cells has been shown to contribute to the occurrence of intimal thickening in coronary bypass grafts and native coronary arteries.^{1,4} Therefore, these data support the concept that the physiological function of VEGF/VPF in mature vessels involves maintenance and/or repair of the luminal endothelium by augmenting endothelial cell NO production.

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Chapter

4

Peroxynitrite Produced by
Hypercholesterolemia-Induced Neointima
Abrogates the Protective Action of Vascular
Endothelial Growth Factor/Vascular
Permeability Factor on Vascular Endothelium

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Submitted

Abstract

Hypercholesterolemia is associated with increased release of nitric oxide (NO) and intimal thickening. Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) also augments NO release, but has been related to reduced intimal thickening.

To investigate this paradox, New Zealand white rabbits (NZWR) were fed a 1% cholesterol diet for either 6 (NZWR-6w, n=10) or 10 weeks (NZWR-10w, n=10). Age-matched Watanabe heritable hyperlipidemic (WHHL) rabbits (n=10) and controls (n=10) were kept on regular chow. At the end of the diet period, the aorta was removed for direct NO measurement, contraction/relaxation experiments, histology (Oil Red O, H&E), and immunohistochemistry (i-NOS, ec-NOS, RAM11, nitrotyrosine).

In the control group no changes were registered. In the NZWR-6w group, (a) intimal thickening was absent, (b) a 2-fold rise in NO release was produced exclusively via ec-NOS, and (c) VEGF/VPF induced a further increase in functional NO. In the NZWR-10w and the WHHL group, (a) intimal thickening with abundant infiltration of macrophages/foam cells had occurred, (b) a 10-fold rise in NO release was produced mainly via i-NOS expressed in intimal cells, (c) peroxynitrite (ONOO⁻), produced by the reaction of NO with the superoxide anion O₂⁻, was evidenced by demonstrating deposition of nitrotyrosine in the neointima, and (d) VEGF/VPF was not capable in restoring the reduced bioavailability of NO.

In conclusion, VEGF/VPF augments functional NO release by endothelial cells. In hypercholesterolemia the formation of peroxynitrite is associated with reduction of the bioavailability of endothelial NO.

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) has a putative function in quiescent adult rabbit endothelium and in human coronary bypass grafts by regulating constitutive endothelial cell nitric oxide (NO) release.^{1,2,3} In addition, VEGF/VPF has a role in repair of endothelium-dependent function in two different models of arterial injury, reducing intimal thickening.^{4,5} The function of VEGF/VPF in the developing primary lesion in a hypercholesterolemic environment has not been reported on.

Previously, it was documented that high cholesterol diet-induced atherosclerosis augments the release of nitrogen oxides in the rabbit aorta.^{6,7} However, the precise mechanism responsible for this observation is still unclear. In the present study, we sought to determine the extent to which VEGF/VPF stimulates the release of NO in the arterial wall that has been exposed to hypercholesterolemia.

Normally, NO release is mediated by the endothelial cell constitutive nitric oxide synthase (ecNOS)/calmodulin complex.^{8,9,10} In this study evidence is provided that NO production is increased by 2-fold in aortic rings of 6 weeks cholesterol-fed rabbits. At this point, the aortic rings still have a normal histological appearance. VEGF/VPF is capable of inducing a further increase in NO production, implicating preservation of functional VEGF-receptors. Furthermore, administration of VEGF/VPF did cause vasorelaxation in aortic rings as opposed to acetylcholine (ACh), which typically fails to induce vasorelaxation in a hypercholesteremic vessel.^{11,12} Interestingly, both VEGF/VPF and ACh caused increased NO production, but only VEGF/VPF had a functional effect on change in vascular tone.

After 10 weeks of cholesterol feeding neointimal thickening had occurred. A 10-fold increase in baseline NO production was registered in these type of vessel rings. VEGF/VPF still augmented NO release, but now failed to cause vasorelaxation. We hypothesized that a simultaneous increase in NO production and excess generation of a superoxide anion (O_2^-) in the neointima of hypercholesterolemic vessels results in the formation of peroxynitrite ($ONOO^-$). As NO is captured in the generation of this potent oxidizing molecule, the regulating function of the endothelium on vascular processes, such as mediating vasorelaxation, is lost. Furthermore, the increase in oxidative stress is a powerful stimulus for initiation and development of atherosclerosis. Therefore, it can be hypothesized that VEGF/VPF preserves endothelial function in hypercholesterolemia, until structural remodeling occurs involving the generation of highly reactive nitrogen oxide radicals.

Methods

Drugs, chemicals, and antibodies

Heterodimeric recombinant human VEGF/VPF, purified from *E. Coli*, was a gift of Napoleon Ferrara and Stuart Bunting, Genentech, South San Francisco, CA. 3-Amino-9-ethylcarbazole (AEC) and 3,3' diaminobenzidine (DAB) was purchased from Biogenex (San Ramon, CA). ACh, N^G -monomethyl-L-arginine (L-NMMA), MOPC-21, and other chemicals were obtained from Sigma Chemical (St. Louis, MO). Krebs solution and Griess reagent were prepared daily. Stock solutions of VEGF/VPF, dissolved in a phosphate-buffered solution (PBS), and ACh, dissolved in sodium acetate buffer (pH 4.0) were refrigerated until they were used. Pellets of L-NMMA were added to the organ bath solution at established concentrations of 0.2 M.

The antibodies used for immunohistochemistry were monoclonal mouse anti-nitrotyrosine (Upstate Biotechnology, Lake Placid NY), monoclonal mouse anti-iNOS and ec-NOS (Transduction Laboratories, Lexington, KY), mouse anti-rabbit macrophage 11 (RAM 11; Dako, Carpinteria, CA), and a monoclonal goat-anti-human VEGF₁₆₅/VPF (R&D System, Minneapolis, MN). Secondary antibodies were included in the Streptavidin Level 2 Kit (Signet, Dedham, MA). All used antibodies have been shown to be specific for the tested receptors.

Cholesterol feeding

Experiments were performed on the thoracic aorta isolated either from New Zealand white rabbits (NZW) rabbits or from age-matched Watanabe heritable hyperlipidemic (WHHL) rabbits. Only male rabbits were used to avoid variations in outcome attributable to gender alone. The experimental protocol described was conducted according to protocols approved by the St. Elizabeth's Institutional Animal Care and Use Committee. Hypercholesterolemia was induced in NZW rabbits by feeding a 1% cholesterol diet for 6 weeks ($n=10$) or for 10 weeks ($n=10$). Age-matched controls ($n=10$) and WHHL rabbits were maintained on standard rabbit chow.

Vessel preparation

Harvesting and preparing the thoracic aorta was conducted as described previously.¹ In short, the rabbits were killed by exsanguination. Blood was collected from all rabbits to determine total cholesterol with an automatic analyzer (Kodak 700, Johnson and Johnson, Rochester, NY). Immediately after cessation of respiration, the thorax was opened and the aorta was excised from aortic valve to the diaphragmatic hiatus. Connective and other adhesive tissue was removed and the vessels were washed initially in PBS. Then, the aorta was subdivided; the proximal part ($n=6$ /group) was used for morphologic studies. Half of it was dehydrated in a graded series of ethanol and paraffin

embedded, the remaining part was embedded in OCT compound (Miles Inc, Elkhart, IN), frozen in liquid nitrogen, and stored at -70°C until further analysis. For Western blotting the entire proximal part of the aorta was frozen ($n=4/\text{group}$).

The adjacent part of the aorta was placed in Krebs buffer (118 mM NaCl, 4.6 mM KCl, 27.2 mM NaHCO_3 , 1.2 mM MgSO_4 , 2.5 mM CaCl_2 , 1.2 mM KH_2PO_4 , and 11.1 mM glucose, pH 7.4), aerated with a 95% O_2 -5% CO_2 gas mixture, and maintained at a constant temperature of 37°C . This part of the aorta was cut at 5 mm intervals to ensure optimal exposure of the endothelium to oxygen and reagents added to the organ chamber. For some experiments, the endothelium was mechanically removed by rubbing the interior of the vessel rings with a wetted cotton swab.

Measurement of NO

Fifteen minutes before administration of the reagents ($t=-15$) the fluid in the organ chamber was replaced to remove any remaining hemoglobin. The reagents were administered at $t=0$ min in a concentrated fashion to avoid significant dilution of the NO or nitrite (NO_2^-) concentration in the organ bath solution. PBS was added for control experiments.

NO was determined at different points in time before and after administration of the reagents to be tested.¹ For each timepoint a 0.7 ml aliquot was sampled from the organ bath solution and added to a 1 ml cuvette with an optical path of 1 cm, prefilled with 0.07 ml of Griess solution (1% sulfanilic acid, 0.1% naphthalene-ethylene diamine in 5% H_3PO_4). Naphthalene-ethylene diamine reacts with nitrogen oxides, the product renders the solution in the cuvette pink. This color displays a spectrophotometric peak at 548 nm. The absorbance at 548 nm was determined with a diode array photospectrometer (8452A, Hewlet Packard, Andover, MA) and was translated into NO concentration using a standard curve which, in our experience, was linear from $0.03125\text{ }\mu\text{M}$ to $0.25\text{ }\mu\text{M}$ NO_2^- . As a blank, Krebs solution, which had not been exposed to the vascular segments, mixed with Griess solution in a similar fashion, was used.

Contraction/relaxation experiments

The 5 mm aortic rings were mounted using two L-shaped 30-gauge stainless steel hooks, one of which was immobile and the other connected by a silk suture to a force displacement transducers (model 7D polygraph, Grass instrument Company, Quincy, MA) for recording isometric tension development. The assay was performed with rings placed in Krebs buffer as described above. Vessels were passively stretched to 2.0 g for isometric force. After 45 min of equilibration, the aortic rings were exposed to 70 mM KCl-solution to assess maximal depolarization. When the contractile response reached a plateau phase, the solution in the organ chamber was replaced by fresh Krebs buffer and again was allowed to equilibrate for 45 min in the presence of $5\text{ }\mu\text{M}$ indomethacin for complete inhibition of cyclooxygenase and subsequent production of vasoactive

prostanoids. The effect of VEGF/VPF or ACh was determined after evoking submaximal tone, defined as approximately 30-50% of the maximal inducible tone caused by KCl, with norepinephrin (NOR) before the cumulative addition of either VEGF/VPF or ACh into the organ bath solution. Data are expressed as percentage of change in NOR-induced vascular tone.

Histology/immunohistochemistry

The part of the aorta that was embedded in paraffin was stained with hematoxylin and eosin and used for immunohistochemistry to show the presence of RAM 11. All other immunohistochemical staining and the Oil Red O reaction for fat were performed on 6 μ m frozen sections.

Tissue was fixed and permeabilized at -20 °C for 5 min in 100% methanol. Endogenous peroxidase activity was blocked with 3% H₂O₂ for 5 min, and tissue was treated with normal horse serum to prevent non-specific binding of biotinylated horse anti-mouse immunoglobulins, which were applied as secondary antibody. Then, the slides were incubated with mouse anti-RAM 11 (overnight at 4 °C, diluted 1:400 in 1% bovine serum albumine (BSA)/PBS), mouse anti-nitrotyrosine (1 hour at 37 °C, diluted 1:100 in 1% BSA/PBS), and mouse anti-inducible nitric oxide synthase (anti-iNOS) or endothelial cell constitutive nitric oxide synthase (ecNOS; overnight at 4 °C, diluted 1:25 in 1% BSA/PBS). For negative controls equal amounts of non-specific mouse anti-rabbit IgG₁ (MOPC-21) were applied. After administration of the secondary antibody and streptavidin-horseradish peroxidase, the slides were incubated with AEC (ecNOS, iNOS, nitrotyrosine) causing a red color or DAB (RAM 11) causing a brown color. The tissue was counterstained with hematoxylin and coverslipped with glycerol gelatin mounting medium.

Western blot

The aortas were cut into small pieces and treated with lysis buffer containing protease inhibitors (PMSF, Pepstatin A, Aprotinin, and Leupeptin). The tissue was homogenized and centrifuged for 20 min at 15,000 g at 4 °C, boiled, and centrifuged again. Protein concentrations were calculated using BSA as a standard. After loading of equal amounts per lane, the proteins were separated by gel-electrophoresis and transferred to 0.2 μ M PVDF-membranes (Bio-Rad Laboratories, Hercules, CA) in 25 mM Tris, 200 mM glycine, 1.3 mM SDS, and 20% methanol at 30 mA for 3 hours. Non-specific protein binding sites were blocked with 10% dry milk for 1 hour. The protein was overlaid with purified goat anti-human VEGF₁₆₅/VPF, diluted 1 μ g/ml. For a negative control non-immune goat IgG₁ was used. Then, biotinylated horse anti-goat immunoglobulin was applied, followed by incubation with streptavidin-horseradish peroxidase. Images were obtained on X-ray film using the ECL kit (Amersham Life Science, Arlington Heights, IL).

Calculations/statistical analysis

The increase in NO caused by the sample-induced decrease in volume of the organ chamber in the presence of a NO-producing source was corrected for according to the formula:

$$C_t = C_{x-2} + (C_t - C_{x-2}) \cdot ((V - \pi \cdot V_s) / V),$$

in which C_t is the NO concentration at time = t min; $x=15$ min; V is the initial volume in the organ chamber (i.e. 10 ml); π is the number of sampling times; and V_s is the sampling volume (i.e. 0.7 ml). To correct for differences in size of vascular segments, the NO production was standardized for surface area (SA). The [NO] per SA in $\mu\text{M}/\text{m}^2$ was documented, assuming that the production of NO was equal across the entire endothelium.

Values are given as means $\pm$ standard error ($M \pm \text{SEM}$). If $n=2$, bars indicate the range of obtained values. The data were evaluated using a 2-factorial (significance over time, significance of drug effect) analysis of variance (ANOVA) for repeated measurements. For comparison of paired values the two-tailed Student t -test was applied. Statistical significance was inferred when $p < 0.05$.

Results

Cholesterol levels

The total serum cholesterol concentrations in the cholesterol-fed NZW and the WHHL rabbits were significantly elevated compared to the rabbits assigned to regular chow (Figure 1). Note the exponential increase in cholesterol levels occurring after 6 weeks of 1% cholesterol feeding.

Measurement of NO

ACh administered to the organ bath solution at $t=0$ min induced an immediate increase in NO release by the aortic rings harvested from regular chow-fed rabbits as opposed to the vascular segments of the 6 weeks cholesterol-fed rabbits where ACh generated higher NO levels only after 9 min (Figure 2). Although NO started to increase almost immediately after ACh administration, more time elapsed to register significant differences because baseline NO release was already elevated by 2-fold.

When VEGF/VPF was administered, NO production was also augmented, but the onset of the increase was slower compared to ACh (Figure 3). Binding of the reagents to different types of receptors, as described previously¹, is a possible explanation of the temporal differences. Removal of CaCl_2 from the Krebs solution abrogated the stimulatory effect of VEGF/VPF, resulting in a NO production that did not differ

Figure 1. Total serum cholesterol concentrations in New Zealand white rabbits and Watanabe Rabbits. HC 1%: high cholesterol diet; NZW: New Zealand white rabbit; reg. ch.: regular chow; w: weeks; WHHL: Watanabe heritable hyperlipidemic rabbit.

significantly from baseline conditions or from unstimulated aortic rings (Figure 4). This observation provides inferential evidence for the involvement of calcium-sensitive ecNOS in the generation of the increased constitutive release of NO in the aortic rings after 6 weeks of cholesterol feeding. Similar results were registered following mechanical removal of the endothelium. Co-administration of 0.2 M L-NMMA at time $t=0$ min also prevented any rise in NO production after stimulation with VEGF/VPF. Moreover, incubating with L-NMMA produced a tendency to diminish NO production, compared to the values obtained for the unstimulated aortic rings.

The baseline production of aortic rings after 10 weeks of a high cholesterol diet is increased by approximately 10-fold compared to the production of vascular segments obtained from rabbits feeding on regular chow (Figure 5). Administration of ACh did not cause an increase in NO production (data not shown). Most likely, the elevated baseline production of NO masks a stimulatory effect of ACh, although it cannot be ruled out that the muscarinic G-protein coupled receptors properties are affected in a rabbit model of hypercholesterolemia.^{13,14,15,16} VEGF/VPF, however, induces a significant rise in NO production at $t=8$ min, which is typically the point in time at which NO release displays a maximum value.¹ The decrease in NO production after 8 min has been thought to be the result of the short half-life time of VEGF/VPF *in vivo* (Napoleon Ferrara,

Figure 2. NO production in response to acetylcholine administration. ACh: acetylcholine; HC: high cholesterol diet; reg.: regular; SA: surface area; *: $p < 0.05$ for increase in time; #: $p < 0.05$ for drug effect at $t = 9$ and $t = 12$ min.

Figure 3. NO production by aortic rings in response to VEGF/VPF administration after 6 weeks of cholesterol feeding. HC: high cholesterol diet; SA: surface area; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; *: $p < 0.05$ for increase in time; #: $p < 0.05$ versus 'regular chow/no VEGF/VPF'.

Figure 4. NO production by aortic rings in response to VEGF/VPF administration after 6 weeks of cholesterol feeding without Ca^{2+} , vascular endothelium, or with L-NMMA. ecNOS: endothelial cell constitutive nitric oxide synthase; HC: high cholesterol diet; L-NMMA: N^G-monomethyl-L-arginine; SA: surface area; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; *: $p < 0.05$ versus 'regular chow; no VEGF/VPF', '1% HC (6 wks); VEGF/VPF 10 $\mu\text{g/ml}$; no endothelium', '1% HC (6 wks); VEGF/VPF 10 $\mu\text{g/ml}$; no Ca^{2+} ', and '1% HC (6 wks); VEGF/VPF 10 $\mu\text{g/ml}$; 0.2 M L-NMMA'.

Figure 5. NO production by aortic rings in response to VEGF/VPF administration after 10 weeks of cholesterol feeding. HC: high cholesterol diet; SA: surface area; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; *: $p < 0.05$ versus regular chow; #: $p < 0.05$ for increase in time; ♦: $p < 0.05$ versus '1% HC (10 wks); no VEGF/VPF' at 8 min.

personal communication). Inferentially, this maximum value at 8 min suggests the preservation of functional VEGF/VPF receptors in a hypercholesterolemic milieu. As opposed to the observed decrease in NO production when CaCl_2 was removed from the organ bath solution containing the aortic rings harvested from rabbits after 6 weeks of cholesterol feeding, the aortic segments of 10 weeks cholesterol-fed rabbits displayed an unaltered NO release when exposed to the same conditions (Figure 6). This observation points to the fact that the produced NO is produced by calcium-insensitive iNOS. Removal of CaCl_2 did abolish the stimulatory effect of VEGF/VPF, which apparently involves ecNOS, but not i-NOS. Mechanical disruption of the endothelium with a wetted cotton swab also failed to significantly diminish NO release, which is in accordance with iNOS, rather than ecNOS, as the involved enzyme in producing NO. Co-administration of L-NMMA reduced NO release by approximately 50% and completely abrogated the stimulatory effect of VEGF/VPF.

The documented increase in baseline NO production in aortic rings of NZW rabbits 10 weeks after a high cholesterol diet was confirmed in vascular segments harvested from the WHHL rabbits, although averaged serum cholesterol concentration was only half the

Figure 6. NO production by aortic rings in response to VEGF/VPF administration after 10 weeks of cholesterol feeding without Ca^{2+} , vascular endothelium, or with L-NMMA. HC: high cholesterol diet; iNOS: inducible nitric oxide synthase; L-NMMA: N^G-monomethyl-L-arginine; SA: surface area; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; *: $p < 0.05$ versus 'regular chow' and '1% HC (10 wks); VEGF/VPF 10 $\mu\text{g}/\text{ml}$; 0.2 M L-NMMA'; #: $p < 0.05$ versus '1% HC (10 wks); no VEGF/VPF', '1% HC (10 wks); VEGF/VPF 10 $\mu\text{g}/\text{ml}$; no endothelium', and '1% HC (10 wks); VEGF/VPF 10 $\mu\text{g}/\text{ml}$; no Ca^{2+} '.

Figure 7. NO production by aortic rings from WHHL rabbits in response to VEGF/VPF administration. HC: high cholesterol diet; iNOS: inducible nitric oxide synthase; L-NMMA: N^G-monomethyl-L-arginine; NZwr: New Zealand white rabbits; SA: surface area; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; Wtb: Watanabe heritable hyperlipidemic rabbits; *: p<0.05 versus NZwr; #: p<0.05 versus NZwr and 'Wtb; no VEGF/VPF'; •: p<0.05 for increase in time; ■: p<0.05 versus 'Wtb; no VEGF/VPF' at t=8 min.

value obtained from diet-induced hypercholesterolemic rabbits (Figure 7). Structural remodeling rather than cholesterol concentration per se might be responsible for the observed differences in quantity and source of NO production.

Contraction/relaxation experiments

Figure 8 shows typical tracings of the effects of cumulative administration of VEGF/VPF and ACh on isolated aortic rings harvested from NZW rabbits after 6 weeks of a high cholesterol diet. Norepinephrin was used to induce the initial contractile tone. VEGF/VPF produced a slowly developing relaxation, reversible with L-NMMA, whereas ACh failed to induce a relaxing effect. However, administration of sodium nitroprusside (SNP) resulted in immediate relaxation, demonstrating that aortic rings of hypercholesterolemic rabbits can dilate in response to NO.

Figure 9 demonstrates the significant difference in vasorelaxation as a result of ACh administration in hypercholesterolemic and regular chow-fed rabbits. In figure 10 VEGF/VPF-induced vasorelaxation is summarized. Note that after 10 weeks of high cholesterol feeding the aortic rings do not dilate anymore after stimulation with VEGF/VPF as opposed to the vascular segments harvested from rabbits who have been

Figure 8. Tracings from contraction/relaxation experiments with aortic rings of NZW after 6 weeks of high cholesterol feeding.

A: Administration of VEGF/VPF. The initial force was induced with a double dose of NOR to reach approximately 50% of the maximal depolarization.

B: Administration of ACh did not cause a relaxing effect in aortic rings that had been exposed to hypercholesterolemia. ACh: acetylcholine; L-NMMA: N^G -monomethyl-L-arginine; NOR: norepinephrin; NZW: New Zealand white rabbits; SNP: sodium nitroprusside; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; w: weeks of a 1% cholesterol diet.

Figure 9. NO-dependent relaxation in aortic rings after administration of ACh.

ACh: acetylcholine; HC: high cholesterol diet; NZW: New Zealand white rabbit; reg. ch.: regular chow; WHHL: Watanabe heritable hyperlipidemic rabbit; *: $p < 0.05$ versus 'NZW/reg. ch.'

Figure 10. NO-dependent relaxation in aortic rings after administration of VEGF/VPF.
HC: high cholesterol diet; NZW: New Zealand white rabbit; reg. ch.: regular chow; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; WHHL: Watanabe heritable hyperlipidemic rabbit; *: $p < 0.05$ versus 'NZW reg. ch.' and 'NZW 1% HC (6 wks).'

fed a high cholesterol diet for only 6 weeks. The aortic rings obtained from WHHL rabbits behaved similar to the rings of the 10 weeks cholesterol-fed rabbits.

Histology/immunohistochemistry

The data obtained from measuring NO were confirmed with immunohistochemistry. Evidence is presented that ecNOS expression, but not iNOS, is present after 6 weeks of cholesterol feeding. Note that the ecNOS appearance includes the cytosol rather than the membrane of endothelial cells (Figure 12). After 10 weeks, when neointimal thickening has emerged, iNOS becomes expressed predominantly throughout the neo-intima. The cells in the neointima are mainly macrophages/foam cells (Figure 13). The deposition of nitrotyrosine, a marker for peroxynitrite generation, is also observed in the basale layers of the neointima. (Figure 14) Note that iNOS expression and deposition of nitrotyrosine correlate as well.

Figure 15. Expression of VEGF/VPF protein in the thoracic aorta of hypercholesterolemic rabbits. VEGF/VPF is without significant differences expressed in all groups ($n=4/\text{group}$). NZW: New Zealand white rabbit; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; w: weeks of a 1% cholesterol diet; W: Watanabe heritable hyperlipidemic rabbit.

Western blot

Previously, several groups postulated that VEGF/VPF had chemotactic properties culminating in infiltration of monocytes into the vessel wall.^{17,18,19} To test the theory that increased baseline NO production by vascular endothelial cells is related with upregulated VEGF/VPF expression in the vascular wall, the quantity of the VEGF/VPF protein was documented in the vessels of hypercholesterolemic and regular chow-fed rabbits. VEGF/VPF appeared to be expressed in all groups without significant differences. The tendency of a slightly elevated expression in the aorta of New Zealand white rabbits that have been fed a 1% cholesterol diet for 10 weeks appeared not to be a consistent finding (Figure 15). From this experiment, it can be concluded that the elevated baseline NO production is not the result of increased VEGF/VPF expression throughout the vessel wall. In addition, the similar expression of VEGF/VPF in all groups does not support the hypothesis that the infiltration of macrophages/foam cells is mediated by VEGF/VPF.

Figure 11. Structural remodeling in hypercholesterolemic rabbits. A-D: Masson trichrome stained sections. E: stained with hematoxylin and eosin. Note that no visible changes have occurred after a 6 week cholesterol diet as opposed to the apparent intimal thickening in NZ after 10 weeks of cholesterol feeding or in WHHL. The scale bar represents 50 μ m. HC: high cholesterol diet; NZ: New Zealand white rabbit; w: weeks.

Figure 12. The expression of ecNOS and iNOS in aortic rings of hypercholesterolemic rabbits. EcNOS is clearly present in NZ after 6 weeks of cholesterol feeding when no intimal thickening is present. The ecNOS in the endothelial layer covering a neointimal lesion, however, is hardly visible. This observation is consistent with the notion that oxLDL accelerates the degradation of ecNOS mRNA. The macrophages/foamcells in neointimal lesions clearly express iNOS as indicated by the small arrows. The larger arrows indicate the internal elastic lamina. The scalebar represents 50 μ m. ec: endothelial cell constitutive; HC: high cholesterol diet; i: inducible; NOS: nitric oxide synthase; NZ: New Zealand white rabbit; oxLDL: oxidized low density lipoprotein; w: weeks; WHHL: Watanabe heritable hyperlipidemic rabbit.

Figure 13. Hypercholesterolemia-induced neointima is predominantly populated by macrophages/foamcells. A: Aortic rings were stained with Oil Red O to localize fat deposits. B-C: sections were also stained for soluble RAM 11 (B-C) to identify the presence of macrophages, also indicated with an arrow. The macrophages are packed together in the neointima, however, the media does not contain macrophages. Fat deposition is colocalized with macrophages pointing to the formation of foamcells. The scalebar indicates 50 μ m. HC: high cholesterol diet; NZ: New Zealand white rabbits; RAM: rabbit antibody macrophages; w: weeks; WHHL: Watanabe heritable hyperlipidemic rabbits.

Figure 14. Deposition of nitrotyrosine in aortic rings of hypercholesterolemic rabbits. Six weeks of cholesterol feeding did not cause structural remodeling. Nitrotyrosine is only visible around small adventitial blood vessels. However, in the neointimal lesion that had emerged after 10 weeks of a high cholesterol diet, nitrotyrosine is clearly visible in the basal layers of the neointima. The macrophages/foamcells in this area express iNOS and the subsequent increase in NO release with simultaneous generation of O_2^- results in the formation of the highly reactive nitrogen oxide radical $ONOO^-$. The generation of $ONOO^-$ is evidenced by the demonstration of nitrotyrosine. The scalebar indicates 50 μ m. HC: high cholesterol diet; iNOS: inducible nitric oxide synthase; NZ: New Zealand white rabbit; O_2^- : superoxide anion; $ONOO^-$: peroxynitrite; wks: weeks.

Discussion

From these experiments it can be concluded that hypercholesterolemia increases the release of NO from the rabbit aorta. This observation is in accordance with the study of Minor and colleagues, who measured NO from rabbit descending thoracic aortas after 5 weeks or 6 months high cholesterol feeding.⁷ Several groups demonstrated a dramatic impairment in the vasodilator activity in these types of vessels despite the marked increase in NO release.^{20,21,22,23} In the present study, evidence is provided that endothelium-dependent vasorelaxation can still be mediated by VEGF/VPF in hypercholesterolemic aortic rings in a dose-dependent fashion. Only after the occurrence of intimal thickening VEGF/VPF fails to induce vasorelaxation. With immunohistochemistry we demonstrated that the neointima predominantly consists of macrophages/foam-cells. These cells account for an increase in NO by almost 10-fold, which results in the formation of other highly reactive nitrogen oxide radicals with detrimental effects on endothelial cells.

Normally, NO is continuously released from vascular endothelial cells, involving the conversion of L-arginine to citrulline mediated by a membrane-bound, NADPH-dependent, eNOS/calmodulin complex.^{8,9,10} The enzymatic activity is responsive to fluctuations in intracellular Ca^{2+} as evidenced by the action of several reagents, such as ACh and VEGF/VPF, capable of increasing endothelial cell NO-release by increasing cytosolic Ca^{2+} .^{1,24,25}

The NO produced in the endothelium exerts many anti-atherogenic features. Endothelium-dependent vasorelaxation is mediated by NO, a potent activator of soluble guanylate cyclase, which causes increased intracellular levels of cyclic GMP and, subsequently, smooth muscle relaxation. Furthermore, also mediated by increased levels of cyclic GMP, NO has an inhibitory effect on cell proliferation and adhesion.^{19,26} It has been reported, that NO reduces vascular smooth muscle cell mitogenesis.^{27,28,29} Platelet and leukocyte adhesion to the vascular endothelium and subsequent chemotaxis are also negatively regulated by NO.^{30,31,32}

Regarding the anti-atherogenic actions of NO, it seems contradictory that the NO-release has increased in the thoracic aortas harvested from NZW rabbits with diet-induced hypercholesterolemia and in Watanabe rabbits with inherited hyperlipidemia as a result of a lack of the low density lipoprotein (LDL) receptor. One explanation for this observation is the concept that only the release of *intact* NO exerts its beneficial actions through increasing cyclic GMP. Several groups have conducted both *in vitro* and *in vivo* experiments that demonstrate an excess generation of the superoxide anion (O_2^-) in cultured cells incubated with native LDL (n-LDL) and in the wall of hypercholesterolemic vessels.^{33,34} This highly reactive oxygen species has been shown to originate in activated macrophages, neutrophils, vascular endothelial cells, and pulmonary type II cells. NO is a free radical gas and reacts readily with O_2^- to yield peroxynitrite (ONOO^-), a potent oxidizing molecule involved in several reactions, such as lipoprotein oxidation, inhibition

Figure 16. The formation of nitrotyrosine after generation of peroxynitrite.

of endothelial cell prostacyclin synthase, increased endothelial cell adhesion to blood-born cells, and nitration of aromatic amino acids.^{35,36,37,38} ONOO^- is only a weak stimulus for soluble guanylate cyclase to produce cyclic GMP.^{39,40} Stimulation of macrophages by cytokines induces the simultaneous production of large amounts of NO and O_2^- , resulting in localized formation of ONOO^- . The invasion of macrophages has been shown to play a crucial role in the initiation of tissue injury in a variety of pathological conditions, particularly in the pathogenesis of atherosclerotic lesion in response to a hyperlipidemic environment.^{41,42,43}

At physiological pH and temperature, ONOO^- has a half-life time of less than 1 sec. Demonstration of ONOO^- *in vivo* is based on identification of stable products of its reaction with several biological compounds. Nitrotyrosine is the stable product of the spontaneous reaction of ONOO^- with tyrosine, involving the substitution of the hydroxyl group at the ortho-position of the aromatic ring in tyrosine with a nitro ($-\text{NO}_2$) group. (Figure 16)^{35,44} The rate of this reaction was determined to be $5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and thus represents one of the faster reactions for ONOO^- reported to date. Furthermore, nitration of tyrosine results in impairment of processes that control signal transduction and regulate cell cycles.^{45,46} Detection of nitrated tyrosine residues by a polyclonal antibody against nitrotyrosine has been verified and has proven to show an excellent relationship between nitrotyrosine and ONOO^- generation.⁴⁷

Endothelial cells in culture exposed to n-LDL produce high rates of both NO and O_2^- as demonstrated by deposition of nitrotyrosine in the neointima. It has been postulated, that n-LDL uncouples L-arginine metabolism from the actual endothelial cell NO-release

resulting in an increased generation of O_2^- by ecNOS.³⁴ After feeding NZW rabbits a high cholesterol diet for 6 weeks, we observe an increase in NO production that specifically originated in the endothelium. Removal of Ca^{2+} from the organ bath solution reduced NO production to levels displayed by rabbits feeding on regular chow. Since only ecNOS, but not iNOS, is sensitive to changes in intracellular Ca^{2+} concentration, it can be concluded that the increase in NO release after 6 weeks of high cholesterol diet is derived only from endothelial cells. In addition, mechanical removal of the endothelium abolished the increased NO production as well. Other support for this observation are the results of immunohistochemistry, which shows no expression of iNOS in the vascular wall of the rabbits after 6 weeks of a high cholesterol diet. However, an increased homogenous expression of ecNOS in the endothelial layer was observed. Until now, we have not been able to demonstrate the generation of O_2^- *in vivo*, which would have been evidenced by the deposition of nitrotyrosine. In addition, as opposed to the situation after 10 weeks of high cholesterol feeding, no intimal thickening has occurred, nor are there other morphological changes compared to the aorta of a normal rabbit.

Interestingly, VEGF/VPF, but not ACh, is capable of inducing vasodilatation in the aortic rings harvested from rabbits 6 weeks after a high cholesterol diet. Evidence for the presence of functional VEGF/VPF receptors is obtained since VEGF/VPF further increased NO release when administered to the vascular segments from hyperlipidemic rabbits in the organ chamber. Taken together with the fact that the release of functional NO depends on the lack of simultaneous O_2^- generation⁴⁸, VEGF/VPF apparently augments NO release without increasing O_2^- production and thus prevent the formation of the potent oxidant ONOO⁻. ACh, however, failed to induce vasorelaxation although it stimulated NO release to significantly higher levels. Most likely, in a hyperlipemic vascular segment the release of non-functional NO was due to inactivation by O_2^- .

The mechanism by which VEGF/VPF reduces the generation of O_2^- remains unclear. VEGF/VPF binds to its tyrosine kinase receptors Flt-1 and Flk-1/KDR, uniquely present on endothelial cells, which dimerize to activate downstream signaling.⁴⁹ Activation of phospholipase $C_{\gamma-1}$ has been shown to cause an increase in cytosolic Ca^{2+} , which is the mechanism for VEGF/VPF to increase ecNOS activity and subsequently to augment NO release.^{24,25} Western blot analysis demonstrates that VEGF/VPF expression is not upregulated in the vessel wall of a hypercholesterolemic rabbit, which is consistent with the observation of Pritchard and colleagues that exposure of endothelial cells in culture to n-LDL did not affect ecNOS activity as measured by the [³H]citrulline assay.³⁴ In addition, the expression of ecNOS mRNA in human vascular endothelial cells *in vitro* did not change when the cells were exposed to increasing concentrations of n-LDL, while shortening of the half-life of ecNOS mRNA significantly decreased its expression when the cells were incubated with oxidized LDL (ox-LDL).⁵⁰ These observations support a protective function of VEGF/VPF on vascular endothelium by augmenting the production of intact NO without simultaneous generation of O_2^- and subsequently of

Figure 17. The protective action of VEGF/VPF on endothelial cells and the detrimental effects of macrophages/foamcells on the vessel wall. VEGF/VPF increases NO without simultaneously stimulating O_2^- production, thus preventing the generation of $ONOO^-$. Macrophages/foamcells are capable of inducing both an increase in NO and O_2^- . The subsequent formation of $ONOO^-$ provides a positive feed-back mechanism for further increase in oxidative stress in the vessel wall. See text for further explanation. ec: endothelial cell constitutive; i: inducible; LDL: low density lipoprotein; n: native; NOS: nitric oxide synthase; O_2^- : superoxide anion; $ONOO^-$: peroxynitrite; ox: oxidized; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor.

$ONOO^-$, because prevention of increasing intracellular oxidative stress reduces the formation of ox-LDL and thus promotes preservation of ecNOS mRNA (Figure 17).

This study does not support the hypothesis that VEGF/VPF mediates chemotaxis of monocytes into the vascular wall, because VEGF/VPF is equally expressed in normal segments and segments with intimal thickening. Ox-LDL and cytokines such as interleukin-1, which are produced by activated macrophages, are known important chemoattractants for monocytes.⁵¹ In addition, ox-LDL enhances the expression of vascular cell adhesion molecule-1 (VCAM-1), which selectively mediates adherence of monocytes to the endothelium.⁵² VCAM-1 expression has been reported to become expressed as soon as one week after cholesterol feeding.⁵³

In endothelial cells, ecNOS is a membrane-bound enzyme. The membrane association is due to the presence of an N-myristoylation sequence.⁵⁴ Myristoylation is the major post-translational modification for ecNOS membrane association. However, this localization of some myristoylated proteins is reversible resulting in membrane-bound proteins becoming cytosolic factors.⁵⁵ Previously, it was reported that n-LDL increases cell membrane rigidity through donating cholesterol.^{56,57} Changes in membrane lipid dynamics as well as other post-translational modifications might promote ecNOS translocation from membrane to cytosol.^{54,58} This might be an explanation for the homogenous staining of ecNOS in the endothelial layer of the aorta in rabbits after 6

weeks of high cholesterol feeding. However, the mechanism by which eNOS causes an increase in NO release without simultaneous O_2^- generation after administration of VEGF/VPF needs still to be clarified.

After 10 weeks of cholesterol diet, structural remodeling has occurred in addition to the merely "functional remodeling" after 6 weeks of cholesterol feeding. The plasma cholesterol concentration has risen by 4-fold, resulting in the development of intimal thickening. Although the expression of VEGF/VPF is not diminished and functional VEGF/VPF receptors still seem to be present, neointimal macrophages/foamcells now are the main source of the increase in baseline NO release. An almost 10-fold increase in NO release is documented in the aortic segments. Removal of Ca^{2+} from the organ bath solution does not result in abrogation of this increase pointing to involvement of the Ca-insensitive iNOS instead of eNOS. Moreover, immunohistochemistry reveals the expression of iNOS throughout the developing neointima, but not in the media.

Interestingly, L-NMMA diminished NO production only by approximately 50%, although it completely abrogated the stimulatory effect of VEGF/VPF on the endothelial cells. The availability of L-NMMA might be better to the endothelium than to the underlying layer of neointimal cells. Furthermore, altered membrane conditions in a hypercholesterolemic environment might interfere with the delivery of L-NMMA to the cytosolic iNOS.

Simultaneous production of O_2^- and NO by the macrophages/foamcells, resulting in the formation of $ONOO^-$, further increasing oxidative stress, is demonstrated by the presence of nitrotyrosine, a marker for $ONOO^-$ deposition.^{36,59} This is supportive by the finding of colocalization of macrophages/foamcells, iNOS expression, and nitrotyrosine in the emerging atherosclerotic lesion of the rabbit aorta after 10 weeks of cholesterol diet.

Similar observations were obtained in age-matched Watanabe rabbits with heritable, instead of diet-induced, hyperlipidemia. Although Watanabe rabbits display only half of the cholesterol levels compared to the 10 week cholesterol-fed rabbits, the hyperlipidemia-induced remodeling appears to be similar. The presence of macrophage/foamcell, and the colocalization with iNOS and nitrotyrosine is completely identical. Furthermore, a comparable amount of the VEGF/VPF protein is demonstrated in the vessel wall and functional VEGF/VPF receptors also are preserved in the Watanabe rabbits. Again, as in the 10 week cholesterol-fed rabbits, the protective function of VEGF/VPF on the endothelium is insufficient to restrict the tissue remodeling caused by the implemented oxidative stress generated by the neointimal macrophages/foamcells.

In conclusion, VEGF/VPF preserves the ability of endothelial cells to produce functional NO, which, when not being inactivated by O_2^- , prevents the subsequent formation of $ONOO^-$, a potent oxidizing molecule involved in the initiation of atherosclerosis in hypercholesterolemic rabbits. When intimal thickening occurs, however, oxidative stress generated by intimal macrophages/foamcells overwhelms the regulating function of the endothelium. Future studies are necessary to investigate the

hypothesis, that amplification of the protective endothelial function, for example by targeting the preserved VEGF/VPF receptors, will delay the development of atherosclerotic lesions in hyperlipemic models.

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Chapter 5

Vascular Endothelial Growth Factor / Vascular Permeability Factor Prevents Peroxynitrite-Induced Apoptosis in Endothelial Cells

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Abstract

Intimal lesions occurring in hypercholesterolemia are associated with the generation of potent oxidizing nitrogen oxides. Since oxidants are known inducers of apoptosis and several growth factors activating protein tyrosine kinase receptors prevent apoptosis, we hypothesized that vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) likewise prevents endothelial cell (EC) apoptosis in hypercholesterolemia.

Cultured ECs and smooth muscle cells (SMCs) were exposed to, (a) graded concentrations of peroxynitrite (ONOO⁻), (b) ONOO⁻ + VEGF/VPF, and (c) ONOO⁻ + VEGF/VPF + the phosphatidylinositol-3' (PI-3) kinase inhibitor, wortmannin. Apoptosis was demonstrated by enzymatic labeling of DNA fragmentation (TUNEL) and quantified. Peroxynitrite induced apoptosis in both cell types. VEGF/VPF significantly reduced the number of apoptotic figures in ECs. This effect was abrogated by co-administration of wortmannin, indicating the requirement for PI-3 kinase. VEGF/VPF exerted no effect on SMC apoptosis.

The generation of peroxynitrite in vivo was demonstrated by nitrotyrosine deposition in aortic intimal lesions from hypercholesterolemic rabbits. Intimal thickening was observed, consisting of SMCs and macrophages/foam cells. In addition, apoptosis was demonstrated by the TUNEL-method, transmission electron microscopy, and agarose gel electrophoresis of genomic DNA.

In conclusion, VEGF/VPF reduces peroxynitrite-induced apoptosis of ECs, requiring PI-3 kinase. VEGF/VPF did not decrease apoptosis of SMCs, thus reducing intimal thickening in hypercholesterolemia.

An important feature of preserving tissue integrity is the regulation of cell death and cell proliferation.¹ Tissue homeostasis is maintained as a result of a complex regulatory system with numerous checks and balances. Physiologic cell death or apoptosis occurs through controlled autodigestion of the cell and/or through ingestion by macrophages, both of which involving activation of endogenous proteases and endonucleases.^{2,3,4,5,6} In many cell types undergoing apoptosis, DNA is degraded into fragments. Consequently, identification of apoptosis in tissue specimens or in cultured cells has been made possible by enzymatic labeling of nuclear DNA fragmentation with terminal deoxynucleotidyl transferase (TdT), based on incorporation of labeled nucleotids at sites of DNA breaks.^{7,8,9}

A variety of extrinsic and intrinsic signals is known to oppose cell proliferation by inducing apoptosis.^{10,11,12} Damage-related inducers of apoptosis are, amongst others, oxidants and radicals.^{13,14,15,16} Previously, we reported on the generation of the potent oxidative free radical peroxynitrite (ONOO⁻) in neointimal lesions of the thoracic aorta in hypercholesterolemic rabbits.¹⁷ Furthermore, evidence was provided that vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) preserved the vasomotor response of hypercholesterolemia-exposed vessels without neointimal thickening.^{17,18} However, the putative function of VEGF/VPF on vascular endothelium is overwhelmed by ONOO⁻-generation in the developing neointima that emerged after the rabbits had been exposed to a high cholesterol diet for a longer period of time.

We now hypothesize that, apart from augmenting nitric oxide (NO), VEGF/VPF displays another protective action in preserving vascular integrity by mediating a survival signal for endothelial cells (ECs).

Recently, Yao and Cooper have demonstrated the requirement for phosphatidylinositol-3' (PI-3) kinase in the prevention of apoptosis by nerve growth factor (NGF) and platelet derived growth factor (PDGF).¹⁹ Interestingly, apoptosis is also prevented by epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), or insulin, all of which are known activators of protein tyrosine kinase receptors.^{20,21,22,23} Consequently, in the present study, we establish the prevention of ONOO⁻-induced apoptosis in vascular endothelial cells by VEGF/VPF, which is another protein tyrosine kinase activating growth factor.^{24,25} In addition, similar to NGF, PDGF, IGF-1, and EGF, evidence is presented that VEGF/VPF mediates cell survival involving a PI-3 kinase signaling pathway.

Methods

Reagents

All reagents and chemicals including the PI-3 kinase inhibitor wortmannin were purchased from Sigma Chemical Co. (St Louis, MO), except peroxynitrite and its

negative control, which was provided by the Alexis Corporation (San Diego, CA). Heterodimeric recombinant human VEGF/VPF, purified from *Escherichia Coli*, was a gift of Napoleon Ferrara and Stuart Bunting, Genentech, South San Francisco, CA. 3-Amino-9-ethyl-carbazole (AEC) was purchased from Biogenex (San Ramon, CA). Culture media were obtained from Life Technologies (Gaithersburg, MD), unless stated otherwise. The antibodies used for immuno-histochemistry were: mouse monoclonal anti-nitrotyrosine (Upstate Biotechnology Inc, Lake Placid, NY), mouse monoclonal CD31 (DAKO, Carpinteria, CA), mouse monoclonal anti- α smooth muscle actin conjugated to alkaline phosphatase (Sigma), and biotinylated GSL I-isolectin B4 (Vector Laboratories, Burlingame, CA). If applicable, secondary antibodies were applied derived from the Ultra Streptavidin Level2 Kit (Signet, Dedham, MA). The specificity of the antibodies is reported in the manual of the providing companies. The In Situ Cell Death Detection Kit (Boehringer Mannheim, Mannheim, Germany) was used for detection of apoptosis.

Cell culture

The thoracic aorta was harvested from regular New Zealand white (NZW) rabbits, NZW rabbits that had been subjected to a high cholesterol diet, and age-matched Watanabe heritable hyperlipidemic (WHHL) rabbits. The aortic ECs were harvested by scraping the luminal side of the excised thoracic aorta. The ECs in the removed fragments were placed in 4-well fibronectin-coated slides (Fisher Scientific, Pittsburg, PA) and cells were allowed to proliferate cultured in M199 medium (Fisher Scientific) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 12 $\mu\text{g}/\text{ml}$ bovine brain extract (Clonetics, San Diego, CA), and antibiotics. ECs were identified with immunohistochemical staining of CD31 (data not shown). They were used for experiments after the second passage.

Rabbit aortic smooth muscle cells (SMCs) were derived from 3 mm aortic segments, that were placed with the luminal side onto fibronectin coated dishes after the endothelium was mechanically removed as described above. The vascular SMCs proliferated in DMEM supplemented with 10% FBS and antibiotics. Cells were maintained in a culture incubator at 37 °C under a 5% CO₂ humidified atmosphere and were used on second or third passage. Cells were maintained at confluence for 2 to 3 days prior to experiments in 4-well fibronectin-coated slides (Fisher Scientific).

ONOO⁻ in 0.3 M NaOH was added in increasing concentrations ranging from 1 nmol/l to 1000 nmol/l. To avoid rapid decomposition, ONOO⁻ was buffered in 50 mmol/l Tris and phosphate-buffered solution (PBS) without Ca²⁺, pH 8.7. At pH 7.4 protonation of peroxynitrite induced rapid decomposition principally to nitrate (NO₃⁻). Before use, the ONOO⁻ concentration was monitored by measuring the increase in absorbance at 302 nm ($E_{302\text{ nm}}=1.67\text{ mmol/l}\cdot\text{cm}$) after addition of 5 μl of stock ONOO⁻ in 3 ml of 1 N NaOH. For control, the same volume of ONOO⁻ was added to the buffer used for the experiments. In order to avoid pH changes in the culture media, small

volumes of ONOO^- were employed. To account for the effects of nitrite, hydrogen peroxide, and NaCl, a negative control solution was administered, containing the decomposed form of ONOO^- prepared from the same stock as the active form. The negative control contained the same concentrations of nitrite, hydrogen peroxide and salt, but had no absorbance at 302 nm under alkaline conditions.

For some experiments, 100 ng/ml VEGF/VPF was added either alone or in combination with 0.1 $\mu\text{mol/l}$ wortmannin. This dose of wortmannin has been shown to specifically inhibit phosphatidylinositol-3' (PI-3) kinase.²²

Analysis of apoptosis in vitro

Analysis of the 4-well slides containing either vascular SMCs or ECs was performed without knowledge of the reagents added to the wells. The total number of cells per well was calculated by counting an area of the well in which no apoptosis was identified. The counting was facilitated by projection of the microscopic image on a large screen (Microscope Slide Projector XM160, Stahl Research Laboratories Inc, Port Chester, NY). Apoptotic cells were discriminated by the presence of condensed dark blue nuclei in contrast to either healthy or necrotic cells.^{7,8,9} All wells were independently counted twice by two different persons. The variation between the two observers and counts was less than 10%. The total number of ECs/well was $77,000 \pm 5,00$; for SMCs $45,000 \pm 3,000$ cells. Values are presented $\pm$ standard error of the mean (SEM).

Animal protocol

Experiments were performed on the thoracic aorta isolated from NZW rabbits or from age-matched Watanabe heritable hyperlipidemic (WHHL) rabbits. Only male rabbits were used to avoid variations in outcome attributable to gender alone. The experimental protocol described was conducted according to protocols approved by the St Elizabeth's Institutional Animal Care and Use Committee. Hypercholesterolemia was induced by feeding a 1% cholesterol diet for 6 (n=6) and 10 weeks (n=6). Age-matched controls (n=6) and WHHL rabbits (n=6) were maintained on standard chow. The NZW rabbits were randomly assigned to the different groups.

Harvesting and preparing the thoracic aorta was conducted as described previously.¹⁸ In short, the rabbits were killed by exsanguination. Blood was collected from all rabbits to determine total serum cholesterol with an automatic analyzer (Kodak 700, Johnson and Johnson, Rochester, NY). Immediately after cessation of respiration, the thorax was opened and the aorta was excised from aortic valve to diaphragmatic hiatus. Connective and other adhesive tissue was removed and the vessels were washed initially in PBS.

Then, the aorta was subdivided; the proximal part (n=4/group), most prone to lesion formation at early stages²⁶, was used for morphologic studies. This part was embedded in OCT compound (Miles Inc, Elkhart, IN), frozen in liquid nitrogen, and stored at -70°C until it was used for immunohistochemistry or nick-end labeling of DNA fragments.

The remaining part was used for transmission electron microscopy after fixing in 2.5% gluteraldehyde (pH 7.3), buffered with 0.1 mol/l sodium cacodylate at 4 °C for at least 1 hour.

Analysis of apoptosis in vivo

Air dried frozen 6 μm sections were fixed with fresh 4% paraformaldehyde (pH 7.4) for 30 min. After 2 washes, the tissue was permeabilized with 0.1% Triton X-100/PBS for 2 min at 4 °C and washed again. Thereafter, the slides were incubated with TdT derived from calf thymus and fluorescein-labeled nucleotides antibody conjugated with alkaline phosphatase for 30 min at 37 °C, washed again, and for 15 min incubated with 5-bromo-4-chloro-3-indolylphosphatase p-toluisine salt and nitroblue tetrazolium, which causes a dark blue color at the site of the incorporated fluorescein-labeled nucleotides. The slides were washed in PBS again and coverslipped with glycerol gelatin mounting medium. Positive controls were obtained by pretreatment with DNase: after immersion in DN-buffer (30 mmol/l Trizma base, pH 7.2; 140 mmol/l sodium cacodylate; 4 mmol/l MgCl_2) for 5 min, the slides were covered with 1 $\mu\text{g}/\text{ml}$ DNase dissolved in DN-buffer at 1:10 dilution. After 10 min, the slides were washed and nick-end labeled as described above. For negative controls, the slides were processed without incubation of TdT.

In arteries harvested from hypercholesterolemic rabbits and their controls kept on regular chow, apoptosis was evaluated by agarose gel electrophoresis of genomic DNA performed to identify ladders of fragmented DNA.

Immunohistochemistry

After DNA nick-end labeling, the 4-well slides containing either SMCs or ECs were further incubated with respectively a mouse monoclonal anti- α smooth muscle actin antibody conjugated to alkaline phosphatase or with biotinylated GSL I-isolectin B4 to respectively identify SMC-actin or EC membrane glycoprotein lectin. Bound primary antibody was detected with 4-chloro-2-methylbenzenediazonium/3-hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate (Fast Red TR/Naphtol AS-MX, Sigma).

For detection of nitrotyrosine, tissue was fixed and permeabilized at -20 °C for 5 min in 100% methanol. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min, and tissue was treated with normal horse serum to prevent non-specific binding of biotinylated horse anti-mouse immunoglobulins, which were applied as secondary antibody. Then, the slides were incubated with mouse anti-nitrotyrosine antibody (1 hour at 37 °C, diluted 1:100 in 1 % bovine serum albumin (BSA)/PBS). For negative controls equal amounts of non-specific mouse anti-rabbit IgG₁ (MOPC-21) was applied. After application of the secondary antibody and streptavidin-horseradish peroxidase, the slides were incubated with AEC causing a red color. The tissue was counterstained with hematoxylin and coverslipped with glycerol gelatin mounting medium.

The Oil Red O reaction for fat was performed on 6 μm frozen sections of the vascular segments. Adjacent sections were stained for α -actin to identify smooth muscle cells.

Transmission electron microscopy

Portions of thoracic aorta from 4 cholesterol-fed, 4 WHHL rabbits, and 4 control rabbits were postfixed with 1% osmium tetroxide. Then, the tissues were dehydrated with increasing concentrations of alcohol, followed by saturation with propylene oxide for 15 min. Thereafter, the tissue was gradually infiltrated with Epon and finally embedded with fresh Epon into molds and heated in a 60 °C oven for 24 hours. Several sections were stained with toluidine blue, and three ultrathin sections of the areas of interest were stained with uranyl acetate and lead citrate for examination with a Philips 300 electron microscope.

Results

Apoptosis in vitro

Peroxyntirite added to either SMCs or ECs in culture causes apoptosis (Figure 1). At the tested ONOO⁻ concentrations no differences were observed between cells harvested from hypercholesterolemic rabbits or rabbits kept on regular chow (data not shown). The percentage of apoptotic cells is maximal at concentrations of 100 nM ONOO⁻. At lower concentrations most cells survive unaffected. At the highest concentrations immediate massive necrotic cell death occurs, importantly diminishing the number of apoptotic cells. VEGF/VPF significantly reduces the percentage of apoptosis in ECs when ONOO⁻ is administered in concentrations from 10 to 100 nmol/l (Figure 2A). Accordingly, VEGF/VPF exerts no significant protective action at ONOO⁻ concentrations of 1000 nmol/l, when necrosis is the most important mode of cell death.

Similar to NGF, PDGF, ECF, and IGF-1, evidence is provided that PI-3 kinase is integral in the pathway by which VEGF/VPF, another growth factor acting on protein tyrosine kinase receptors, prevents apoptosis. When ECs are co-incubated with VEGF/VPF and 0.1 $\mu\text{mol/l}$ PI-3 kinase inhibitor wortmannin, the protective action that VEGF/VPF exerts in reducing the percentage of apoptotic death is almost completely abrogated (Figure 2B).

As expected, VEGF/VPF has no significant effect on SMCs, confirming that the VEGF/VPF-receptors *Flk-1/KDR* and *flt-1* are uniquely present on ECs²⁴ (Figure 3).

Apoptosis in vivo

The *in vitro* findings were confirmed *in vivo*, using a hypercholesterolemic rabbit model, which previously has been shown to involve neointimal thickening.¹⁷ In the intimal lesion

Figure 1. Peroxynitrite (ONOO⁻, 100 nM) induces apoptosis in representative examples of cultures with smooth muscle cells (SMCs) or endothelial cells (ECs). Only dark blue condensed nuclei were discriminated for quantification. Cells in which nuclei were not longer visible (D) were not identified as apoptotic cells.

Figure 2A. VEGF/VPF protects endothelial cells in a confluent monolayer from undergoing apoptosis when exposed to peroxynitrite for 48 hours. *: $p<0.05$ versus 'ECs/peroxynitrite'. ECs: endothelial cells; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; control refers to a solution containing the decomposed form of peroxynitrite prepared from the same stock solution as the active form; n represents the number of wells exposed to defined reagents.

Figure 2B. Wortmannin (0.1 μM), a phosphatidylinositol 3'-kinase inhibitor, significantly abolishes the effect of VEGF/VPF on endothelial cells. *: $p<0.05$ versus other values at indicated concentrations. ECs: endothelial cells; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor, n represents the number of wells exposed to defined reagents.

Figure 3. Percentage of apoptotic cells in a of rabbit aortic smooth muscle cells exposed to peroxynitrite for 48 hours. SMCs: smooth muscle cells; VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; control refers to a solution containing the decomposed form of peroxynitrite prepared from the same stock as the active form; *n* represents the number of wells exposed to defined reagents.

both smooth muscle cells and foam cells are observed; however, lipid-laden cells are absent in the media (Figure 4). At the site of the neointima significant quantities of peroxynitrite were produced as evidenced by the formation of nitrotyrosine (Figure 5A/C). The oxidative stress imposed on the thoracic aorta by persistent hypercholesterolemia is associated with the occurrence of apoptotic cell death in the neointima, but not in the media (Figure 5B/D). These findings were both present in WHHL rabbits and NZW rabbits with diet-induced hypercholesterolemia. Nitrotyrosine deposition and apoptotic nuclei were absent in the control animals (data not shown).

Transmission electron microscopy was performed at several levels of the thoracic aorta harvested from cholesterol-fed rabbits and rabbits maintained on regular chow to identify cells with ultrastructural changes of apoptosis. Again, apoptotic cells illustrating typical features such as condensation of nuclear chromatin and cell shrinkage without apparent damage of other cellular organelles were present in the neointima, but not in the media (Figure 6).

DNA laddering, another sign of apoptosis, was observed in the vessels harvested from WHHL and NZW rabbits after 10 weeks of high cholesterol feeding (Figure 7). In rabbits kept on regular chow or on a 6 weeks high cholesterol diet the occurrence of intimal thickening was absent. In these vessels DNA laddering was also absent (data not shown).

Figure 4. The occurrence of smooth muscle cells and foam cells in the neointima of a cholesterol-fed rabbit aorta. A: Smooth muscle cells (SMCs) are identified by a red color representing α -actin. The density of SMCs in the neointima is lower than in the media. The arrow indicates the internal elastic lamina (IEL) on top of which the intimal lesion has occurred. The spacebar indicates 50 μ m. The spacebar indicates 100 μ m. B: Foam cells are stained red with Oil Red O. The arrow indicates the IEL.

Discussion

From this study, it can be concluded that ONOO^- applied to vascular SMCs and ECs in culture induces apoptosis. However, VEGF/VPF abrogated the occurrence of apoptosis in ECs. Furthermore, ONOO^- produced by hypercholesterolemia-induced neointimal lesions appears to be associated with apoptotic cell death *in vivo*.

Oxidative stress, leading to the formation of free radicals, has been implicated to generate cell toxicity in several acute and chronic diseases. In particular, activated macrophages in the vascular wall of vessels that have been exposed to hypercholesterolemia are capable to produce large quantities of NO and O_2^- simultaneously in an equimolar fashion,^{27,28} resulting in the formation of ONOO^- and its conjugate acid, peroxyntitrous acid (ONOOH),^{29,30} both of which dramatically enhance toxicity of either NO or O_2^- alone.^{31,32,33} It has been demonstrated *in vitro* that $\text{ONOO}^-/\text{ONOOH}$ oxidizes DNA

Figure 5. Peroxynitrite induces apoptosis in the hypercholesterolemia-induced neointima. Nitrotyrosine, a marker for peroxynitrite formation, predominantly is present in the neointima of the hypercholesterolemic rabbit aorta (A/C). The occurrence of apoptosis is present in the same region (B/D). This colocalization is in accordance with *in vitro* experiments, where peroxynitrite induced apoptosis. Arrows indicate the internal elastic lamina on top of which the neointima has formed. The spacebar indicates 50 μm . NZ: New Zealand white rabbits; WHHL: Watanabe heritable hyperlipidemic rabbits; HC: high cholesterol diet; w: weeks; TUNEL: Terminal deoxynucleotidyl transferase dUTP Nick End Labeling.

Figure 6. Ultrastructural identification of apoptotic nuclei in the rabbit hyper-cholesterolemia-induced neointima. A: 3900x; B 8900x. The vertical arrows indicate apoptotic nuclei, the horizontal arrows indicate normal nuclei. Note that the apoptotic nuclei are compacted and show condensation of chromatin. They are surrounded with cytoplasmic organelles without visible abnormalities. The white round artefacts indicate spots of lipid deposition. Lipid is washed out during fixation with glutaraldehyde. HC: high cholesterol diet; NZ: New Zealand white rabbit; w: weeks; WHHL: Watanabe heritable hyperlipidemic rabbit.

Figure 7. DNA laddering in vessels from rabbits with hypercholesterolemia-induced intimal thickening. Agarose gel electrophoresis shows DNA fragmentation in vessels harvested from cholesterol-fed animals in which neointimal thickening had occurred; lane 1: New Zealand white rabbits (NZwr), regular chow; lane 2: NZwr, 6 weeks 1% cholesterol diet; lane 3: NZwr, 10 weeks 1% cholesterol diet; lane 4: Watanabe heritable hyperlipidemia rabbits; C: DNA control; MW: Molecular weight marker.

bases, thus inhibiting DNA or protein synthesis.^{34,35} In addition, ONOO[•]/ONOOH causes nitration of tyrosine residues,^{36,37} which has been shown to interfere with mitochondrial Mn superoxide dismutase, reducing the ability of cells to cope with additional oxidative stress.³⁸ Finally, it has been reported that nitrotyrosine significantly abolishes phosphorylation of tyrosine residues in ECs *in vitro*, possibly affecting normal signal transduction.^{39,40}

Recently, several groups have suggested that oxidative stress is a key component of the apoptotic pathway in various cell types.^{41,42} For example, virus-induced apoptosis in prostate carcinoma cells was completely abrogated by the thiol antioxidant N-acetylcysteine, one of the most effective inhibitors of transcription factor NF- κ B.⁴³ We showed the occurrence of apoptosis when vascular SMCs and ECs were exposed to oxidative stress both *in vitro* and *in vivo*. This observation is in accordance with the mechanism proposed by Schreck and Baeuerle, in which oxygen radicals are a common second messenger utilized by multiple stimuli to activate NF- κ B, which, under certain conditions, culminates in apoptotic cell death.^{44,45} In addition, overexpression of the proto-oncogene bcl-2, which has been shown to prevent apoptosis, functions in an antioxidant pathway by decreasing the generation of oxygen radicals.^{46,47}

In contrast, exposure to high concentrations of ONOO^- results in necrotic cell damage, which, in the case of ECs, can not be ameliorated by VEGF/VPF. Furthermore, apoptosis developed over many hours after administration of ONOO^- , whereas massive necrotic cell death could be documented within an hour. This observation explains the decrease in apoptotic figures at higher doses of ONOO^- .

Interestingly, we observed that VEGF/VPF abrogated significantly the occurrence of apoptosis after administration of ONOO^- . VEGF/VPF binds to its protein kinase receptors, *Flk-1/KDR* and *Flt-1*, uniquely present on vascular ECs, which must dimerize to activate downstream signaling.²⁴ However, evidence has been presented that protein kinases and protein phosphatases function as targets for oxidants and antioxidants. Anderson and colleagues have documented that oxidation of protein tyrosine kinase by reactive oxygen species culminates in the induction of NF- κ B, and the subsequent occurrence of apoptosis.^{48,49} Application of tyrosine kinase inhibitors abrogated this effect.

Alternatively, stimulation of tyrosine kinase receptors with NGF, PDGF, EGF, IGF-1, and, as shown in the present study, VEGF/VPF mediates cell survival by increasing PI-3 kinase activity. This effect was abolished in the presence of a PI-3 kinase inhibitor. Taken together, it seems likely that the balance of agents acting on protein tyrosine kinase influences the fate of the cell: its resulting signal transmits either survival or apoptosis (Figure 8). Further support supplied by Burton, who reported that control

Figure 8. Schematic representation of survival/apoptosis signaling in the rabbit hypercholesterolemia-induced neointima. Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) exerts a protective effect on endothelial cells by preventing them from undergoing apoptosis. The transmission of this survival signal involves activation of phosphatidylinositol 3'-kinase (PI-3 kinase). The redox balance of the cell at a given time is crucial for cell survival. An increase in oxidative stress may overcome the effect of VEGF/VPF and may culminate in apoptosis by activating nuclear factor- κ B (NF- κ B).

of cell proliferation versus apoptosis is mediated by receptor-determined events that often lead to the generation of reactive oxygen species, causing alternative responses depending on the specific cellular redox balance at a given time.⁵⁰

In the vascular wall of the hypercholesterolemic rabbit apoptosis occurs in the neointima, but not in the media. The localization of apoptotic figures coincides with the deposition of nitrotyrosine, which is the stable and specific footprint of ONOO⁻ and indicates the severity of oxidative stress. The formation of the highly reactive nitrogen oxides is likely to push the tissue redox balance towards mediating apoptosis.

In mature organisms, cell number is controlled as a result of the net effects of cell proliferation and cell death. Regulation of cell death is a crucial event in the developing neointima in this hypercholesterolemic model, since it allows for the elimination of cells that have been produced in excess and eventually account for pathological conditions such as impaired tissue perfusion by luminal narrowing. VEGF/VPF exerts its protective action by reducing free radical-induced apoptosis only on ECs, but not on SMCs, thus promoting cell loss in the neointima and reduction of additional cell adhesion and infiltration.

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Chapter 6

General Discussion

Introduction

From the data described in this thesis it can be concluded that vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) augments nitric oxide (NO) release in the endothelium of mature vessels. This observation has two important implications. First, the presence of functional VEGF/VPF-receptors on adult endothelium, but not on other vascular cells, can be inferred, and, second, VEGF/VPF has a role in vascular biology besides mediating vasculogenesis in the developing embryo^{1,2} or, in the adult, angiogenesis in pathological tissue especially in conjunction with ischemia.^{3,4,5}

NO has been the subject of numerous studies, since this simple free radical gas is involved in many biological phenomena, such as vascular relaxation,⁶ inhibition of platelet⁷ and leukocytes⁸ adhesion to vascular endothelium, DNA modifications and repair,⁹ transcriptional regulation,¹⁰ and inhibition of mitogenesis of vascular smooth muscle cells.¹¹ The release of NO by the endothelium is mandatory for preservation of vascular integrity. Consequently, disruption of vascular endothelium causes vasospasm, thrombus formation, infiltration of inflammatory cells, and proliferation/migration of smooth muscle cells.^{12,13} Also, degenerative changes of blood vessels occurring in aging, hypertension, and diabetes mellitus have been associated with dysfunctional endothelium, characterized by reduced endothelial cell (EC) NO production.^{14,15,16,17,18}

VEGF/VPF

The data presented in this thesis are supportive for the concept that VEGF/VPF mediates a maintenance/repair role on quiescent mature vascular endothelium (Figure 1). The biological activity of VEGF/VPF and the subsequent increased availability of functional NO released by the endothelium affects vessel function and patency and as a result protects the blood supply of depending organs. It can be hypothesized, that VEGF/VPF is a crucial growth factor/cytokine in preservation or restoration of sufficient tissue oxygen supply.

The biological activity of VEGF/VPF on mature quiescent vascular endothelium promotes normal tissue metabolism and physiology. In addition, VEGF/VPF has been

Figure 1. Schematic representation showing the opposite effects of NO depending on the type of NOS. VEGF/VPF: vascular endothelial growth factor/vascular permeability factor; NOS: nitric oxide synthase; i: inducible; ec: endothelial cell constitutive; O_2^- : superoxide anion; $ONOO^-$: peroxynitrite; SMC: smooth muscle cell; EC: endothelial cell.

shown to accelerate angiogenesis in ischemic tissue,¹⁹ thus restoring adequate oxygen supply. Gene transfer of a plasmid encoding VEGF₁₆₅/VPF into the femoral artery of patients with severe peripheral artery disease has been reported to be a useful therapeutical application.²⁰ Other clinical applications of VEGF₁₆₅/VPF in ischemia-related disorders, such as severe trivascular coronary artery disease, or in expedited wound healing still require investigation.

Hypercholesterolemia

An ongoing assault on the vascular endothelial function was designed in a rabbit model of hypercholesterolemia to test the concept that VEGF/VPF can exert a repair/maintenance function. Before the occurrence of intimal thickening, VEGF/VPF, but not acetylcholine, appeared to be capable of inducing normal vasorelaxation as a result of NO release by the endothelial cells. However, after being exposed to higher levels of cholesterol for a longer period of time, a neointimal lesion consisting of macrophages/foamcells and smooth muscle cells had a detrimental effect on endothelial function, which could not be reversed by VEGF/VPF (Figure 1).

This observation has been related to the generation of highly reactive nitrogen oxides, mediated by macrophages/foamcells in the neointima. Especially the formation of peroxynitrite, a condensation of NO and the superoxide anion, has been shown to

markedly increase oxidative stress.²¹ In this thesis, evidence is provided that oxidative stress causes apoptosis in the neointimal cells of a hypercholesterolemia-induced intimal lesion. However, binding of VEGF/VPF to its receptors, which are only present on ECs,^{22,23} mediates a survival signal and consequently reduces the incidence of apoptotic figures. Because of its narrow target cell specificity, oxidative stress-induced smooth muscle cell apoptosis was not affected by VEGF/VPF. The mechanism of action of VEGF/VPF in hypercholesterolemia might consist of promoting EC survival and reducing neointimal cellularity, thus conserving normal vascular anatomy and function.

It has not been investigated whether VEGF/VPF might expedite recovery of EC function when cholesterol concentration returns to lower levels. Also, the effects of VEGF/VPF on endothelial function in patients suffering from complications of diabetes mellitus and/or hypertension are yet unknown. Survival of ECs and restoration of constitutive EC NO release may be crucial steps in preventing the occurrence of, (a) hypertension as a result of increased vascular tone;²⁴ (b) vascular thrombosis and occlusive artery disease with subsequent jeopardized tissue perfusion;²⁵ (c) adhesion of monocytes to the endothelium and infiltration in the subendothelial area, which is considered to be the initial event in atherosclerosis;²⁶ and (d) mitogenesis of vascular smooth muscle cells, their migration to the luminal site, and the formation of (additional) intimal thickening.²⁷

Recent studies demonstrate the beneficial effects of dietary supplementation of L-arginine in a rabbit model of hypercholesterolemia.²⁸ L-arginine constitutes the substrate for NO synthase (NOS), yielding NO during conversion to citrullin. In this thesis, evidence is provided that in hypercholesterolemia both EC constitutive NOS (ec-NOS) and inducible NOS (i-NOS) are expressed in the vascular wall. Especially i-NOS has been demonstrated in intimal cells and has been associated with the formation of highly reactive nitrogen oxides that impair the bioavailability of functional NO. Since binding sites for L-arginine have been reported on both ec-NOS and i-NOS,²⁹ dietary supplementation might exert detrimental effects on endothelial function in patients with established vascular disease in which i-NOS is likely to be present in intimal cells. Only normal blood vessels in which i-NOS is absent are likely to improve in endothelial function when increased L-arginine levels will become available exclusively for ec-NOS. For example, patients who underwent vascular bypass surgery might display an improved graft patency with a high L-arginine diet.

Future investigations

There is now considerable evidence that NO production is abnormal in patients with heart failure.³⁰ Increased myocardial NO production as a result of expression of i-NOS by cytokines such as TNF- α contributes to reduced contractility and myocyte injury. Further support is provided by the observation that progressive heart failure is a feature of established septic shock.³¹ Overproduction of i-NOS mediated NO leads to marked vasodilatation and loss of normal cardiac function. Likewise, in humans with left

ventricular dysfunction, intracoronary infusion of the NO synthase inhibitor L-NMMA potentiated the inotropic response to peripheral infusion of dobutamine.³² Also, after cardiac transplantation allograft rejection occurs after the expression of large amounts of i-NOS in response to cytokines.³³ Finally, it has been documented that i-NOS is abundantly expressed in patients with idiopathic dilated cardiomyopathy contributing to the detrimental effects of this disease.³⁴ However, NO release from vascular endothelium seems to be preserved and this maintains tissue perfusion by attenuating the vasoconstrictive impulses by various neurohumoral factors.

The constitutive production of NO through ec-NOS activity has not been associated with altered myocardial contractility. However, evidence has been provided that immune-mediated stimulation of i-NOS leads to an excess of NO capable of inhibiting the positive inotropic and chronotropic responses to β -adrenergic receptor stimulation.³⁵ In addition, further increase in NO production mediated by muscarinic cholinergic stimulation of the heart is, at least partially, responsible for parasympathetic slowing of the heart rate and additional inhibition of β -adrenergic contractility.³⁶ Thus, similar to the effects of hypercholesterolemia on the vascular wall, the maintenance function that ec-NOS mediated NO exerts on preserving tissue integrity can become overpowered by the cytokine-stimulated NO release via i-NOS with subsequent tissue injury.

It remains to be investigated whether selective enhancement of the putative ec-NOS activity and simultaneous inhibition of i-NOS activity favourably modifies the course of these conditions (Figure 1). Consequently, the signaling pathway involving the increased transcription of NOS induced with cytokines needs to be identified. Then, therapeutical interventions might be targeted at this specific pathway, at influencing ec-NOS m-RNA expression and stability, or at inhibiting i-NOS function. Recently, some evidence became available that 21-aminosteroidazaroids might inhibit the formation of highly reactive nitrogen oxides species, such as peroxynitrite, and consequently reduce lethal cell injury during periods of energy depletion.³⁷ It has been suggested that these type of reagents selectively attenuate the expression of i-NOS in a dog model of ischemic liver injury.³⁸ In contrast to α -methylprednisolone, which exerts similar effects, azaroids are devoid of glucocorticoid actions, which is an appealing aspect for future investigations. In addition, in this thesis it has become evident that VEGF/VPF selectively stimulates ec-NOS activity as a result of the unique target cell specificity of this growth factor. For example, Von der Leyen and colleagues reported on *in vivo* transfer of the ec-NOS gene to augment constitutive NO production, which resulted in reduced mitogenesis of smooth muscle cells following balloon injury.³⁹ This might constitute a novel approach in preventing restenosis after balloon angioplasty.

Conclusions

In conclusion, EC-derived NO has a crucial role in preserving the integrity of blood vessels. NO mediated through i-NOS rather exerts cytotoxic effects. Promoting ec-NOS

activity and simultaneously inhibiting i-NOS expression and/or activity might constitute a new approach in the therapy of atherosclerosis-related vascular disease, heart failure, allograft rejection or in the prevention of long-term complications of hypertension and/or diabetes mellitus.⁴⁰ Drugs are now being developed to selectively inhibit i-NOS expression. Furthermore, VEGF/VPF has been shown to selectively increase ec-NOS activity in quiescent endothelial cells of mature blood vessels. In addition, VEGF/VPF has been shown to mediate a survival signal in ECs. More clinical trials are warranted to test the therapeutical potential of this unique growth factor.

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Summary

Vascular endothelial cells have been shown to play a crucial role in preserving the patency of blood vessels throughout life. Nitric oxide (NO) produced by endothelial cells is an important secreted factor that mediates a great number of phenomena in vascular biology, all contributing to normal vessel anatomy and function.

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is a protein produced by vascular cells that is capable of selectively augmenting NO production by vascular endothelium. This observation provides inferential evidence for the expression of the VEGF-receptors, *Flk-1/KDR* and *Flt-1*, on mature, quiescent endothelium. More support for this notion is provided by the demonstration of both receptor m-RNA and the expressed proteins in human blood vessels used for coronary bypass grafting.

NO is continuously released by healthy vascular endothelium. This free radical gas can be produced in other cell types as well, however, this occurs only after the expression of an inducible nitric oxide synthase (i-NOS). We now show that in a rabbit model of hypercholesterolemia, i-NOS becomes abundantly present in neointimal macrophages/foam cells. The produced NO by this type of cells reacts with simultaneously produced superoxide anions (O_2^-) to generate peroxynitrite ($ONOO^-$), another potent oxidizing molecule. The generation of peroxynitrite, however, tremendously increases oxidative stress in the vessel wall. Peroxynitrite has been shown to cause numerous detrimental cellular effects and importantly interferes with normal endothelial function.

In hypercholesterolemia, VEGF/VPF appeared to preserve endothelial function. Only after the occurrence of intimal thickening and the generation of peroxynitrite the putative function of VEGF/VPF becomes overwhelmed. Evidence is provided that VEGF/VPF protects ECs from peroxynitrite-induced apoptosis. Since the VEGF/VPF-receptors are uniquely expressed on ECs, this protective effect is not exerted on neointimal cells. Consequently, apoptotic figures were extensively demonstrated in the neointima. The reduction of cellularity in the intima is likely to promote vascular patency.

NO mediated via i-NOS has been shown to be involved in the pathophysiology of heart failure, septic shock, and allograft rejection. Also, reduced ec-NOS activity has been reported in vascular endothelium of patients with essential hypertension or diabetes mellitus. Future studies are necessary to test the hypothesis that selectively augmenting ec-NOS mediated NO, for example with VEGF/VPF, and simultaneously inhibiting i-NOS mediated NO release might have a beneficial effect in these highly prevalent cardiovascular disorders.

Samenvatting

De endotheelcellen van bloedvaten zijn van doorslaggevend belang voor goed doorgankelijke bloedvaten als men ouder wordt. Stikstofoxide (NO), dat uit endotheelcellen afkomstig is, vervult een belangrijke rol in het behoud van een normale functie en anatomie van bloedvaten.

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), een eiwit dat onder meer in bloedvaten wordt aangemaakt, verhoogt de productie van stikstofoxide (NO) in endotheelcellen. Hieruit kan bovendien worden afgeleid dat receptoren voor VEGF/VPF, *Flk-1/KDR* en *Flt-1*, aanwezig zijn op niet geactiveerde endotheelcellen. Voorts wordt in dit proefschrift aangetoond dat deze receptoren aanwezig zijn op bloedvaten die als omleidingen worden gebruikt bij operaties voor kransslagadervernuwing. VEGF/VPF kan dus een nuttige werking hebben bij het behoud van een goede doorgankelijkheid van deze omleidingen.

NO wordt in kleine hoeveelheden continu geproduceerd door endotheelcellen. NO kan ook in grote hoeveelheden door andere cellen worden aangemaakt, echter alleen nadat een bepaald enzym, "induceerbaar-NO synthase" (i-NOS), tot uitdrukking is gekomen onder invloed van prikkels uit de omgeving. In dit proefschrift wordt aangetoond, dat in konijnen met verhoogde cholesterolspiegels i-NOS overvloedig wordt gezien in macrofagen/schuimcellen, die gelocaliseerd zijn op plaatsen waar bloedvatvernuwing optreedt. De hoge concentraties NO die op deze plaatsen worden geproduceerd reageren met gelijktijdig gevormde zuurstofradicalen (O_2^-), waardoor peroxynitriet ($ONOO^-$) ontstaat. Peroxynitriet heeft een buitengewoon sterk oxiderend vermogen en oefent daardoor zeer schadelijke effecten uit op de vaatwand.

VEGF/VPF blijkt bloedvat-endotheel echter te kunnen beschermen tegen de effecten van verhoogd cholesterol. Pas nadat bloedvatvernuwing is opgetreden en peroxynitriet wordt gevormd, verliest VEGF/VPF zijn beschermende functie. Wel blijkt uit dit proefschrift dat VEGF/VPF voorkomt dat endotheelcellen te gronde gaan onder invloed van peroxynitriet. Doordat de receptoren voor VEGF/VPF uitsluitend aanwezig zijn op endotheelcellen, zullen andere cellen betrokken bij vaatvernuwing wel te gronde gaan, waardoor het dichtslibben van bloedvaten wordt geremd.

NO dat geproduceerd wordt in cellen waar i-NOS tot uitdrukking is gekomen blijkt ook een rol te spelen bij het ontstaan van hartfalen, septische shock, en afstotingsreacties na orgaantransplantatie. Ook is het zo, dat verminderde afgifte van NO door endotheelcellen wordt gevonden bij patiënten met hoge bloeddruk of suikerziekte. Onderzoek zal moeten uitwijzen of het versterken van de NO productie in endotheelcellen en het selectief afremmen van i-NOS activiteit bovengenoemde aandoeningen, die vaak voorkomen, gunstig kan beïnvloeden.

Dankwoord

Bij de voltooiing van dit proefschrift wil ik Dr. G.J.H. den Ottolander bedanken voor zijn doortastende wijze waarop hij mij in de klinische gezondheidszorg heeft geïntroduceerd. Zijn betrokkenheid bij het onderwijs van medisch studenten heeft in ruimere zin een belangrijk stempel gedrukt op het klinisch denken van meerdere generaties medici.

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Prof. Dr. M.J.A.P. Daemen is een onmisbare schakel geweest bij het kritisch omvormen van mijn onderzoeksresultaten tot een degelijk geheel waarbij ruime aandacht werd besteed aan de continuïteit in de verschillende hoofdstukken soms zelfs tot in de kleinste mogelijke details.

Uiteraard noem ik veel te weinig mensen. Ik moet natuurlijk alle medewerkers van de Vakgroep Cardiologie en ook de verpleegkundigen van het AZM noemen, omdat zij samen bijdragen aan een sfeer waarin wetenschappelijk onderzoek, klinische zorg en een goede onderlinge verstandhouding hand in hand kunnen gaan.

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Curriculum Vitae

behorende bij

Marinus Cornelis van der Zee
geboren 25 september 1960
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Na middelbaar onderwijs aan het Christelijk Gymnasium te Leeuwarden, afgerond met een β -diploma in 1980, werd de militaire dienstplicht vervuld van 1980-1981. Aansluitend werd gestart met de studie in de Geneeskundige Wetenschappen te Gent (B), waar het kandidaatsexamen werd verkregen in 1984. De studie werd voortgezet aan de Erasmus Universiteit te Rotterdam. Hier werd het artsexamen behaald in 1988. Hierna werd gewerkt als arts-assistent in het toenmalige Bergweg Ziekenhuis op de afdeling Interne Geneeskunde onder Dr. G.J.H. den Ottolander. In 1990 werd begonnen met de opleiding tot cardioloog in het Academisch Ziekenhuis Maastricht met Prof. Dr. H.J.J. Wellens als opleider. Van 1995-1996 werd stage gelopen aan het St. Elizabeth's Medical Center te Boston bij Jeffrey M. Isner. Hier werd het promotie-onderzoek verricht. De opleiding tot cardioloog werd afgerond op 1 maart 1997; aansluitend wordt gewerkt als cardioloog bij het Academisch Ziekenhuis Maastricht.

Diploma's

1980	VWO- β Christelijk Gymnasium; Leeuwarden
1988	Artsexamen Erasmus Universiteit; Rotterdam
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1996	Research Fellow Tufts University, School of Medicine; Boston
1997	Cardioloog; Maastricht

Publicaties

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