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Nonclassic Endogenous Novel Regulators of Angiogenesis

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Abstract——**Angiogenesis, the process through which new blood vessels arise from preexisting ones, is regulated by several "classic" factors, among which the most studied are vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2). In recent years, investigations showed that, in addition to the classic factors, numerous endogenous peptides play a relevant regulatory role in angiogenesis. Such regulatory peptides, each of which exerts well-known specific biological activities, are present, along with their receptors, in the blood vessels and may take part in the control of the "angiogenic switch." An in vivo and in vitro proangiogenic effect has been demonstrated for erythropoietin, angiotensin II (ANG-II), endothelins (ETs), adrenomedullin (AM), proadrenomedullin N-terminal 20 peptide (PAMP), urotensin-II, leptin, adiponectin, resistin, neuropeptide-Y, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), and substance P. There is evidence that the angiogenic action of some of these peptides is at least partly mediated by their stimulating effect on VEGF (ANG-II, ETs, PAMP, resistin, VIP and PACAP) and/or FGF-2 systems (PAMP and leptin). AM raises the expression of VEGF in endothelial cells, but VEGF blockade does not affect the proangiogenic action of AM. Other endogenous peptides have been reported to exert an in vivo and in vitro antiangiogenic action. These include somatostatin and natriuretic peptides, which suppress the VEGF system, and ghrelin, that antagonizes FGF-2 effects. Investigations on "nonclassic" regulators of angiogenesis could open new perspectives in the therapy of diseases coupled to dysregulation of angiogenesis.**

I. Introduction

Angiogenesis, the process through which new blood vessels arise from preexisting ones, plays a pivotal role during embryonal development and later, in adult life, in several physiological (e.g., corpus luteum formation) and pathological conditions, such as tumors and chronic inflammation, in which angiogenesis itself may contribute to the progression of disease (Folkman, 1995). Angiogenesis is regulated, under both physiological and pathological conditions, by numerous "classic" factors, among which are vascular endothelial growth factor (VEGF[1]), fibroblast growth factor-2 (FGF-2), transforming growth factors (TGFs), angiopoietins, platelet-derived growth factor (PDGF), thrombospondin-1, and angiostatin. Several excellent reviews on this topic are available (Cross and Claesson-Welsh, 2001; Ribatti et al., 2002b; Suhardja and Hoffman, 2003; Turner et al., 2003; Ferrara, 2004; Hoeben et al., 2004; Simons, 2004; Tait and Jones, 2004; Presta et al., 2005; D'Andrea et al., 2006; Folkman, 2006; Ren et al., 2006; Rüegg et al., 2006).

In recent years, evidence has accumulated that, in addition to the classic factors, many other endogenous peptides play an important regulatory role in angiogenesis, especially under pathological conditions. Although some articles surveyed the angiogenic regulatory action of some of these peptides (see sections III. and IV.), a comprehensive review of the "nonclassic" angiogenesis regulators has not yet been published. Thus, after a brief account of the classic angiogenetic mechanisms, we will survey the role played by the nonclassic proangiogenic and antiangiogenic endogenous peptides under physiological and pathological conditions, as well as their possible signaling mechanism(s) and their interaction(s) with the classic angiogenic factors. Finally, the new possible therapeutic perspectives opened by investigations of nonclassic angiogenic mechanisms will be discussed shortly.

[1] Abbreviations: VEGF, vascular endothelial growth factor; FGF-2, fibroblast growth factor-2; TGF, transforming growth factor; PDGF, platelet-derived growth factor; EC, endothelial cell; MMP, matrix metalloproteinase; VSMC, vascular smooth muscle cell; R, receptor; ECM, extracellular matrix; ET, endothelin; AM, adrenomedullin; EPO, erythropoietin; HIF, hypoxia-inducible transcription factor; JAK, Janus kinase; STAT, signal transducer and activator of transcription; PI3K, phosphatidylinositol 3-kinase; CAM, chorioallantoic membrane; ANG, angiotensin; ACE, angiotensin-converting enzyme; GPR, G protein-coupled receptor; AT_1-R, angiotensin II-type 1 receptor; AT_2-R, angiotensin II-type 2 receptor; KO, knockout; NO, nitric oxide; NOS, nitric-oxide synthase; PD123319, *S*-(+)-1-([4-(dimethylamino)-3-methylphenyl]methyl)-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo(4,5-*c*)pyridine-6-carboxylic acid; ET_A-R, endothelin type A receptor; ET_B-R, endothelin type B receptor; HUVEC, human umbilical vein endothelial cell; BQ788, *N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine; BQ123, cyclo(D-Asp-Pro-D-Val-Leu-D-Trp); ABT-627, [2*R*-(4-methoxyphenyl)-4*S*-(1,3-benzodioxol-5-yl)-1-(*N,N*-di(*n*-butyl)aminocarbonyl-methyl)-pyrroli-dine-3*R*-carboxylic acid]; PGE2, prostaglandin E_2; PAMP, proadrenomedullin N-terminal 20 peptide; CRLR, calcitonin receptor-like receptor; CGRP, calcitonin gene-related peptide; RAMP, receptor-activity-modifying protein; AM_1-R, adrenomedullin type 1 receptor; AM_2-R, adrenomedullin type 2 receptor; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; UT-R, urotensin II receptor; ACT058362, palosuran, 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt; Ob-R, leptin receptor; ERK, extracellular signal-regulated kinase; adipoR, adiponectin receptor; PK, protein kinase; HAEC, human aortic endothelial cell; NPY, neuropeptide-Y; Y_1-R to Y_6-R, neuropeptide-Y type to 6 receptor; DPPIV, dipeptidylpeptidase IV; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC_1-R, pituitary adenylate cyclase-activating polypeptide type 1 receptor; $VPAC_1$-R, vasoactive intestinal peptide/pituitary adenylate cyclase-activating polypeptide type 1 receptor; $VPAC_2$-R, vasoactive intestinal peptide/pituitary adenylate cyclase-activating polypeptide type 2 receptor; PD98059, 2′-amino-3′-methoxyflavone; NK_1-R to NK_3-R, neurokinin type 1 to 3 receptor; PLC, phospholipase C; sst1-R to sst5-R, somatostatin type 1 to 5 receptor; BAEC, bovine aortic endothelial cell; GHS-R, growth hormone secretagogue receptor; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide.

II. Classic Mechanisms of Angiogenesis Regulation

A. General Overview

Angiogenesis, a term applied to the formation of capillaries from preexisting vessels, i.e., capillary and postcapillary venules, is based on endothelial sprouting or intussusceptive (nonsprouting) microvascular growth (Risau, 1997). The latter represents an additional and/or alternative mechanism and is not dependent on local endothelial cell (EC) proliferation or sprouting: a large sinusoidal capillary divides into smaller capillaries, which then grow separately (Djonov et al., 2000).

Sprouting angiogenesis is a multistep, highly orchestrated process, which involves not only vessel sprouting, but also cell migration, proliferation, tube formation, and survival (Risau, 1997). It develops through five steps: 1) basement membrane degradation by the action of proteolytic enzymes, such as matrix metalloproteinases (MMPs) and plasminogen activators secreted by ECs, resulting in the formation of tiny sprouts penetrating the perivascular stroma; 2) migration of the ECs at the sprout tip toward the angiogenic stimulus; 3) proliferation of the ECs below the sprout; 4) canalization, branching, and formation of vascular loops, leading to the development of a functioning circulatory network; and 5) perivascular apposition of pericytes and vascular smooth muscle cells (VSMCs) to support the abluminal side and de novo synthesis by ECs and pericytes of the basement membrane constituents.

As the vascular system develops, the initial plexus becomes remodeled into a complex and heterogeneous array of blood vessels, including larger vessels, such as arteries and veins (after forming a media and adventitia), and smaller vessels, such as venules, arterioles and capillaries (after association with pericytes). Differentiation of arteries and veins was thought to be exclusively governed by hemodynamic forces, molding these vessels from the primary vascular plexus. However, the discovery that members of the ephrin family are differentially expressed in arteries and veins from very early stages of development (i.e., before the development of functional circulation), was one of the first indications that artery-vein identity is intrinsically programmed: Ephrin-B2 marks arterial ECs and VSMCs, whereas ephrin-B4 marks veins (Wang et al., 1998).

Pericytes are adventitial cells located within the basement membrane of capillary and postcapillary venules. Because of their multiple cytoplasmic processes, distinctive cytoskeletal elements, and envelopment of ECs, pericytes are generally considered to be contractile cells that stabilize the vessel wall and participate in the regulation of microcircular blood flow (von Tell et al., 2006). They may also influence EC proliferation, survival, migration, and maturation (von Tell et al., 2006). The balance between the number of ECs and pericytes seems to be highly controlled. Potential regulators include soluble factors acting in an autocrine and/or paracrine manner, mechanical forces secondary to blood flow and blood pressure, and homotypic and heterotypic cell contacts. For example, PDGF is involved in EC to pericyte signaling, stimulating pericyte migration and proliferation (Lindahl et al., 1997). Moreover, pericytes may differentiate into VSMCs (Rhodin and Fujita, 1989). Targeted disruption of the PDGF-BB gene resulted in a defective development of the VSMCs (Levéen et al., 1994).

The vascular system is highly heterogeneous and nonuniform in different organs and tissues (Ribatti et al., 2002a). It is widely accepted that the organotypic differentiation of ECs is dependent on interactions with stromal parenchymal cells in target tissues. Heterogeneity develops partly through interactions of endothelium with the organ and tissue environment via either soluble factors or cell-cell interactions, leading to a particular phenotype of the endothelium. Interactions between different microvascular and surrounding tissue cells play a major role in determining vascular structure and function. These interactions may occur through the release of cytokines and the synthesis and organization of matrix proteins on which the endothelium adheres and grows. The organ microenvironment can directly contribute to the induction and maintenance of the angiogenic factors (Ribatti, 2006).

B. The Angiogenic Switch

Angiogenesis is controlled by the balance between molecules that have positive and negative regulatory activity (Pepper, 1997). This concept led to the notion of the "angiogenic switch," which depends on an increased production of one or more positive regulators of angiogenesis (Ribatti et al., 2007). EC turnover in the healthy adult organism is low, the quiescence being maintained by the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli. In pathological situations angiogenesis may be triggered not only by the overproduction of proangiogenic factors, but also by the down-regulation of inhibitory factors. Various regulatory elements control the switch to the vascular phase.

1. Genetic Factors. In transgenic mice containing an oncogene in the β-cells of the pancreatic islets, angiogenic activity was observed in a subset of hyperplastic islet cells before the onset of tumor formation (Ribatti et al., 2007). In a tumor model of transgenic mice containing the genome of the bovine papilloma virus type I, the switch to the angiogenic phenotype was associated with the ability to export FGF-2 from the cells (Kandel et al., 1991). In cultured human fibroblasts, the angiogenic switch has been reported to be controlled by the tumor suppressor gene *p53*, which regulates the synthesis of thrombospondin-1 and is down-regulated during tumorigenesis (Dameron et al., 1994).

2. Secretion of Growth Factors. Numerous inducers of angiogenesis have been identified (Table 1), among which FGF-2 and VEGF are the most potent. FGF-2 is a heparin-binding polypeptide that induces proliferation, migration, and protease production in cultured ECs and neovascularization in vivo (Basilico and Moscatelli, 1992). It interacts with ECs through tyrosine kinase-FGF receptors (Rs) and low-affinity, high-capacity heparan sulfate proteoglycan Rs on the cell surface and in the extracellular matrix (ECM) (Rusnati and Presta, 1996). However, FGF-2 genetically deficient mice possess a normal vasculature and apparently do not display defects related to impaired angiogenesis (Ortega et al., 1998).

VEGF is an angiogenic factor in vitro and in vivo and a mitogen for ECs with effects on vascular permeability. It plays a role in the control of blood vessel development and pathological angiogenesis and is expressed when angiogenesis is high, and its levels are low when angiogenesis is absent (Ferrara, 2004; Hoeben et al., 2004; Ribatti, 2005). VEGF and VEGF Rs are the first EC-specific signal transduction pathway activated during vascular development and are critical molecules in the formation of the vascular system, as evidenced in embryos homozygous and heterozygous for a targeted null mutation in their genes (Ferrara et al., 2003). VEGF and its Rs function in a paracrine manner: VEGF expression is elevated in tissues of the developing embryo at the onset of their vascularization, and ingrowing vascular sprouts express high levels of VEGF Rs (Ferrara et al., 2003).

Other angiogenic factors involved in the switch are TGF-β1, PDGF-B, and angiopoietins 1 and 2. When mesenchymal cells are treated with TGF-β1, they express VSMC markers, indicating differentiation toward a VSMC lineage, and the differentiation can be blocked by antibodies against TGF-β1 (Hirschi et al., 1998). TGF-β1 has been also reported to direct neural crest cells toward a VSMC lineage (Shah et al., 1996). PDGF-B is secreted by ECs, presumably in response to VEGF and facilitates recruitment of mural cells. PDGF-B gene mutation may cause failure of pericyte recruitment (Lindhal et al., 1997). Angiopoietins 1 and 2 play a role in vascular stabilization. The former is associated with developing vessels and its absence leads to defects in vascular remodeling (Thurston, 2003); the latter antagonizes angiopoietin-1 action, causing destabilization of preexisting vessels. It is found in tissues such as ovary, uterus, and placenta that undergo transient or periodic growth and vascularization, followed by regression (Maisonpierre et al., 1997).

3. Recruitment of Inflammatory Cells Releasing Angiogenic Factors. The stromal microenvironment is essential for cell proliferation and angiogenesis through its provision of survival signals, secretion of growth and proangiogenic factors, and direct adhesion molecule interactions. For example, tumor cells are surrounded by an infiltrate of inflammatory cells, namely lymphocytes, neutrophils, macrophages, and mast cells, which communicate via a complex network of intercellular signaling pathways mediated by surface adhesion molecules, cytokines, and their Rs. Evidence is accumulating that mast cells play an important role in angiogenesis: FGF-2, VEGF, and PDGF stimulate migration of mast cells that produce tryptase, which in turn degrades ECM to provide space for neovascular sprouts (Feoktistov et al., 2003; Hiromatsu and Toda, 2003). In this connection, it seems of interest to recall that findings indicate that

TABLE 1
Main features of classic proangiogenic and antiangiogenic factors

Factor	Receptors	Signaling Pathways[a]	Angiogenic Activity[b]				
			In Vitro Assays (ECs)		Differentiation (Capillary Tube Formation)	In Vivo Assays (New Vessel Formation)	
			Proliferation	Migration		CAM	Rabbit Cornea
VEGF	VEGF-R_1, VEGF-R_2	+ PLCγ/PKC + MAPK	S	S	S	S	S
FGF-2	FGF-R_1, FGF-R_2, FGF-R_3, FGF-R_4	+ MAPK	S	S	S	S	S
TGF-β	TGF-β-R_1, TGF-β-R_2	+ ALK-1 and −5 + SMAD1/5 and 2/3	I	N	S	S	S
PDGF	PDGF-R	+ PI3K + SFK + Ras GAP	N	S	=	S	S
Angiopoietin-1	Tie 2	+ PI3K + Pac GTPase	N	S	S	S	S
Thrombospondin-1	VEGFLRP-1	+SRCK	I	I	I	I	I
Angiostatin	$\alpha v\beta 3$ Integrin	−MAPK	I	I	I	I	I
Endostatin	$\alpha 5\beta 1$ Integrin, VEGF-R_2	−MAPK	I	I	I	I	I

[a] +, stimulation; − , inhibition.
[b] I, inhibition; N, no effect; S, stimulation; =, no findings available.

mast cells were found to express and release angiogenic regulatory peptides, such as endothelin (ET)-1 (Maurer et al., 2004; Hültner and Ehrenreich, 2005) and adrenomedullin (AM) (Belloni et al., 2005, 2006; Tsuruda et al., 2006; Zudaire et al., 2006) (see sections III.C. and III.D.1.).

4. Mobilization of Angiogenic Cytokines from Extracellular Matrix. FGF-2, VEGF, and TGF-β are stored in the heparin-like glycosaminoglycans of ECM and may be released after ECM degradation by proteinases secreted by tumor and inflammatory cells (Mignatti and Rifkin, 1993). Tumor and inflammatory cells also secrete proteinase inhibitors, thereby making it likely that the degree of ECM degradation and ensuing angiogenesis stimulation by released cytokines depends on the level of proteinase/proteinase inhibitor equilibrium (Pepper et al., 1994).

5. Interactions of Adhesion Receptors with Matrix Metalloproteinase-2. The adhesion receptor $\alpha v\beta_3$ is selectively expressed on growing blood vessels (Brooks et al., 1994). $\alpha v\beta_3$ Integrin binds activated MMP-2 to the surface of ECs, facilitating ECM degradation (Brooks et al., 1996). Thus, the adhesion receptor $\alpha v\beta_3$ may act cooperatively with MMP-2 to promote EC functions necessary for angiogenesis, such as cell adhesion and migration (Li et al., 2003a; van Hinsbergh et al., 2006).

6. Classic Endogenous Inhibitors of Angiogenesis (Table 1). Thrombospondin-1 was the first protein to be recognized as a naturally occurring inhibitor of angiogenesis (Good et al., 1990) (Table 1). It is a heparin-binding protein that is stored in ECM, is able to inhibit proliferation of ECs from different tissues (Taraboletti et al., 1990), and destabilizes contacts among ECs (Iruela-Arispe et al., 1991). Tumors grow significantly faster in thrombospondin-1-null mice than in wild-type animals (Lawler, 2002).

Angiostatin has been identified as a 38-kDa internal fragment identical in amino acid sequence to the first four kringle structures of plasminogen (O'Reilly et al., 1994). Angiostatin inhibits growth of primary tumors by up to 98% and is able to induce regression of large tumors and to maintain them at a microscopic dormant size (O'Reilly et al., 1996).

Endostatin isolated by O'Reilly et al. (1997) is the 20 kDa C-terminal proteolytic fragment of the basement membrane component collagen XVIII. Endostatin has been proposed to interfere with VEGF and FGF-2 pathways (Taddei et al., 1999; Yamaguchi et al., 1999), to induce EC apoptosis (Dhanabal et al., 1999), and to inhibit MMP (Kim et al., 2000).

7. The Validity of in Vitro and in Vivo Assays as Predictors of Effects on Angiogenesis Relevant to Physiology and Pathophysiology. One of the major problems in angiogenesis research is the difficulty of finding suitable methods for assessing the angiogenic response. A single assay that is optimal for all situations has not yet been described, and ideally it should be easy, reproducible, quantitative, and cost-effective and should permit rapid analysis. To full understand and interpret the effects of a particular test substance on the process of angiogenesis, it is necessary to use more than one in vitro assay and to use different sources of ECs. Only this procedure can ensure that the results seen in vitro translate across to the in vivo conditions, where other cells and ECM proteins are involved in the process of angiogenesis.

C. Tumor Angiogenesis

It is generally accepted that tumor growth is angiogenesis-dependent and that any increment of tumor growth requires an increase in vascular growth (Ribatti et al., 1999b). Tumor angiogenesis is an uncontrolled and unlimited process essential for tumor growth, invasion, and metastasis, which is regulated by the interactions of numerous mediators and cytokines with pro- and antiangiogenic activity. Tumors lacking angiogenesis remain dormant indefinitely.

New vessels promote growth by conveying oxygen and nutrients and removing catabolites, whereas ECs secrete growth factors for tumor cells and a variety of ECM-degrading proteinases that facilitate invasion. An expanding endothelial surface also gives tumor cells more opportunities to enter the circulation and metastasize, whereas their ability to release antiangiogenic factors may explain the control exerted by primary tumors over metastasis. Growth of solid and hematological tumors consists of an initial avascular and a subsequent vascular phase (Ribatti et al., 1999b, 2004; Vacca and Ribatti, 2006). Assuming that the latter process is dependent on the angiogenesis and the release of angiogenic factors, the acquisition of angiogenic capability can be seen as an expression of progression from neoplastic transformation to tumor growth and metastasis.

III. Nonclassic Endogenous Stimulators of Angiogenesis

A. Erythropoietin

Erythropoietin (EPO) is a 30.4-kDa glycoprotein, which plays a crucial role in the maintenance and stimulation of erythropoiesis and erythrocyte differentiation. EPO is produced from peritubular fibroblast-like cells of the kidney cortex after birth and during the fetal life from hepatocytes. Its gene expression is induced by hypoxia-inducible transcription factors (HIFs). EPO acts via two R molecules that mainly activate Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. Evidence has accumulated that EPO also exerts a marked proangiogenic effect during embryonic and adult life, as well as enhances tumor growth by promoting angiogenesis and decreasing apoptosis (Ghezzi and Brines, 2004; Jelkmann, 2004; Koury, 2005; Rossert and Eckardt, 2005; Hardee et al., 2006). Admin-

istration of recombinant human EPO to patients with head and neck and breast cancers expressing EPO Rs may promote tumor growth via the induction of cell proliferation and angiogenesis (Henke et al., 2003; Leyland-Jones, 2003). Nevertheless, several preclinical studies have shown a beneficial effect of EPO on delaying tumor growth through reduction of tumor hypoxia and the deleterious effects of hypoxia on tumor growth, metastasis, and treatment resistance (Farrell and Lee, 2004), and a meta-analysis did not find an unfavorable effect on overall survival of the treated cancer patients (Bohlius et al., 2005). However, it is important to underline a strong-enough caution on the use of EPO in patients with malignancies, according to the directive of the US Food and Drug Administration.

EPO R mRNA expression was detected in human ECs (Anagnostou et al., 1994). EPO was found to stimulate proliferation and migration of cultured mature ECs and to lower the apoptotic rate (Anagnostou et al., 1990; Dimmeler and Zeiher, 2000; Jaquet et al., 2002; Ribatti et al., 2003b). The same effects were reported in cultured neonatal ECs, in which EPO also induced capillary-like tube formation (Ashley et al., 2002), and in embryonic ECs, in which EPO promoted differentiation into the mature phenotype (Heeschen et al., 2003; Müller-Ehmsen et al., 2006). Evidence has been provided that these effects were mediated by the EPO R-mediated activation of JAK/STAT and PI3K/Akt pathways (Haller et al., 1996; Ribatti et al., 1999a; Dimmeler and Zeiher, 2000; Mahmud et al., 2002). The proangiogenic effect of EPO has been confirmed in vivo in the chick embryo chorioallantoic membrane (CAM) assay (Ribatti et al., 1999a, 2003b), and in experimental models of myocardium and hind limb ischemia (Calvillo et al., 2003; Heeschen et al., 2003; Parsa et al., 2003).

The angiogenic potential of EPO has been reported to be similar to that of FGF-2 (Ribatti et al., 1999a) and VEGF (Jaquet et al., 2002). Of interest, findings suggested that EPO could stimulate angiogenesis in vitro through an autocrine mechanism involving the proangiogenic peptide ET-1 (see section III.C.): EPO enhanced ET-1 release from ECs, and its angiogenic effect was blunted by an anti-ET-1 antibody (Carlini et al., 1993, 1995).

B. Angiotensin II

Angiotensin (ANG) II is a well-known octapeptide hormone that regulates blood pressure, plasma volume, and electrolyte balance mainly through its stimulating action on aldosterone and vasopressin release, cardiovascular tissue growth, and neuronal sympathetic activity. It is the active component of the classic renal renin-angiotensin system, for which circulating kidney-derived renin cleaves liver-derived angiotensinogen to the decapeptide ANG-I, that in turn is transformed, mainly in the lung, by angiotensin-converting enzyme (ACE) to ANG-II. ANG-II may also be produced locally by several tissue renin-angiotensin systems. ANG-II acts through two main subtypes of G protein-coupled receptors (GPRs), referred to as AT_1-R and AT_2-R, that both are abundantly expressed in the vasculature (Matsusaka and Ichikawa, 1997; Touyz and Schiffrin, 2000).

ANG-II was found to be angiogenic in vivo in the CAM and in the rabbit cornea assay (Fernandez et al., 1985; Le Noble et al., 1991), and the bulk of the findings indicated that this effect was mediated by the AT_1-R, with AT_2-R playing an opposite action which was overcome by AT_1-R activation. ANG-II was shown to stimulate the growth of quiescent ECs via AT_1-Rs, but in the presence of proangiogenic factors it dampened EC growth via AT_2-Rs (Stoll et al., 1995). The AT_2-R blockade enhanced the angiogenic effect of ANG-II in rat subcutaneous sponge granuloma (Walsh et al., 1997). Sasaki et al. (2002) provided evidence that AT_1-Rs could play an important role in ischemia-induced angiogenesis. Well-developed collateral vessels and neoangiogenesis were observed in wild-type mice in response to hind limb ischemia, whereas the response was markedly reduced in AT_1-R KO mice. Ischemia-induced angiogenesis was also impaired in wild-type mice by AT_1-R blockade by a selective antagonist. Moreover, the suppression of inflammatory cell infiltration by the AT_1-R blockade could provide an unique strategy against angiogenic disorders, including malignant tumors. In fact, infiltration of macrophages and T lymphocytes promote tumor related-angiogenesis (Balkwill and Mantovani, 2001).

Silvestre et al. (2002) reported that the ischemic/non-ischemic hind limb angiographic ratio and blood flow were markedly higher in AT_2-R KO mice compared with wild-type animals. ANG-II was found to be cosecreted with ET-1 by ECs, suggesting its possible autocrine/paracrine mechanism of action (Kusaka et al., 2000).

ANG-II was shown to induce VEGF expression in VSMCs, which may stimulate EC proliferation, migration, and angiogenesis (Williams et al., 1995; Chua et al., 1998; Otani et al., 1998; Richard et al., 2000). The VEGF involvement in the AT_1-R-mediated angiogenic response of ischemic tissues has been demonstrated. The infiltration of inflammatory mononuclear cells, including macrophages and T cells, was suppressed in the ischemic hind limb of AT_1-R KO mice. Double immunofluorescence staining revealed that infiltrated inflammatory cells expressed VEGF, and the expression of VEGF and monocyte chemoattractant protein-1 was also decreased in KO mice (Sasaki et al., 2002). VEGF-mediated angiogenesis was impaired by AT_1-R blockade in the cardiomyopathic hamster heart, because this procedure markedly lowered VEGF mRNA expression, and capillary and microvascular density (Shimizu et al., 2003). No differences in VEGF protein expression were observed in the ischemic hind limb of wild-type and AT_2-R KO mice, and ANG-II increased it in both strains. Of interest, endothelial nitric-oxide synthase (NOS) protein levels were higher in AT_2-R KO mice than in the wild type controls,

indicating that AT_2-R down-regulated NO production (Silvestre et al., 2002). These last investigators also provided evidence that the antiangiogenic effect of AT_2-Rs was connected with the activation of apoptotic process in vascular cells.

Sporadic findings have been obtained, suggesting an antiangiogenic effect of endogenous ANG-II. In fact, in a rabbit model of hind limb ischemia the blockade of ANG-II production induced by the treatment with the ACE inhibitor enalapril led to an increase of angiogenesis (Fabre et al., 1999). Accordingly, in nude mice inoculated with alginate beads encapsulating human and mouse carcinoma-derived cell lines, the daily administration of enalapril induced a marked increase of angiogenesis within 11 days (Walther et al., 2003). However, these investigators observed that in transgenic mice overexpressing ANG-II, the angiogenic response in alginate bead implants was markedly increased, the response being abolished by the AT_2-R antagonist PD123319 and unaffected by the AT_1-R antagonist losartan. In keeping with this finding, the angiogenic response of implants was reduced in AT_2-R KO mice. In light of these findings, Walther et al. (2003) advanced the "unorthodox" hypothesis that ANG-II regulates in vivo angiogenesis acting through both AT_1-Rs and AT_2-Rs, which exert an inhibitory and a stimulatory effect, respectively.

C. Endothelins

ETs are a family of hypertensive 21-amino acid peptides, mainly secreted by ECs. This family includes three distinct isoforms, named ET-1, ET-2, and ET-3, which derive by the post-translational cleavage of inactive precursors, the big-ETs, by specific endopeptidases, referred to as endothelin-converting enzymes. ETs act via two main classes of GPRs, named ET_A-Rs and ET_B-Rs, whose potency in binding ETs is as follows: ET_A-R, ET-1 = ET-2 $\ggg$ ET-3; and ET_B-R, ET-1 = ET-2 = ET-3 (Rubanyi and Polokoff, 1994; Nussdorfer et al., 1999; Davenport, 2002). ETs and their receptors are present in a variety of tissues, where they play important physiological and pathophysiological roles, mainly concerning cardiovascular system (Kedzierski and Yanagisawa, 2001; Rossi et al., 2001; D'Orléans-Juste et al., 2002).

Human umbilical vein ECs (HUVECs) were found to express high levels of ET-1 and ET_B-R mRNAs and low levels of ET_A-R mRNA (Salani et al., 2000b; Bagnato et al., 2001). These cells also actively produced and secreted ET-1 (Fujitani et al., 1992; Flynn et al., 1998). Secretion of costored ANG-II and ET-1 by coronary rat ECs was also reported, and the process was inhibited by NO and enhanced by NOS inhibition (Kusada et al., 2000). Evidence has also been provided that ECM regulated ET-1 secretion by HUVECs: collagen IV dampened ET-1 secretion, whereas collagen I, acting via the activation of integrin and tyrosine kinase, stimulated it (González-Santiago et al., 2002). A potential autocrine role for endogenous ET-1 has also been suggested, inasmuch as ET-1 via the ET_B-R was shown to increase its own synthesis (Saijonmaa et al., 1992).

ET-1 and ET-3, acting via the ET_B-R, promoted in vitro EC proliferation (Vigne et al., 1990; Morbidelli et al., 1995; Noiri et al., 1997, 1998; Goligorsky et al., 1999) and migration (Ziche et al., 1990; Noiri et al., 1997). HUVECs cultured on Matrigel in the presence of ET-1 migrated throughout and aligned to form capillary-like tubular structures, and the effect was inhibited by the selective ET_B-R antagonist BQ788, but only weakly impaired by the ET_A-R antagonist BQ123, thereby confirming the main involvement of the ET_B-R in the angiogenic action of ET-1 (Salani et al., 2000b). Recent findings indicated that ET-1 stimulated HUVEC proliferation via ET_B-Rs coupled to Ca^{2+}-activated large conductance potassium channels, whose activation induced hyperpolarization of the cell membrane and consequently raised Ca^{2+} influx (Kuhlmann et al., 2005). ET-1 was found to act as an antiapoptotic factor for ECs and VSMCs, thus contributing to the maintenance of the integrity of newly formed blood vessels (Shichiri et al., 1997, 2000; Wu-Wong et al., 1997).

The most striking angiogenic effect was seen when ET-1 was combined with VEGF. Whereas unable to stimulate blood vessel growth in the chick embryo CAM (Ribatti et al., 1999a) and in a rat sponge model (Hu et al., 1996), ET-1, in association with VEGF, showed clear proangiogenic activity in the Matrigel plug implanted into mice (Salani et al., 2000b). ET-1-producing Chinese hamster ovary cells grafted onto CAM induced a clear-cut angiogenic effect, which was prevented by the mixed ET_A/ET_B-R antagonist bosentan and the endothelin-converting enzyme-1 inhibitor phosphoramidon. Chinese hamster ovary/ET-1-mediated effect was also prevented by an inhibitor of VEGF tyrosine kinase Rs, thereby confirming the involvement of VEGF in the ET-1 angiogenic response (Cruz et al., 2001). VEGF increased both the expression of ET-1 mRNA in and ET-1 secretion from ECs (Matsuura et al., 1998). ET-1, acting predominantly via the ET_A-R, stimulated both the expression of VEGF mRNA in and VEGF secretion from VSMCs, as well as enhanced VEGF-induced EC proliferation and migration (Pedram et al., 1997a,b; Okuda et al., 1998). Thus, VEGF and ET-1 have reciprocal stimulatory interactions, which may result in concomitant proliferation of ECs and VSMCs.

In cancer, VEGF and ET-1 have been reported to be up-regulated by various stimuli, including hypoxia, growth factors, and inflammatory cytokines (Okuda et al., 1998; Molet et al., 2000; Yamashita, 2001). Overexpression of ET-1 and its Rs was found in lung cancer, Kaposi's sarcoma, colon cancer, astrocytomas, and glioblastomas (Stiles et al., 1997; Ahmed et al., 2000; Egidy et al., 2000a,b; Asham et al., 2001; Bagnato et al., 2001; Fagan et al., 2001; Bagnato and Spinella, 2003). ABT-627, a potent ET antagonist (Verhaar et al., 2000), dis-

played antitumor activity and decreased neovascularization in vivo against established ovarian cancer xenografts in nude mice (Rosanò et al., 2001).

ET-1/VEGF interactions have been demonstrated to occur also in tumors. In primary and metastatic ovarian carcinomas, there was a highly significant correlation between ET-1 expression and microvascular density, as well as between ET-1 and VEGF expression (Salani et al., 2000a). The high amount of ET-1 released by ovarian carcinoma cells into ascitic fluid was responsible primarily for EC migration, acting via the ET_B-R, as demonstrated by its inhibition by BQ788. The significant inhibition of migration observed by coincubating HUVECs with BQ788 and anti-VEGF antibodies suggested that ET-1 and VEGF might have a complementary and coordinated role during neovascularization in ovarian carcinoma (Salani et al., 2000a). When tested in ovarian carcinoma-derived cell lines, ET-1 increased VEGF mRNA expression and induced VEGF production in a time- and dose-dependent fashion and did so to a greater extent during hypoxia (Salani et al., 2000a; Spinella et al., 2002). There is also evidence that ET-1 promoted VEGF production through HIF-1α: after ET-1 stimulation, the HIF-1α protein level increased in ovarian carcinoma cells, and the HIF-1α transcription complex was formed and bound to the hypoxia-responsive element-binding site (Spinella et al., 2002). These actions of ET-1 were mediated by the ET_A-R, because BQ123 reversed the stimulation of VEGF production (Spinella et al., 2002). ET-1-induced ET_A-R activation stimulated prostaglandin-E_2 (PGE_2) production and increased the expression of PGE_2 R type 2 and type 4. Cyclooxygenase-1 and -2 inhibitors blocked ET-1-induced PGE_2 and VEGF release by ovarian carcinoma cells. Thus, the conclusion was drawn that PGE_2 contributed to the tumor progression by promoting angiogenesis and that this effect was mediated by VEGF (Spinella et al., 2004).

D. Proadrenomedullin-Derived Peptides

AM and proadrenomedullin N-terminal 20 peptide (PAMP) are produced by the post-translational proteolytic cleavage of a 185-amino acid prohormone, the pre-pro-AM. AM (a 52-amino acid peptide in humans) and PAMP exert potent long-lasting and transient hypotensive effects, respectively. Although originally isolated from human pheochromocytomas, AM and PAMP have been subsequently shown to be synthesized in several tissues and organs, including blood vessels and heart. AM acts via selective Rs derived from the calcitonin receptor-like receptor (CRLR), which may act as either a calcitonin gene-related peptide (CGRP) or an AM R, depending on its interactions with the members of a family of single transmembrane domain proteins, named receptor-activity-modifying proteins (RAMPs): $RAMP_1$ generates CGRP Rs from CRLRs, whereas $RAMP_2$ and $RAMP_3$ produce AM Rs, called AM_1-R and AM_2-R, respectively. CGRP(8–37) and AM(22–52) have been identified as AM_1-R and AM_2-R antagonists (Hinson et al., 2000; López and Martínez, 2002; Poyner et al., 2002; Julián et al., 2005; García et al., 2006). PAMP binding sites are well distinct from AM Rs but have not yet been fully characterized, although recent findings seem to suggest that corticostatin $MrgX_2$-R may act as PAMP R (Kamohara et al., 2005; Nothacker et al., 2005). Nevertheless, evidence indicates that PAMP(12–20) behaves as a potent antagonist of PAMP Rs (Belloni et al., 1999).

1. Adrenomedullin. AM exerts several biological actions, including regulation of fluid and electrolyte homeostasis (Samson, 1999; Nussdorfer, 2001) and protective action on the cardiovascular system (Kato et al., 2005). Moreover, evidence that AM possesses a clearcut proangiogenic effect under both physiological and pathophysiological conditions has accumulated, and reviews on this topic have already been published (Nikitenko et al., 2002, 2006; Nagaya et al., 2005; Ribatti et al., 2005).

A genetically determined absence of AM may be one of the causes of nonimmune hydrops fetalis and hemorrhage, as a result of cardiovascular abnormalities and disturbance of angiogenesis and lymphangiogenesis (Caron and Smithies, 2001; Shindo et al., 2001). AM has been reported to exert its angiogenic activity via AM_1-Rs and AM_2-Rs, which activate mitogen-activated protein kinase (MAPK) and Akt cascades and focal adhesion kinase (Kim et al., 2003b; Miyashita et al., 2003; Fernandez-Sauze et al., 2004), as well as play an anti-inflammatory role in controlling VEGF-induced adhesion molecule gene expression and adhesiveness toward leukocytes in ECs (Kim et al., 2003a). AM augmented vascular collateral development in response to acute ischemia (Abe et al., 2003, 2006; Iwase et al., 2005) and enhanced capillary-like tube formation by HUVECs cultured on Matrigel and blood vessel formation in the CAM assay, the effect being counteracted by AM(22–52) (Ribatti et al., 2003a). AM gene transfer was found to induce therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia (Tokunaga et al., 2004). Miyashita et al. (2006) and Xia et al. (2006) showed that AM administration improved vascular regeneration in the ischemic rat brain. Using immortalized human microvascular ECs, Schwarz et al. (2006) showed that AM increased cAMP production, stimulated MAPK p42/p44, and enhanced EC migration but not proliferation, all these effects being inhibited by AM(22–52). Moreover, AM raised the expression of both CRLR and $RAMP_2$ mRNAs, suggesting up-regulation of AM_1-Rs. The role of this receptor subtype in the mediation of the AM proangiogenic effect has been confirmed by the demonstration that $RAMP_2$ gene silencing by short-interfering-RNA technology impaired the ability of HUVECs cultured on Matrigel to form capillary-like tubules in response to AM (Albertin et al., 2006).

Evidence has been provided that AM up-regulated the expression of VEGF in both in vitro and in vivo models

(Iimuro et al., 2004; Albertin et al., 2005; Schwarz et al., 2006). Using laser Doppler perfusion imaging, Iimuro et al. (2004) showed that AM stimulated recovery of blood flow to the affected limb in a mouse hind limb ischemia model, partly by promoting local expression of VEGF. Immunostaining for the EC marker CD31 revealed that this enhanced flow reflected increased capillary density. In EC and fibroblast cocultures, AM raised VEGF-induced capillary formation, and in EC cultures it increased VEGF-induced Akt activation. Iimuro et al. (2004) also demonstrated that heterozygous AM KO mice treated with AM(22–52) displayed reduced capillary development, and the administration of either AM or VEGF favored blood flow recovery and capillary formation. However, blocking antibodies to VEGF did not significantly inhibit AM-induced in vitro capillary-like tube formation by ECs (Fernandez-Sauze et al., 2004), suggesting that AM does not act directly through up-regulation of VEGF.

The detection of high levels of AM expression in various types of cancer cells suggests that this peptide is involved in tumor growth (Forneris et al., 2001; Li et al., 2001; Oehler et al., 2001; Martínez et al., 2002; Jimenez et al., 2003; Mazzocchi et al., 2004; Zudaire et al., 2006). In addition, AM expression and AM Rs have been detected in several carcinoma-derived cell lines (Hata et al., 2000; Belloni et al., 2001). AM produced by tumor cells is thought to inhibit their hypoxic death as an antiapoptotic factor (Oehler et al., 2001; Abasolo et al., 2006). AM was found to be up-regulated by hypoxia (Cormier-Regards et al., 1998; Nakayama et al., 1998), through the HIF-1α (Nguyen and Claycomb, 1999; Garayoa et al., 2000; Frede et al., 2005). Oehler et al. (2001) studied the role of AM in endometrial carcinoma cells under hypoxia and found that AM conferred resistance to hypoxic cell death in an autocrine/paracrine manner, the antiapoptotic effect being probably mediated by the up-regulation of the Bcl-2 oncogene.

AM overexpressing tumors are characterized by increased vascularity (Oehler et al., 2002, 2003), and an increased expression of AM mRNA in ovarian tumors has been statistically associated with a poor prognosis (Hata et al., 2000). Martínez et al. (2002) stably transfected human breast cancer-derived cell lines expressing low basal levels of AM, with an expression construct that contained the coding region of human AM gene or with an empty expression vector. Cells overexpressing AM displayed a more heterogenous morphology and increased angiogenic potential both in vitro and in vivo compared with those transfected with the empty vector. AM and VEGF have been reported to be the most widely expressed angiogenic factors in uterine leiomyomas (Hague et al., 2000). Leiomyomas displayed a higher vascular density and EC proliferative activity than normal myometrium and endometrium, and the expression of AM, but not of VEGF, correlated with the vascular density in these tumors.

Ribatti et al. (2003a) demonstrated that vinblastine was angiostatic in the angiogenic response induced by AM in two assays, namely capillary-like tube formation by HUVECs cultured on Matrigel and in vivo CAM vasculogenesis. They suggested that these findings implicate AM as a promoter of tumor growth and a possible target for anticancer strategies, such as the use of vinblastine at very low, nontoxic doses.

2. Proadrenomedullin N-Terminal 20 Peptide. In vivo and in vitro angiogenic assays showed that PAMP was more effective than AM and VEGF, inasmuch as PAMP acted at femtomolar concentrations and these latter peptides only in the nanomolar range. Some differences were observed in the actions of AM and PAMP (Martínez et al., 2004, 2006). In in vitro assays with human dermal microvascular ECs, PAMP did not affect cell growth, whereas AM and VEGF enhanced it. PAMP and VEGF, but not AM, stimulated EC migration. Finally, PAMP was less effective than AM and VEGF in inducing tubular organization in Matrigel cultured ECs. PAMP lowered by approximately 50% Ca^{2+} influx induced by ATP, and the effect was reversed by PAMP(12–20). PAMP increased VEGF, FGF-2, and PDGF mRNA expression in ECs, as revealed by real-time PCR. In in vivo assays with silicon tubes filled with Matrigel implanted in mice, the PAMP-R antagonist PAMP(12–20) completely blocked angiogenesis induced by PAMP or human lung cancer-derived cell line. Moreover, PAMP(12–20) lowered tumor growth rate in xenograft-implant experiment with this cell line, whereas PAMP was ineffective. According to Martínez et al. (2004), this last finding could be due to a rapid cleavage of PAMP by tumor cells, which saturates PAMP Rs on ECs. It is concluded that PAMP is a potent proangiogenic factor and tumor growth promoter, which may act either directly on ECs or indirectly by inducing the production of classic angiogenic promoters. The PAMP-R antagonist PAMP(12–20) could be used in antineoplastic therapeutic strategies.

E. Urotensin-II

Urotensin-II is a cyclic 11-amino acid (human) or 15-amino acid (rodents) peptide, originally isolated from the fish urophysis, which exerts a potent systemic vasoconstrictor and hypertensive effect. Urotensin-II has been identified as an endogenous ligand of the orphan GPR-14, which has been renamed urotensin R (UT-R) (Ames et al., 1999; Davenport and Maguire, 2000). Urotensin-II and UT-R are widely expressed in the heart and large arteries, and many lines of evidence led to the conclusion that urotensin-II plays a role in the physiology and pathophysiology of cardiovascular system (Douglas and Ohlstein, 2000). Urotensin-II has also been shown to exert a marked mitogenic action on many cell phenotypes, and the expression of urotensin-II and UT-R has been detected in several tumor-derived cell lines (Yoshimoto et al., 2004). A UT-R antagonist has

been identified and named palosuran (ACT058362) (Clozel et al., 2004).

Rat neuromicrovascular ECs were found to express urotensin-II and UT-R mRNAs and proteins, as revealed by PCR and immunocytochemistry. FGF-2 raised the EC proliferation rate, whereas urotensin-II did not. However, urotensin-II markedly stimulated the formation of capillary-like tubes by ECs cultured on Matrigel, and image analysis showed that the effect of this peptide was of the same order of magnitude as that of FGF-2. Accordingly, urotensin-II, added to CAM, induced a strong angiogenic response. Both in vitro and in vivo proangiogenic effects of urotensin-II were counteracted by Palosuran, indicating that it was mediated by the UT-R (Spinazzi et al., 2006).

F. Adipokines

The development of the vascular bed in adipose tissue is tightly connected to both the number and size of adipocytes, and adipose tissue serves as an important conduit for growing blood vessels. Immortalized preadipocyte cell lines were found to promote the formation of highly vascularized fat pads after injection into nude mice (Green and Kehinde, 1979), and adipocytes are known to secrete several cytokines, such as VEGF and tumor necrosis factor-α (Zhang et al., 1997). Hence, it is conceivable that adipocytes may modulate the growth of the vasculature in a paracrine manner (Mohamed-Ali et al., 1998). In addition to secreting the above-mentioned classic proangiogenic cytokines, adipocytes produce three other regulatory peptides, the adipokines leptin, adiponectin and resistin, which seem to be involved in angiogenesis modulation under both physiological and pathological conditions.

1. Leptin. Leptin, a 167-amino acid peptide in humans, is the protein product of the *ob* gene transcription, that acts through specific Ob-Rs, of which several isoforms (from Ob-Ra to Ob-Rf) have been described. Leptin is an adipose tissue-secreted hormone, which is involved in the regulation of satiety, metabolic rate, and thermogenesis (Ahima and Flier, 2000; Sweeney, 2002). In situations of continuous adipose-tissue growth, it is documented that angiogenesis is present (Crandall et al., 1997). Because obesity in humans is associated with an elevation of leptin in the plasma and the adipose tissue, it is tempting to speculate that the leptin-mediated cross-talk between adipocytes and ECs promotes angiogenesis.

ECs have been reported to express functionally active Ob-Ra and Ob-Rb, which mediated their leptin-induced proliferation, through the activation of STAT-3 and extracellular signal-regulated kinases (ERK) 1/2. Leptin also induced angiogenesis in vivo in the CAM and in the rat cornea assays (Bouloumié et al., 1998; Sierra-Honigmann et al., 1998). Ribatti et al. (2001) confirmed that leptin was able to stimulate angiogenesis when applied onto the chick CAM and showed that the angiogenic response was similar to that obtained with FGF-2. The stimulating property of leptin was specific, as the exposure to anti-leptin antibodies significantly inhibited the angiogenic response. However, the application to CAM of anti-FGF-2 antibodies reduced by approximately 40% the angiogenic effect of leptin, indicating that the activation of endogenous FGF-2 at least in part mediated leptin action. Evidence has been provided that leptin-induced new blood vessels were fenestrated, playing a critical role in the maintenance and regulation of vascular fenestration in the adipose tissue. In fact, leptin caused a rapid vascular permeability response when administered intradermally, which might provide a mechanism by which the excess amount of leptin would be exported into the circulation (Cao et al., 2001). Leptin has been found to be produced at a high level also in the placenta, a highly angiogenic tissue, where it could increase the exchange of small molecules between the maternal circulation and the fetus by the induction and maintenance of vascular permeability (Masuzaki et al., 1997).

Rather contrasting findings were reported by Cohen et al. (2001), who demonstrated that leptin induced the expression of angiopoietin-2 in adipose tissue without concomitant increase in VEGF, thereby providing a strong angiostatic rather than angiogenic signal. They proposed that induction of angiopoietin-2 by leptin in adipocytes is one of the events leading to adipose tissue regression, because this induction coincided with initiation of apoptosis in adipose tissue ECs.

The possible effects of leptin on tumor angiogenesis have not been investigated. However, findings showed that leptin not only stimulated proliferation of a mouse mammary carcinoma-derived cell line both cultured in vitro and implanted in syngeneic mice, but also enhanced the expression of VEGF and VEGF-R2, via PI3K, JAK/STAT, and ERK1/2 signaling pathways (Gonzalez et al., 2006).

2. Adiponectin. Adiponectin is an adipose tissue-derived peptide, which in humans exists in a full-length and a globular form (230- and 147-amino acid residues, respectively). The former represents almost all adiponectin in plasma, the latter being generated by the proteolytic cleavage of the C-terminal region of the full-length form. Two adiponectin Rs have been recently identified: adipoR_1, the receptor for globular adiponectin, and adipoR_2, the receptor for full-length adiponectin. Adiponectin is a regulator of energy homeostasis and plays a role in the obesity-induced insulin resistance and related complications (Wolf, 2003; Kadowaki and Yamauchi, 2005).

Adiponectin has been supposed to play a role in vascular remodeling: it was down-regulated in obesity-linked diseases, such as coronary artery disease in type 2 diabetes (Kumada et al., 2003), and its overexpression exerted an anti-inflammatory effect on the vasculature and reduced atherosclerotic lesions in a mouse model

(Arita et al., 2002; Okamoto et al., 2002). Of interest, the anti-inflammatory and antiatherogenic action of adiponectin may be linked to its inhibitory effect on interleukin-8 expression in and release from ECs, an effect mediated by the inhibition of the tumor necrosis factor-α-induced nuclear factor-κB-dependent pathway through the activation of the cAMP-protein kinase (PK) A and PI3K-Akt cascades (Kobashi et al., 2005).

Adiponectin has been reported to activate adenosine monophosphate kinase in ECs, leading to enhanced in vivo angiogenesis in murine Matrigel plug and rabbit cornea assays and inhibition of caspase 3-mediated apoptosis in HUVECs cultured in vitro (Kobayashi et al., 2004; Ouchi et al., 2004). Adiponectin has also been found to play a role in the ischemia-induced angiogenesis: in the ischemic limb, as a result of the excision of the femoral artery and vein, the blood flow returned to 80% of that of the nonischemic limb at day 28 after surgery in wild-type mice, whereas the flow recovery was impaired in adiponectin KO animals. This proangiogenic action seemed to be mediated by the stimulation of AMP kinase-dependent signaling within the skeletal muscle of ischemic limb (Shibata et al., 2004).

Surprisingly, opposite findings have been obtained by Bråkenhielm et al. (2004). They showed that adiponectin inhibited EC migration and proliferation in vitro and neoangiogenesis in vivo in the CAM and cornea assays, as well as decreased angiogenesis and induced apoptosis in tumors, obtained by implanting T241 fibrosarcoma cells in mice. However, a cross-talk between adiponectin and FGF-2 has been suggested to occur in ECs of hepatocellular carcinoma, supporting tumor angiogenesis and growth (Adachi et al., 2006). In fact, FGF-2 was found to induce the expression of the proangiogenic cell adhesion molecule T-cadherin (Ivanov et al., 2004) in tumor but not in normal liver ECs, and T-cadherin is a R for adiponectin (Hug et al., 2004).

3. Resistin. Resistin, an adipose tissue-produced peptide (92 amino acid residues in humans), belongs to a family of proteins found in the inflammatory zone, called FIZZ. Resistin links obesity to diabetes, and it is considered a predictive factor for coronary atherosclerosis (Steppan et al., 2001; Bełtowski, 2003; Verma et al., 2003; Reilly et al., 2005).

Resistin has been reported to promote VSMC proliferation via the ERK1/2 and PI3K pathways (Calabro et al., 2004) and to stimulate in vitro angiogenesis (Mu et al., 2006). Resistin stimulated proliferation, migration, and capillary-like tube formation in cultured human aortic ECs (HAECs). It also up-regulated VEGF-R1, VEGF-R2, and MMP-1 and MMP-2 expression, as mRNA and protein in HAECs, as well as elicited a transient activation of ERK1/2 and MAPK p38. These cascades seemed to mediate the proangiogenic effect of adiponectin, as their selective inhibitors suppressed adiponectin-induced HAEC proliferation and migration (Mu et al., 2006).

G. Neuropeptide-Y

Neuropeptide-Y (NPY) is a 36-amino acid peptide that belongs to a family of highly conserved peptides, including peptide-YY and pancreatic polypeptide. NPY is widely distributed in the nervous system, where it is thought to act as a neurotransmitter, being mainly released from sympathetic nerve fibers. NPY, along other members of its family, binds GPRs, referred to as Y-Rs. Six subtypes of Y-Rs have been identified (from Y_1-R to Y_6-R), and NPY preferentially binds Y_1-R, Y_2-R, and Y_5-R subtypes. The physiological functions of NPY include the regulation of blood pressure, appetite, and feeding, the modulation of learning and memory, and the control of brain-endocrine axes (Balasubramaniam, 1997; Michel et al., 1998; Cerdá-Reverter and Larhammar, 2000; Spinazzi et al., 2005).

NPY has been reported to stimulate proliferation of rat aorta VSMCs, acting via Y_1-Rs and Y_2-Rs (Shigeri and Fujimoto, 1993; Zukowska-Grojec et al., 1993). The effect of NPY was bimodal, showing two peaks of proliferation at 10^{-12} and $10^{-8}/10^{-7}$ M concentrations. The first peak was mimicked by Y_2-R agonists and suppressed by Y_2-R antagonists, and the second peak was mimicked by Y_1-R agonists and partially blocked by Y_1-R antagonists (Zukowska-Grojec et al., 1998a). NPY has also been shown to stimulate ERK1/2 activity in rat coronary ECs in primary culture (Zukowska-Grojec et al., 1998a). Further studies revealed that NPY at low concentrations ($10^{-12}/10^{-11}$ M) promoted in vitro angiogenesis by enhancing adhesion, migration, proliferation, and capillary-like tube formation by HUVECs. It also stimulated in vivo angiogenesis in a murine Matrigel plug assay, its potency being similar to that of FGF-2. HUVECs expressed both Y_1-R and Y_2-R mRNAs, but the in vitro proangiogenic action of NPY seemed to be mainly mediated by the Y_2-R subtype, because it was mimicked and suppressed by Y_2-R agonists and antagonists, respectively (Zukowska-Grojec et al., 1998b). The main involvement of Y_2-Rs has been confirmed by the demonstration that the in vivo and in vitro angiogenic effect of NPY was impaired in Y_2-R KO mice (Lee et al., 2003). Moreover, a selective Y2-R agonist enhanced collateral-dependent blood flow in a rat model of peripheral artery disease (bilateral occlusion of the femoral artery distal to the inguinal ligament) (Cruze et al., 2007). More recent findings showed that NPY promoted in vitro angiogenic activity of HUVECs through not only Y_1-Rs and Y_2-Rs but also Y_5-Rs. The effect required the participation of all three R subtypes, the Y_5-R probably acting as an enhancer (Movafagh et al., 2006). It has also been shown that NPY-induced vessel growth decreased markedly with age in mice (from 2 to 18 months of age), the impairment being associated with the down-regulation of Y_2-R mRNA (Kitlinska et al., 2002).

HUVECs expressed not only Y_1-Rs, Y_2-Rs, and Y_5-Rs but also NPY, as mRNA and protein, and the NPY-

converting enzyme dipeptidylpeptidase IV (DPPIV). DPPIV terminated the Y_1-R binding activity of NPY by cleaving the Tyr^1–Pro^2 bond and transforming it to NPY(3–36), an agonist of Y_2-Rs (Zukowska-Grojec et al., 1998b). DPPIV expression decreased in mouse ECs with aging (Kitlinska et al., 2002). On the basis of these results, these investigators proposed that "endothelium is not only the site of action of NPY, but also the site of an autocrine NPY system, which, together with sympathetic nerve" terminals, may play a pivotal role in angiogenesis regulation.

The proliferogenic effect of NPY on ECs and VSMCs might be implicated in the development and progression of postangioplasty restenosis and atherosclerosis (Kuo and Zukowska, 2007). A common polymorphism of the prepro-NPY gene with Leu^7 to Pro^7 substitution has been reported to highly correlate with elevated total and low-density lipoprotein cholesterol levels and increased carotic artery intima-media thickening (Niskanen et al., 2000; Karvonen et al., 2001). This contention has been experimentally supported by findings showing that NPY accelerated postangioplasty occlusion of rat carotid artery (Li et al., 2003b). Y_1-R, Y_2-R, and to a lesser extent Y_5-R mRNAs were up-regulated within 24 h in operated artery compared with the contralateral vessel and DPPIV mRNA was down-regulated, thereby enhancing the Y_1-R-mediated VSMC proliferative effect of NPY. The increased expression of Y_1-Rs and Y_5-Rs but not Y_2-Rs persisted until 14 days after the operation. The local application of the NPY dose dependently increased neointima formation and media thickening. The treatment with Y_1-R and Y_5-R antagonists prevented carotic occlusion, suggesting a new possible therapeutic strategy for avoiding postangioplastic restenosis.

Of great interest, NPY, via both Y_2-Rs and Y_5-Rs, has been reported to play a major role in promoting the growth and neovascularization of neuroblastomas (Kitlinska, 2007). Aggressive neuroblastomas highly expressed NPY and its Rs, and high plasma levels of NPY were frequently correlated with a poor clinical outcome.

H. Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are members of a family of structurally related peptides that includes secretin, glucagon, glucagon-like peptides, growth hormone-releasing hormone, gastric inhibitory peptide, parathyroid hormone, and exendins. VIP is a highly basic 28-amino acid C-amidated peptide, whereas PACAP is a basic 38-amino acid C-amidated peptide. They display a high degree of homology in their N-terminal sequence, and act via GPRs, named PACAP/VIP Rs. Three subtypes of PACAP/VIP Rs have been identified, whose names and binding potency are as follows: 1) PAC_1-R, PACAP $\ggg$ VIP; 2) $VPAC_1$-R, VIP $\geq$ PACAP; and 3) $VPAC_2$-R, VIP = PACAP. VIP and PACAP and their Rs are widely distributed in the body and exert multiple actions, including the modulation of immune and inflammatory responses (Harmar et al., 1998; Vaudry et al., 2000; Conconi et al., 2006).

VIP and PACAP(1–27) were found to increase VEGF expression in lung cancer cells, through a mechanism involving the activation of the PKA- but not the ERK1/2-dependent cascade (Casibang et al., 2001; Moody et al., 2002). Similarly, VIP and PACAP(1–27) have been shown to raise VEGF mRNA expression within 60 min in the androgen-responsive prostate carcinoma-derived cell line LNCaP, and the effect was due to an increase at the transcriptional level, because VEGF mRNA stability was decreased. The effect was mediated by the $VPAC_1$-R, because the agonists of this R but not of $VPAC_2$-R, mimicked VIP and PACAP(1–27) action. Moreover, it occurred via the activation of PKA-, PI3K-, and ERK1/2-dependent pathways, because it was suppressed by H89, wortmanin, and PD98059 exposure (Collado et al., 2004, 2005). Hypoxia up-regulated VIP expression in LNCaP cells, and VIP raised the expression of PAC_1-Rs and $VPAC_1$-Rs and decreased that of $VPAC_2$-Rs. VIP did not affect the expression of HIF-1α, but increased its translocation from the cytosolic compartment to the nucleus (Collado et al., 2006).

Using a rat sponge model, Hu et al. (1996) showed that daily (for up 14 days) injections of high doses of VIP (1 nmol) evoked intense neovascularization, as assessed by ^{133}Xe clearance technique (for blood flow estimation) and morphometry. Lower doses of VIP (10 pmol) were ineffective but when administered with a subthreshold dose of interleukin-1α evoked an angiogenic response similar to that observed with the higher doses of VIP.

I. Substance P

Substance P is a C-amidated decapeptide that belongs, along with neurokinin-A, neurokinin-B, and neuropeptide-K, to the tachykinin family. Tachykinins act via three GPR subtypes, referred to as NK_1-R, NK_2-R, and NK_3-R. Substance P preferentially binds the NK_1-R, which activates the phospholipase C (PLC)/PKC-dependent cascade (Nussdorfer and Malendowicz, 1998; Harrison and Geppetti, 2001). Substance P is released from the peripheral terminals of sensory nerve fibers, and evidence has been provided that it may mediate acute inflammatory responses by inducing, via NK_1-Rs, vascular permeability, plasma extravasation, and edema (Richardson and Vasko, 2002).

Substance P and a selective NK_1-R agonist were found to enhance capillary growth in vivo in a rabbit cornea assay, and to stimulate proliferation and migration in vitro of different EC types, including HUVECs. NK_2-R and NK_3-R agonists were ineffective, whereas substance P antagonists blocked the response (Ziche et al., 1990). Fan et al. (1993) confirmed the in vivo proangiogenic action of substance P in a rat sponge assay and showed that the effect was suppressed by a selective NK_1-R

antagonist. The angiogenic effects of substance P were prevented by N^G-nitro-L-arginine, suggesting the involvement of NOS/NO-dependent signaling (Ziche et al., 1994).

In vivo experiments showed that endogenous substance P could be implicated in the neoangiogenesis connected with neurogenic inflammation (Seegers et al., 2003). Substance P and capsaicin, which induces substance P release from sensory nerve terminals, were injected in the rat knee and animals were sacrificed 24 h later. Both chemicals increased vascular density in synovia and EC proliferation. The coinjection of a selective NK_1-R antagonist attenuated the effect of substance P and capsaicin, whereas that of a NK_2-R antagonist was ineffective.

J. Summary

The main features of nonclassic proangiogenic factors are summarized in Table 2. The bulk of evidence indicates that a major role is played by EPO, Ang-II, ET-1, AM, and NPY, which all display intense in vitro and in vivo proangiogenic activity and promote tumor growth and vascularization. The proangiogenic activity of PAMP, urotensin-II, adipokines, VIP/PACAP, and substance P seems to be of minor relevance, but this may depend on the fact that it has been far less investigated. ANG-II, ET-1, AM, PAMP, resistin, and VIP up-regulate the VEGF/VEGF-R system, whereas leptin and adiponectin exhibit positive interactions with angiopoietin-2 and FGF-2, respectively. The possible interactions of EPO with the classic angiogenic factors have not been studied, but findings suggest that it enhances ET-1 expression in and release from ECs. Selective R antagonists are available for many nonclassic proangiogenic factors (e.g., ANG-II, ET, AM, PAMP, urotensin-II, NPY, and VIP), which could make them possible targets of antineoplastic therapeutic strategies.

IV. Nonclassic Endogenous Inhibitors of Angiogenesis

A. Somatostatin

Somatostatin is a regulatory peptide, which had been initially described as a hypothalamic inhibiting releasing hormone of the pituitary growth hormone. Two biologically active forms of somatostatin, which derive from the C-terminal portion of prosomatostatin, are recognized: somatostatins 14 and 28. Somatostatin acts via five subtypes of GPRs, referred to as sst1-R, sst2-R, sst3-R, sst4-R, and sst5-R. Somatostatin and its Rs are widely distributed in tissues and organs, where they exert multiple actions, including inhibition of cell growth and angiogenesis, especially in neoplastic tissues (Patel, 1999; Csaba and Dournaud, 2001; Garcia de la Torre et al., 2002; Olias et al., 2004).

Investigations carried out with PCR and immunocytochemistry techniques showed that human blood vessels expressed high levels of sst1-Rs and low levels of sst2-Rs and sst4-Rs (Curtis et al., 2000). They also showed that HUVECs expressed sst1-R and sst4-R mRNAs and also sst2-R mRNA after repeated passages. In partial contrast with these findings, other studies reported that sst2-Rs were expressed in the proliferating angiogenic sprouts of human vascular endothelium, but not in quiescent ECs. They were present at high density in proliferating blood vessels of tumors (Watson et al., 2001) and could represent an elective target in antineoplastic therapy (Gulec et al., 2001). There is proof that the sst2-R-mediated antiangiogenic action of somatostatin could either be direct, involving the inhibition of EC proliferation (Danesi et al., 1997), or indirect, being mediated by the suppression of production of growth factors, including VEGF (Cascinu et al., 2001; Mentlein et al., 2001).

Further findings indicated the prevalent involvement of sst3-Rs in the antitumoral effect of somatostatin (Florio et al., 2003). Somatostatin exerted an antiproliferative action on bovine aortic ECs (BAECs) and the EC line EAhy926 (originated by fusion of HUVECs with A549 cell line), and the effect was abolished by a selective sst3-R antagonist. However, the inhibition of proliferation probably occurred via a synergism with other R subtypes, because BAECs, expressing sst1-Rs, sst3-Rs, and sst5-Rs, were more sensitive than EAhy926 cells, expressing only sst3-Rs. Human embryo kidney-derived cells were implanted into nude mice, and the angiogenesis and growth of the tumor was impaired by the peritumor injection of somatostatin, which acted via sst3-Rs negatively coupled to ERK1/2 and NOS.

B. Ghrelin

Ghrelin is a 28-amino acid peptide, that acts as an endogenous ligand of the growth hormone secretagogue R (GHS-R). Two subtypes of GHS-Rs have been identified: the fully functional GHS-R1a and the biologically inactive GHS-R1b. Ghrelin and its Rs are widely expressed in tissues and organs. Although the main biological effects of ghrelin are thought to be the stimulation of pituitary growth hormone release and food intake, the peptide has be found to exert many other actions, including a protective effect on cardiovascular system (Kojima et al., 2001; van der Lely et al., 2004; Davenport et al., 2005; Kojima and Kangawa, 2005; Camiña, 2006; Cao et al., 2006).

Ghrelin and GHS-Rs have been shown to be expressed in HUVECs (Conconi et al., 2004). Earlier investigations reported that ghrelin inhibited doxorubicin-induced apoptosis of porcine aortic ECs, via the ERK1/2 and PI3K/Akt signaling cascades (Baldanzi et al., 2002). However, further studies did not confirm this observation: ghrelin did not affect the basal apoptotic rate of HUVECs cultured in normal growth medium (Belloni et al., 2004) or raise it in cultured rat brain microvascular ECs and HUVECs. Moreover, ghrelin suppressed the

TABLE 2
Main features of nonclassic proangiogenic and antiangiogenic factors

Factor	Receptors	Signaling Pathways[a]	Angiogenic Activity[b]								Tumor Growth and Vascularization	Interaction with Other Factors[c]
			In Vitro Assays (ECs)				In Vivo Assay (New Vessel Formation)					
			Differentiation	Proliferation	Migration	Differentiation (Capillary Tube Formation)	CAM	Rabbit Cornea	Sponge Model (Matrigel Plug)	Experimental Ischemia		
EPO	EPO-RA, EPO-RB	+ PI3K/Akt + JAK/STAT		S	S	S	S	=	=	S	S	↑ ET-1
ANG-II	AT_1-R, AT_2-R	+ NOS/NO ?	=S	SI	S=	S=	S I	SI	SI	S [I], [S][d]	S (?)=	↑ VEGF
ET	ET_A-R, ET_B-R	+ BK_{ca} + PGE_2/COX	I	S	S	S	S	=	S	=	S	↑ VEGF
AM	AM_1-R, AM_2-R	+ AC + Akt + MAPK	I	S [N]	S	S	S	=	=	S	S	↑ VEGF (?)
PAMP	?	?	=	=	=	S	=	=	S	=	S	↑ VEGF/FGF-2/↑ PDGF (?)
Urotensin-II	UT-R	?	=	N	=	S	S	=	=	=	S (?)	?
Leptin	Ob-Ra, Ob-Rb	+ MAPK + STAT-3	=	S	=	=	S	S	=	=	=	↑ angiopoietin-2 ↑ VEGF (?)
Adiponectin	$AdipoR_1$, $AdipoR_2$	+ AMP kinase	I	[I]	[I]	=	[I]	S[I]	S	S	S (?)	↑ FGF-2 (?)
Resistin	?	+ PI3K + MAPK	=	S		S	=	=	=	=	=	↑ VEGF
NPY	Y_1-R/Y_2-R (Y_5-R)	+ MAPK	=	S	S	S	=	=	S		S	?
VIP (PACAP)	$VPAC_1$-R	+ PKA + PI3K + MAPK	=	=	=	=	=	=	S	=	=	↑ VEGF
Substance-P	NK_1-R	+ PLC/PKC + NOS/NO	=	S	S	=	=	S	S	=	=	?
Somatostatin	sst-2R, sst-3R (sst-1R/sst-5R)	− MAPK − NOS/NO	=	I	=	=	=	=	=	=	I	↓ VEGF
Ghrelin	GHS-R1a	− MAPK − PI3K/Akt	S	I	=	I	I	=	=	=	=	?
Natriuretic peptides	A-R/C-R (B-R)	− MAPK − JNK	=	I	I	I	=	=	=	=	=	↓ VEGF

BK_{ca}, Ca^{2+}-activated large conductance potassium channel; COX, cyclooxygenase; AC, adenylate cyclase; JNK, cJun N-terminal kinase.
[a] >+, stimulation; − , inhibition.
[b] I, inhibition; N, no effect; S, stimulation; =, no findings available.
[c] ↑, up-regulation; ↓, down-regulation.
[d] Brackets indicate uncommon findings.

antiapoptotic effect of FGF-2 (Baiguera et al., 2004; Conconi et al., 2004). Ghrelin was found to lower the proliferative activity of rat ECs and HUVECs and to exert a marked inhibitory action on capillary-like tube formation by these types of ECs cultured on Matrigel. It also counteracted the angiogenic effect of FGF-2 in the CAM assay (Baiguera et al., 2004; Conconi et al., 2004). The proliferogenic effect of ghrelin on ECs was annulled by the GHS-R antagonist D-Lys^3-growth hormone releasing peptide-6, and the peptide did not increase lactate dehydrogenase release from cultured ECs, indicating that the antiangiogenic action of ghrelin was mediated by the GHS-R and did not ensue from an aspecific toxic action. Baiguera et al. (2004) demonstrated that FGF-2 enhanced tyrosine kinase and ERK1/2 activities in rat brain ECs. Ghrelin significantly decreased tyrosine kinase and ERK1/2 activities, and effectively counteracted the effect of FGF-2, thereby strongly suggesting that the mechanism underlying its antiangiogenic action involved the inhibition of these cascades.

C. Natriuretic Peptides

Natriuretic peptides are a family of small proteins that modulate salt and water balance and vascular biology. This family includes atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), which in humans are 28-, 32-, and 53-amino acid residue peptides, respectively. ANP and BNP are predominantly synthesized in the heart, whereas CNP is produced by ECs (Levin et al., 1998). Natriuretic peptides act via at least three subtypes of receptors: the A-R and B-R subtypes are positively coupled to guanylate cyclase, thereby stimulating cGMP production and subsequently activating PKG and modulating potassium channels; the C-R subtype, also named clearance receptor, is not coupled to or inhibits guanylate cyclase. Their binding potency is as follows: A-R subtype, ANP = BNP $\gg$ CNP; B-R subtype, CNP selective; and C-R subtype, ANP = BNP = CNP (Silberbach and Roberts, 2001).

Evidence has been provided that natriuretic peptides inhibited human EC proliferation (Itoh et al., 1992). Further studies showed that ANP and CNP impaired basal and ET- or hypoxia-stimulated VEGF production in human ECs and VSMCs cultured in vitro, as well as their growth (Pedram et al., 1997a,b). These investigators subsequently reported that natriuretic peptides, acting via either A-Rs and B-Rs or C-Rs, blocked VEGF signaling in primary cultures of BAECs (i.e., ERK1/2-dependent c-Jun N-terminal kinase and to a lesser degree MAPK p38), thereby suppressing VEGF-induced EC proliferation, migration, and capillary-like tube formation on Matrigel (Pedram et al., 2001). Hence, natriuretic peptides can be considered one of the first described endogenous inhibitors of VEGF-modulated angiogenesis.

Investigations have also demonstrated that ANP mRNA was up-regulated in rat ventricular myocardium in the early phases of ischemia, this being preceded by an increase in HIF-1α expression. Accordingly, hypoxia or HIF-1α activation was found to induce ANP gene expression in rat neonatal cardiomyocytes and the rat ventricular myoblast cell line H9c2 (Chun et al., 2003). The up-regulation of ANP and BNP expression in ischemic myocardium have been confirmed in humans, but this was not associated with enhanced VEGF gene expression (Rück et al., 2004). Nevertheless, the overexpression of anti-angiogenic natriuretic peptides could be one of the causes of the relative inefficiency of spontaneous neoangiogenesis in some patients with chronic heart ischemia (angina pectoris), as well as of their poor response to clinical trials with proangiogenic agents.

D. Summary

The main features of nonclassic antiangiogenic factors are shown in Table 2. Available findings identify somatostatin as a major suppressive factor of tumor growth and vascularization, but experimental studies on its in vitro and in vivo antiangiogenic action are surprisingly almost completely lacking. The antiangiogenic activity of ghrelin has been well demonstrated both in vitro and in vivo assays, but investigations on its possible antineoplastic action have not yet been carried out. The same is true for natriuretic peptides, whose antiangiogenic action has been studied only in vitro. Of interest, somatostatin and perhaps natriuretic peptides have been reported to down-regulate the VEGF system, thereby suggesting an indirect mechanism of action.

V. Conclusions and Perspectives

The preceding sections of this review have shown that in recent years evidence has accumulated, indicating that several endogenous peptides are expressed along with their Rs in the blood vessels and may regulate angiogenesis under both physiological and pathological conditions. Briefly, EPO, ANG-II, ETs, AM, PAMP, urotensin-II, leptin, adiponectin, resistin, NPY, VIP, PACAP, and substance P were found to stimulate angiogenesis, whereas somatostatin, ghrelin, and natriuretic peptides were found to inhibit it. Findings also indicated that the proangiogenic action of many of these peptides could be at least partly mediated by the stimulation of VEGF and FGF-2 systems and the antiangiogenic action of somatostatin and natriuretic peptides by the suppression of VEGF system (Table 2).

It is well established that the angiogenic phenotype results from the imbalance between positive and negative regulator factors, so that the contribution of each classic and/or nonclassic angiogenic factor may play a different role in the definition of the angiogenic phenotype. Increased production of angiogenic stimuli and/or reduced production of classic and/or nonclassic angiogenic

inhibitors may lead to abnormal neovascularization, such as that occurring in cancer, chronic inflammatory diseases, diabetic retinopathy, macular degeneration, and cardiovascular disorders.

The development of a clinical trial requires the identification and characterization of the physiological targets involved in angio-stimulatory and angio-inhibitory activities. Much research effort has been concentrated on the role of angiogenesis in cancer, and inhibition of angiogenesis is a major area of therapeutic development for the treatment of this disease. New pathophysiological concepts generated in the past few decades have given rise to the development of a large variety of new drugs to interfere with angiogenesis. The target of antiangiogenic therapy is the vascular EC rather than the tumor cell. In fact, whereas conventional chemotherapy, radiotherapy, and immunotherapy are directed against tumor cells, antiangiogenic therapy is aimed at the tumor vasculature and will either cause tumor regression or keep the tumor in a state of dormancy. The predominant mode of action of antiangiogenic agents clinically tested to date in cancer trials has been cytostatic. Many of the cytostatic agents under clinical investigation have been shown to have reversibility of their activity upon removal of the agent. This implies that the benefit of administration may be limited to the time during which a physiologically concentration of the agent is available. Preclinical and clinical studies have made it increasingly clear that strategies that target tumor blood vessel networks ultimately will be most effective if used in conjunction with, or adjuvant to, conventional anticancer therapies.

Detailed knowledge of the mechanism of action and expression as well as the interactions of the new nonclassic endogenous regulators of angiogenesis with their Rs will provide new insights that are essential for the future development of chemical compounds that can modulate the activity of these new nonclassic endogenous regulators and may have potential for antitumor activity. In fact, tumors and other angiogenic pathologies exploit redundant mechanisms to induce angiogenesis, and neutralization of multiple factors, including both classic and nonclassic regulators, may be required to suppress tumor growth. In this context, a paradigmatic example is represented by the characterization of ET-1 and AM and their Rs as new vascular markers in tumors, providing a better understanding of novel targeting approaches for cancer treatment. The recognition by clinicians of the angiogenesis-modulatory properties of common drugs is important in the development and future application of angiogenesis-inhibitory therapies. The linkage between the laboratory and the clinic, which brought this important new development to the patient, must be maintained to further our understanding of the role of angiogenesis in normal physiology and disease, to develop and validate intermediate and surrogate markers of benefit, and to advance to the optimal use of antiangiogenic molecules.

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