Current perspectives

The molecular mechanisms of angiogenesis: a new approach to cardiovascular diseases

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Although the role of new blood vessel formation in cancer and its development have been well documented, the strategy to manipulate angiogenesis in order to restore blood flow in the ischemic myocardium, a novel form of therapy currently undergoing clinical trials, has received less attention. Recent advances in our understanding of the stimuli and of the control mechanisms regulating the development of new blood vessels in coronary heart disease have led to an improved picture of the compensatory healing process that accompanies myocardial ischemia and infarction. However, we have to remind that, together with life- and tissue-saving effects, the angiogenetic process might alter the natural course of the consequences and organ manifestation of arterial diseases as in the atherosclerotic plaque.

The purpose of this review is to provide an overview on the molecular mechanisms involved in the angiogenetic process. Angiogenesis during ontogenesis, neoangiogenesis (adult new vessel formation), arteriogenesis and the related regulators will be analyzed. Moreover, the role of neoangiogenesis in plaque development and instability will be discussed. Due to the introductory nature of this review and the large number of studies on neovascularization in ischemic limbs this topic has been omitted.

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Warm blooded species critically depend on the high rate of oxygen delivery to all tissues for their metabolic needs. Oxygen uptake in mammals is 20 to 50 times higher than in ectotherm animals[1]. Since oxygen extraction in basal conditions is close to the maximum in most tissues, oxygen uptake during various life activities mainly depends on a very efficient and well regulated vascular system. In mammalian hearts, with elevated aerobic requirements, capillary density is quite constant and ranges from 2.7 to $5 \times 10^5/cm^2$, with an intercapillary distance of 11÷18 μm and a capillary to muscle fiber ratio very close to 1:1[2].

On this basis, two considerations may arise: a) control of the development of the vascular system needs very efficient genetic and molecular mechanisms to achieve such a complex network, and b) any pathological process limiting the flow in an artery may lead to ischemia and eventually to cell death in the vascular territory perfused by the artery itself.

The understanding of the molecular mechanisms underlying the formation of the vascular tree started quite recently in 1970s with the pioneering work of Judah Folkman[3] on neoplastic angiogenesis. However, the existence of a rich vascular neoformation in neoplasia was already recognized at the beginning of the century[4] and the presence of intercoronary connections, known as collateral arteries, both in normal and in diseased hearts, had already been described in the 17th century.

To date our knowledge of the mechanisms of vascular tree formation has expanded tremendously with the discovery of several factors and of the mechanisms leading to vessel and capillary formation and stabilization. New vessel formation is not limited to fetal life but is a leading process in some physiological and many pathological conditions, such as ovarian cycle, wound healing, tumor growth, rheumatoid arthritis, retinal neovascularization, and cardiovascular diseases.

The terms commonly used to define the processes of vascular development and growth are listed in table I[3,5-10].

Vessel formation and growth

Vasculogenesis. As suggested by Leonardo da Vinci, the sprouting of a vessel represents the principal mechanism of blood ves-

Table I. Terminology for vessel formation and growth.

Vasculogenesis and early angiogenesis[5,6]	Development of primitive vascular system, sprouting and non-sprouting angiogenesis
Adult angiogenesis[3,7]	Sprouting of new capillaries from pre-existing vessels
Arteriogenesis (recapitulated vasculogenesis)[8-10]	Growth of arteries from preexisting arterioles (collateral artery growth)

sel formation during ontogenesis. However, recently developed techniques that permit alteration of genomic sequences and manipulation of developing embryonic tissues, have provided an important insight into molecular and genetic elements that regulate vascular development. The vascular system is formed before the heart starts beating rather than sprouting from the heart as speculated by Leonardo. On the contrary, vessel formation in the adult occurs from preexisting vessels in response to tissue demand. The *de novo* organization of endothelial cells into vessels (formation of the early vascular plexus) in the absence of any preexisting vascular system is referred to as vasculogenesis and only occurs in the early embryo. The continued expansion of the vascular tree as the result of endothelial cell sprouting from existing vessels denoted as angiogenesis, occurs in avascular regions of the embryo (both in the yolk sac and in the embryo) and is repeated many times in the mature animal most commonly during wound healing and tumor metastasis (adult angiogenesis)[5,7]. Therefore, during ontogenesis the formation of the early vascular plexus can be divided into vasculogenesis (formation of the primary vascular plexus) and sprouting or non-sprouting angiogenesis (involved in vascularization of organs or tissue such as the heart, lung and yolk sac). How the pattern of the vascular tree is established or which factors govern the site of sprouting or the route taken by migrating endothelial cells during angiogenic expansion is still unclear.

Genetic programs regulating early vascular development. The best insight into molecular events required to initiate and maintain vascular development has come from a detailed analysis of the mouse embryo in which the genes for specific growth factors and their receptors have been inactivated. Such experiments show that initiation of vascular development required both basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF)[6]. The details of these growth factors and their receptors will be discussed below. However, it must be pointed out that the decision for a vessel to become a vein or an artery appears to be under the control of yet another growth factor belonging to the VEGF family. This factor also binds to the VEGF receptor 3[11] which is expressed later in the development only on the endothelial cells that will become veins or lymphatic vessels.

Once the vascular plexus is formed (vasculogenesis), expansion of the vascular tree continues by endocardial and ventricular development and formation of the vascular wall (by sprouting and non-sprouting angiogenesis). This process is controlled by two members of the *tie* family named *tie-1* (tyrosine kinase with immunoglobulin and epidermal growth factor homology domain) and *tie-2* or *tek* (tunica interna endothelial cell kinase)[12]. Two ligands, termed angiopoietin-1[13] and angiopoietin-2[14] are specific to *tie-2* and are synthesized by cells surrounding the developing vessels. Targeting mutation of the genes for either *tie-2* or angiopoietin-1 results in embryos with abnormal hearts and vessels with poorly formed walls[15]. This has led to the suggestion that angiopoietin-1 acts to stimulate the production of growth factors that, in turn, stimulate the differentiation of surrounding mesenchyme into pericytes or smooth muscle cells required for vessel wall formation[16]. This is consistent with the phenotype of a *tie-2* in humans that leads to smooth muscle cell deficiencies around small vessels and microaneurysms[17]. Thus, these observations indicate that both *tie-1* and *tie-2* are required for continued vascular branching and vessel remodeling subsequent to the action of VEGF.

Although genetic manipulation has provided important insight there is much that we do not know about vascular development. Random genetic mutation introduced into the zebrafish has also generated many surprising and fascinating cardiovascular anomalies. Indeed, zebrafish can be induced to develop with hearts that do not contain an endocardium although the remainder of the vascular system appears to be functional[18]. It is likely therefore that a combination of genetic and developmental biology will identify new genes and suggest new paradigms thus extending our knowledge on cardiovascular diseases.

Origin of the vascular endothelium. Molecular events involved in endothelial cell differentiation to the early mesoderm remain uncertain; however the early vascular plexus originates from the mesoderm by the differentiation of angioblasts. Factors belonging to the FGF family, act on the mesoderm to induce the formation of a bipotential precursor known as *hemoangioblast* of a two-cell type: the *angioblast* and the *hemopoietic cell*[5]. Recently the angioblast has also been identified in adults[19]. Within the embryo the first angioblasts arise from the lateral mesodermal plate and cardiac crescent[5]; some cells migrate into the forming brain, whereas others assemble into the endocardium of the early heart tube. Other angioblasts form a plexus of endothelial cells at the base of the primitive heart tube that assemble in the vitelline vessels, allowing blood cells from the yolk sac to circulate within the body of the embryo[5].

True sprouting and non-sprouting angiogenesis. After the primary vascular plexus is formed (Fig. 1), more endothelial cells are generated which can form new capillaries by sprouting or by splitting from their vessels of origin in a process termed angiogenesis (Fig. 1). There are at least two different types of angiogenesis: true sprouting of capillaries from preexisting vessels, and non-sprouting angiogenesis or intussusception that has been revised by Risau[5]. These processes are highly regulated and the complex sequence of events has been established in other organs[16] and is assumed to be similar during coronary vascular growth. Briefly, proteolytic degradation and dissociation of the extracellular matrix architecture initially occur as a result of elaboration of proteases by endothelial cells. This process is followed by chemotactic migration and proliferation of endothelial cells. Subsequently, organization of adjacent endothelial cells leads to the formation of a lumen followed by pericyte migration, determined by basement membrane and functional maturation of the endothelium[16].

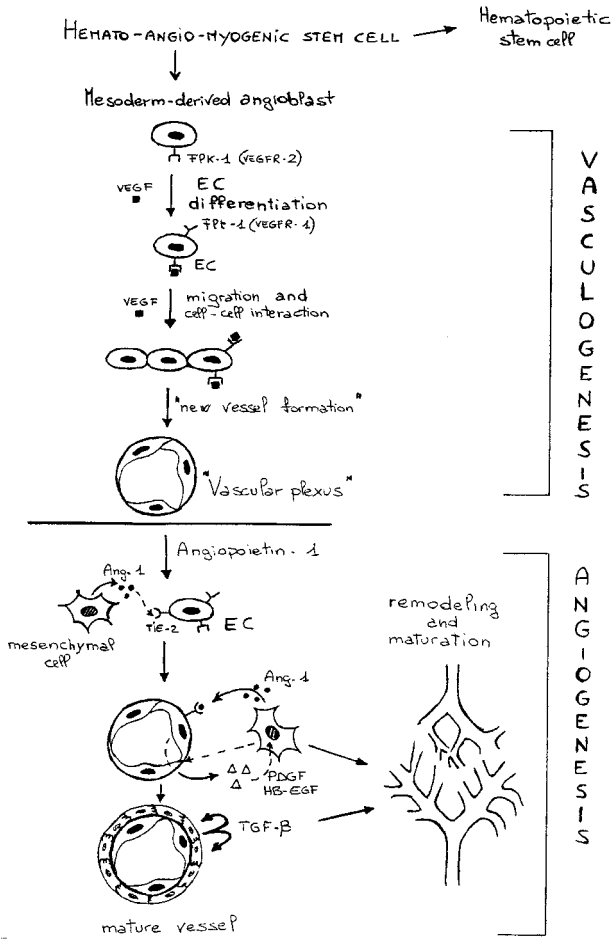

Figure 1. *The formation of the primitive blood vessels during ontogenesis. Vasculogenesis refers to the formation of the early vascular plexus. The bipotential precursor, the hemoangioblast, and the differentiated vascular endothelial cell precursor, the angioblast, as well as the growth factors involved in the process are depicted. Angiogenesis refers to the expansion, both in the yolk sac and embryo, of the primary vascular plexus. The expression of additional growth factors is required for maturation and remodeling during later organogenesis. Ang = angiopoietin; EC = endothelial cells; EGF = endothelial growth factor; PDGF = platelet-derived growth factor; TGF = transforming growth factor; VEGF = vascular endothelial growth factor; VEGFR = vascular endothelial growth factor receptor.*

Vessel maturation. Further maturation of blood vessels, which mature or regress along with the tissue or organ they supply, depends on both intra and extraluminal factors. Although initially only dependent on the oxygen level, the vascular system is later shaped by forces generated by the circulation. Shear stress affects endothelial cells, inducing modification of cell-cell as well as cell-extracellular-matrix junctions and up regulating growth factors such as platelet-derived growth factor (PDGF)-BB and transforming growth factor (TGF)-β[20]. Simultaneously, the vascular wall matures and the *tie-2*/angiopoietin system appears to govern maturation and stabilization of blood vessels. "Stabilization" of blood vessels is a stage when remodeling ceases, new branches do not develop and luminal size is constant apart from physiological vasodilation and vasoconstriction; pericytes and smooth muscle cells differentiate, endothelial cells stop to proliferate[20] and elastogenesis begins in elastic arteries.

Angiogenesis. Adult endothelial cells are quiescent, however they can form new vessels via neoangiogenesis (Fig. 2). In pathological conditions and in wound healing, angiogenesis is a complex process accompanied by, and possibly requiring, inflammation. Although the sequence of events resembles that of embryonic angiogenesis, additional events in established quiescent vessels are probably needed. Schematically angiogenesis can be divided into the following five discrete steps: 1) release of angiogenic factors; 2) release of proteolytic enzymes; 3) endothelial cell migration and capillary morphogenesis; 4) endothelial cell proliferation; and 5) microvessel differentiation. Hypoxia represents the major stimulus.

Release of angiogenic factors. A large number of angiogenic factors has been characterized and detected in sites where angiogenesis takes place. These angiogenic factors can act directly by stimulating endothelial cells and/or indirectly via accessory cells like macrophages and/or stromal cells. Thus, candidate angiogenic factors have often been found to have opposite actions *in vivo* to those expected from *in vitro* studies. Examples include TGF-β[21] and tumor necrosis factor (TNF)-α[22], both of which inhibit endothelial cell growth *in vitro* but are stimulatory *in vivo*. The *in vivo* picture is further clouded by the release of many angiogenic factors from the extracellular matrix which might also have another level of control through binding to soluble growth factor receptors within the stroma[23].

Release of proteolytic enzymes. The capillary basement membrane and surrounding extracellular matrix are then degraded by a variety of proteolytic enzymes, including the plasminogen activators[24] and the matrix

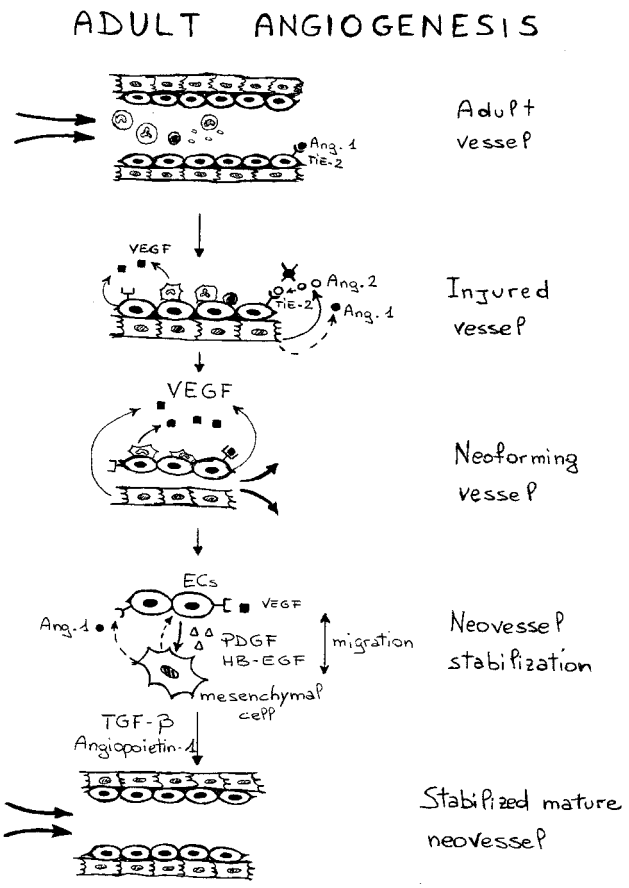

Figure 2. *Adult angiogenesis or neoangiogenesis. Sprouting of new capillaries from preexisting vessels. This process is mainly dependent on hypoxia and seems to require molecular events involved in inflammation. A scheme of the process and the associated factors are reported. Abbreviations as in figure 1.*

metalloproteinases[25], secreted by both endothelial and paracrine cells. The generation of the broad specificity protease plasmin by urokinase-type plasminogen activator is fundamental since it is able to degrade most matrix components either directly or by activation of other latent enzyme systems. However, remodeling of extracellular matrix is also accomplished through the action of metalloproteinase members of a multigene family of metal-dependent enzymes. These proteases have been classified into different categories originally based on their substrate specificity[25]. The regulation of metalloproteinase activity occurs at several levels including gene transcriptional control and proenzyme activation and inhibition of activated metalloproteinases by endogenous inhibitors. These native metalloproteinase inhibitors comprise a family of proteins generally referred by the acronym TIMPs (tissue inhibitors of metalloproteinases). During proteolysis, angiogenic stimulators and inhibitors are also released from matrix or paracrine cells which then influence the ability of endothelial cells to express components of these systems and to synthesize matrix[25]. Thus, matrix remodeling is a careful balance between signals for synthesis and degradation.

Endothelial cell migration and capillary morphogenesis. Dissolution of the underlying basement membrane and extracellular matrix permits endothelial cells from postcapillary parent venules to migrate and form sprouts. Endothelial sprouts elongate by intercalation of endothelial cells before fusion of microvilli at the sprout tips to form a network of interconnecting loops. Little is known about the control of these processes; however migration has reported to be modulated by a variety of cytokines and growth factors including interleukin (IL)-3[26], IL-6[27], IL-8[28], thrombopoietin[29], VEGF[30], TNF-α[28], PDGF[31], hepatocyte growth factor[32], FGF-1[33] and FGF-2[34], platelet activating factor[35], as well as physical forces[36], vasoactive hormones[37], TIMPs[38] and high density lipoproteins[39].

Recent studies have demonstrated that the expression of adhesion molecules belonging to the selectin and integrin families on endothelial cells is important not only in inflammation and metastasis but also plays an important role in capillary morphogenesis[40]. *In vitro* selectins are required for normal capillary formation[41] and *in vivo* up-regulation of these molecules is present where neovascularization is most active[42]. In particular, E-selectin, an endothelial membrane glycoprotein, is known for its ability to promote adhesion of leukocytes to cytokine-activated endothelial cells[43]. However, the *in vitro* observation that anti-E-selectin or anti-sialylated fucosylated oligosaccharides (structures to which E-selectin can bind) inhibits the formation of tube-like structures, supported its role in angiogenesis[44]. In addition to selectins several integrins have been described to be selectively expressed during angiogenesis[43]. Indeed, both *in vitro* and *in vivo* angiogenesis is severely perturbed by the interruption of integrin function[45].

Endothelial cell proliferation and vessel differentiation. During endothelial sprout formation, cell division occurs to supply the elongating vessel. Endothelial cells in normal tissues are quiescent and divide every 7-10 years[46]. However in pathological conditions autoradiographic studies suggested that endothelial cells can rapidly proliferate[47]. Although the FGF is considered to be the endothelial mitogen *per antonomasia*, a vast array of growth factors and cytokines have been identified as potent endothelial cell mitogens[26-32].

The final stages of vessel formation might also be referred to as "remodeling" and appear to define a process through which a forming vessel becomes stable[20]. Benjamin et al.[48] reported that the association of forming vessels with mural cells marks the end of the period of growth factor dependence and appears to signal vessel stabilization. In support of this idea, ultrastructural studies documented that a basement membrane is deposited only after endothelial cell-mural cell association has occurred[49].

Arteriogenesis. Atherosclerosis of the heart leads to a progressive narrowing of the coronary arteries, result-

ing in regional myocardial ischemia. Nature has created a mechanism able to adapt to regional ischemia. This consists in the development of a functional collateral circulation. Many terms have been used to define this type of vessel growth, such as collateral development, collateral dilation, non-sprouting angiogenesis, recapitulated vasculogenesis; however, currently the most widely accepted is "arteriogenesis"[50].

Arteriogenesis refers to the growth of arteries from preexisting arterioles characterized by fully developed anatomical hallmarks. Unlike vessels formed through angiogenesis, these arterioles remodel into functional arteries rather than capillary sprouts and their growth does not depend upon ischemia.

Collateral circulation: an adaptable growth. The anatomical and physiological aspects of collateral circulation have been extensively investigated in the past. The first demonstration of collateral anastomoses in the coronary circulation is generally ascribed to Lower[51]. In his classical *Tractatus de Corde* in 1669, by using a technique of fluid injection, he described the presence of "the vessels which carry blood to the heart" and found that "here and there they communicate by anastomoses. As a result, fluid injection into one of them spreads at one and the same time through both".

Since then, the existence of a collateral circulation in the heart has alternated between assertors and contestants. However, the universal acceptance of such connections came from Fulton's work in 1965[52], who demonstrated the existence of collaterals and their functional significance in normal and diseased hearts. Indeed, the collateral circulation exists and bears a pathophysiological meaning not only in the coronary circulation, but also in other vascular districts such as the limbs, the kidneys and the brain[53].

To date, the main features of collateral circulation can be summarized as follows:

• collateral circulation is a well defined aspect of the coronary artery anatomy and shows a wide interspecies variability, ranging from the absence of anatomically demonstrable collaterals in the rabbit and pig, to a well developed collateral circulation in the guinea pig[54];

• man stands in between, since the presence of collaterals during fetal life has been demonstrated[55]. Moreover, collateral vessels can be angiographically identified in adults in the absence of any recognized abnormality of the main arteries[56]. In the fetal[55] and adult[54] heart the anastomoses are chiefly localized in the interventricular septum and in the subendocardial layers; however, anastomoses are also present between epicardial arteries. Moreover, the level of collateral flow during ischemia depends on the age and pathology of each individual;

• collateral vessels can be divided into preexisting (as found in organs with a completely normal circulation) and transformed (when subjected to growth stimuli)[54]. Little is known about the structure of preexisting collateral arteries, both in the skeletal and cardiac muscle; however in the canine heart they range between 20 and 60 μm. Like the arterioles, collaterals consist of endothelium and some layers of smooth muscle[54] and have been considered as remnants of the complex three-dimensional branching process of the vascular network during embryogenesis, that do not reach full development[8];

• one common feature of collateral development is that vessels grow not only in width but also in length giving the typical corkscrew aspect. Indeed, when an epicardial artery is slowly occluded, "the collateral vessels increase by a factor of 20 times their internal diameter and the tissue mass increases by 50-fold[8]";

• collateral arteries usually develop far from the ischemic area. In the leg the site of artery occlusion and of the development of collateral circulation may be as far as 70 cm from the ischemic or necrotic region[57]. Similarly, in the heart collateral vessels develop in the epicardial regions or away from the ischemic myocardium, and the growth process still persists when ischemia has been resolved[53], indicating spatial as well as temporal dissociation.

Molecular mechanisms involved in arteriogenesis. The correlation between the molecular events underlying arteriogenesis and anatomical changes was first reported by Schaper[9] in the early 1970s, who demonstrated an active DNA synthesis and enhanced proliferation of cells of growing collaterals such as endothelial and smooth muscle cells. Although angiogenesis and arteriogenesis share some common pathways, they must be considered as two different processes. Indeed, while angiogenesis is mainly stimulated by local tissue hypoxia/ischemia, promoting up-regulation of both angiogenic factors and their receptors, arteriogenesis never occurs in ischemic areas[57] and is not associated with hypoxia-induced gene transcription[10] or with VEGF secretion[58].

A hallmark of the collateral growth is the increase in shear stress. Shear stress is one of the two main vectors in which the force acting on the vessel wall can be resolved. Its direction is parallel to the long axis of the vessel and represents the frictional force acting on the endothelial surface. It has been reported that, before and after femoral artery ligation, shear stress in preexisting collaterals increases by about 200-fold. This type of force, directly acting on endothelial cells, starts a process very similar to inflammation (Fig. 3). Under the influence of such a massive increase in shear stress, the major change in the newly recruited collateral vessels is endothelial cell activation. This occurs via the opening of chloride channels that, in turn, leads to modification of the volume and the swelling of cells and via the expression of adhesion molecules such as intracellular adhesion molecule-1. At the same time other signals such as transcription of gene encoding for monocyte-chemoattractant protein-1 (MCP-1), PDGF, and nitric oxide synthase, are triggered. Such a gene transcription par-

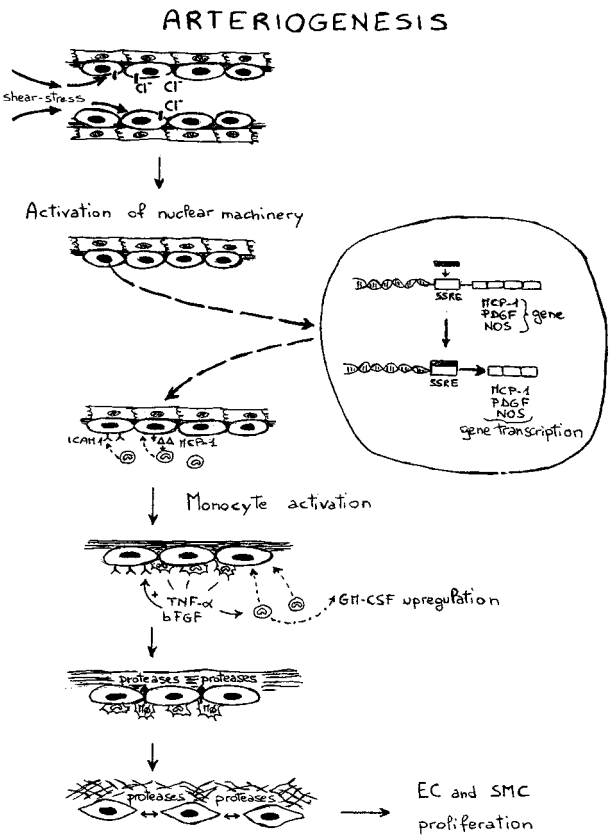

Figure 3. *Arteriogenesis. The process consists in the development of a functional collateral circulation. It refers to the growth of arteries from preexisting arterioles. They differ from vessels formed through angiogenesis because, unlike capillary sprouting, arterioles remodel into functional vessels in response to the increase in shear stress. The main molecular events are reported. FGF = fibroblast growth factor; GM-CSF = granulocyte-macrophage colony stimulating factor; MCP = monocyte-chemoattractant protein; NOS = nitric oxide synthase; SMC = smooth muscle cells; TNF = tumor necrosis factor. Other abbreviations as in figure 1.*

tially depends on the binding of a protein to a sequence located in the promoter region denoted as shear stress responsive elements. In response to MCP-1 surface expression, monocytes are recruited into the collateral vessels and become activated as denoted by the release of several soluble molecules including TNF-α, bFGF and granulocyte-macrophage colony stimulating-factor. In addition to a survival factor role these cytokines act as matrix remodeling regulators to create space for expanding vessels and as mitogens for endothelial and smooth muscle cells. At the end of both the remodeling phase and the wave of mitosis the vessels gain all the characteristics of normal arteries except for the content of matrix proteins between smooth muscle layers[59].

The remarkable interest in collateral vessels depends upon their potential ability to become large conductance arteries which can potentially rescue blood flow to ischemic areas. However this adaptive process is rather slow and unable to fully compensate for the flow lost because of the occlusion of native coronary arteries. Several efforts are thus still needed to improve their efficiency in the ischemic myocardium.

Angiogenic growth factors

Vascular endothelial growth factors. VEGF or vascular permeability factor has been characterized either as a heparin binding angiogenic growth factor, displaying high specificity for endothelial cells, or as a protein promoting extravasation[60]. Five human VEGF mRNA species encoding VEGF isoforms of 121, 145, 165, 189, and 206 amino acids are produced by alternative splicing of the VEGF mRNA ($VEGF_{121-206}$) to form active disulfide-linked homodimers. The heparin and heparan-sulfate binding ability distinguishes the different VEGF isoforms. Subsequently, additional growth factors, belonging to the VEGF family that share common receptors with VEGF have been identified[60].

The role of vascular endothelial growth factor in vasculogenesis and angiogenesis. The role of VEGF as the principal regulator of vasculogenesis comes from studies that have used the targeted gene disruption in mice. Mice lacking even one of the two VEGF alleles die before birth because of defects in the development of the cardiovascular system[61]. These observations indicate that cardiovascular system development depends on a precise VEGF concentration and that a decreased amount of VEGF leads to fatal consequences. Similarly, targeted disruption of both alleles of genes encoding the VEGF receptors (VEGFR1[61] and VEGFR2[62]) results in severe abnormalities of blood vessel formation mainly associated with defects in endothelial cell differentiation and migration[62,63].

Vascular endothelial growth factor tyrosine kinase receptors. Two different tyrosine kinase receptors denoted as VEGFR1 and VEGFR2 have been cloned and characterized[64,65]. Along with VEGFR3 which is expressed in lymph vessels[66] and bind VEGF-C and VEGF-D, these receptors form a subfamily distinguished by the presence of seven immunoglobulin-like domains in their extracellular domain and a split tyrosine kinase domain in the intracellular part[67]. The VEGFR1 and VEGFR2 are predominantly expressed on endothelial cells; however few additional types of cells express one or both of these receptors (Table II)[68,69]. The VEGFR1 and VEGFR2 are probably activated by all VEGF isoforms but fulfill somewhat different functions *in vivo*, as targeted disruption experiments have revealed[62,63].

Neuropilin-1: the $VEGF_{165}$-specific receptor. Together with VEGFR1 and VEGFR2, endothelial cells express isoform specific receptors that can bind to $VEGF_{165}$ but not to $VEGF_{121}$ and that are not related to the VEGFR1 and VEGFR2[70]. These receptors are encoded by neuropilin-1 and 2 genes[71,72]. Neuropilin-1 was identified as a receptor for several types of the collapsin/semaphorin family[60,72]. Semaphorins were initially characterized as factors that act as repellents of nerve growth cones and mediate neural cell guidance[71,72]. Gene disruption stud-

Table II. Angiogenic growth factors and related receptors.

Growth factors	Cell receptors	Target cells
VEGF-A	VEGFR-1 (flt-1) VEGFR-2 (flk-1/KDR) Neuropilin-1 Neuropilin-2	Endothelial cells, monocytes, coagulation system, hemopoietic stem cells
VEGF-B	VEGFR-1 (flt-1) Neuropilin-1	Endothelial cells
VEGF-C	VEGFR-2 (flk-1/KDR) VEGFR-3 (flt-4)	Lymphatic endothelial cells, endothelial cells
VEGF-D	VEGFR-2 (flk-1/KDR) VEGFR-3 (flt-4)	Endothelial cells, lymphatic endothelial cells
PIGF-1 PIGF-2	Flt-1 Neuropilin-1	Human placenta, exravillous trophoblast, endothelial cells[68]
FGF-1 (acid)	FGFR-1, 2, 3, 4	Smooth muscle cells, endothelial cells, others
FGF-2 (basic)	FGFR-1, 2	Smooth muscle cells, endothelial cells, others
FGF-4	FGFR-1, 2	Endothelial cells, megakaryocytes, others
FGF-5	Unknown	Endothelial cells, CNS, hair follicles
HGF	c-met	Endothelial cells, epithelial cells, others
PDGF-BB	PDGFR-β	Smooth muscle cells, endothelial cells, neutrophils, connective tissue
EGF	EGFR (c-erbB)	Endothelial cells, epithelial cells, gastric glands
TGF-α	EGFR (c-erbB)	Endothelial cells, epithelial cells, gastric glands
TNF-α	TNFR-1 TNFR-2	Neutrophils, endothelial cells, muscle cells, T-lymphocytes, B-lymphocytes, hepatocytes, adipocytes
PAF	PAF-R	Platelets, endothelial cells, smooth muscle cells, granulocytes
TPO	c-Mpl	Megakaryocytes, platelets, endothelial cells
IL-3	IL-3R	Hemopoietic cells from different lineages, CNS, endothelial cells, smooth muscle cells[69]
IL-6	IL-6R	Endothelial cells, T-lymphocytes, B-lymphocytes, hepatocytes
IL-8	IL-8R (2)	Endothelial cells, lymphocytes, monocytes, neutrophils, basophils
Angiopoietin-1	tie-2	Endothelial cells

CNS = central nervous system; EGF = endothelial growth factor; FGF = fibroblast growth factor; HGF = hepatocyte growth factor; IL = interleukin; PAF = platelet activating factor; PDGF = platelet derived growth factor; PIGF = placenta growth factor; R = receptor; TGF = transforming growth factor; TNF = tumor necrosis factor; TPO = thrombopoietin; VEGF = vascular endothelial growth factor; VEGFR = vascular endothelial growth factor receptor.

ies indicate that neuropilin-1 represents an important regulator of blood vessel development as mouse embryos, lacking a functional neuropilin-1 gene, die because their cardiovascular system fails to develop properly[73]. This finding leads to the fascinating conclusion that the process of axon guidance and development of a network of capillary tubes share, at least, some common molecular mechanisms.

Mechanisms involved in hypoxia-mediated vascular endothelial growth factor production. In normal myocardium VEGF mRNA is constitutively expressed[74]; however, an increased level of VEGF mRNA transcription has been detected after transient ischemic insult and in similar pathological conditions characterized by hypoxia. Indeed, hypoxia as well as hypoglycemia are major stimulators of VEGF expression[74]. The mechanism that regulates VEGF production by oxygen availability is similar to another oxygen-sensitive gene: the erythropoietin gene[75]. Hypoxia-induced transcription of VEGF mRNA is apparently mediated, at least in part, by the binding of hypoxia-inducible factor (HIF)-1 to an HIF-1 binding site located in the VEGF promoter[76]. In addition to the induction of transcription, hypoxia promotes post-transcriptional events such as the stabilization of VEGF mRNA. This occurs via proteins, such as the HuR mRNA binding protein that binds to sequences located in the 3' untranslated region of VEGF mRNA[77]. Moreover, a macrophage-derived peptide, which was found in the wound fluid[78] as well as along the border of myocardial infarction[79], the cathelin-related proline-and arginine-rich peptide (PR39), has been described as a potent post-transcriptional regulator of VEGF expression[80]. However, it turns out that some mechanisms leading to elevated VEGF production actually short-circuit the normal hypoxia-depending pathway. In addition to the ligand, both VEGFR1 and VEGFR2 can be up regulated under hypoxia both at the transcriptional level *in vivo*[81] and on the protein level *in vitro*[82].

Fibroblast growth factors. The FGF family of genes currently comprises 18 members. They exhibit a high degree of cross-species homology[83]. They all require cell surface heparan-sulfate proteoglycans to bind to their tyrosine kinase receptors. The two prototypes FGF1 (acid) (aFGF) and FGF2 (basic) (bFGF) are the best characterized[83]. Many of the FGF genes were identified as oncogenes, and a forced secretion of FGF1 *in vivo*, by gene transfer methods (uptake of the gene by the host cell), leads to the creation of prominent cellular hyperplasia including exaggerated angiogenesis[83]. It is well established that secreted members of the FGF family remain bound to the extracellular matrix; therefore similar to VEGF, they have a very short half-life about 3 min after intravenous injection[84]. The intravenous administration of various growth factors is limited by hypotension, due in part to the release of nitric oxide[85]. It is of interest, however, that the angiogenic potential of FGF2 appears to be independent of its effect on nitric oxide release[85]. This is in contrast with the inhibition of VEGF-induced angiogenesis by nitric oxide synthase inhibition in a rabbit model in which cells from a VEGF transfected breast cancer cell line were implanted in the cornea[86]. Moreover, despite the angiogenic potential, as endothelial cell mitogens and stimulators of migration, FGFs also act as trophic factors in the myocardium[87]. FGF1 and FGF2 have been localized in normal cardiac myocytes[88], suggesting that they can act as autocrine factors[89] to regulate normal cellular processes. However, evidence suggests that FGFs play a role during the adaptive response to myocardial ischemia[90]. Finally, FGFs can also stimulate endothelial production of various proteases, including plasminogen activator and collagenase that can digest extracellular matrix.

The tyrosine kinase fibroblast growth factor receptors. The FGFs elicit their effects on cells by forming a ternary complex that includes the ligand, a high-affinity tyrosine receptor and heparan-sulfate proteoglycans. At present, four alternative spliced tyrosine kinase receptors (FGFR1, 2, 3 and 4) comprise the FGFR gene family[91]. The overall amino acid sequence identity among members of the family is between 56 and 71%. FGFRs all have several features in common including an extracellular domain consisting of three immunoglobulin-like domains, a heparin binding domain, an acid box domain, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase domain that is split by an insert sequence[91]. In several vertebrate species, the spatio-temporal patterns of expression of the different FGFRs mRNA indicate that these tyrosine kinase receptors may have tissue-specific as well as developmental stage-specific functions[91].

Other angiogenic growth factors

Other growth factors, in or near the blood vessel walls that can induce endothelial cell proliferation include: TGF-α, hepatocyte growth factor, platelet activating factor, the hematopoietic growth factors IL-3, thrombopoietin and erythropoietin, IL-8, and, in microvascular endothelial cells, PDGF. Each of these factors interacts with a specific receptor on the endothelial cell membrane. However, because of space limitations data concerning these factors are summarized in table II.

The atherosclerotic plaque: role of neovascularization

Atherosclerosis is now viewed as a progressive inflammatory disease in which inflammatory cells, activated smooth muscle cells, lipids and extracellular matrix accumulate on the arterial wall resulting in plaque growth[92]. However, considerable evidence indicates that vasa vasorum, that constitute a potential reservoir for post-natal angiogenesis, also contribute to the growth and development of neointima thickening.

The role of vasa vasorum: an old and a novel story. The inner part of the wall of large blood vessels normally does not contain intrinsic vasculature. As Geiringer[93] suggested a half-century ago vasa are required to extend beyond the adventitia into the media when arterial wall thickness exceeds 0.5 mm. Thus, like malignant tumors, atheromatous lesions, beyond a certain size, contain an increased number of vasa, indicating that intimal angiogenesis occurs as part of an adaptive change recently addressed as vascular remodeling. This idea is supported by early observations of Koester[94] in 1876 and Winternitz et al.[95] in 1938 who noted that vascularization of the intima was associated with atherosclerosis. These observations were later reconsidered when Barger et al.[96] demonstrated an important network of adventitial microvessels associated with atherosclerotic plaques. The potential clinical relevance was then outlined by Patterson[97] who proposed that coronary thrombosis was the result of intimal capillary rupture. Despite these critical observations little is known about the mechanisms involved in intraplaque microvessel formation. However, one current hypothesis of neovascularization in atherosclerosis is that small vessels develop from adventitial vasa vasorum[98]. This is sustained by the observations that in normal vessels, the microvascular network of vasa vasorum is confined to the adventitia and outer media. In vessels with atherosclerotic involvement, this network becomes more abundant and extends into the intima of atherosclerotic lesions[94-96]. Moreover, it has recently been reported that plaque regression is associated with either loss of vasa vasorum or reduction in blood flow through the vasa to the coronary intima and media[98].

Proposed mechanisms of intraplaque new vessel formation. Recent developments in the understanding of the molecular mechanisms involved in angiogenesis

sustain the possibility that intraplaque vessels may be the response of plaque ischemia[99]. Thus, developing primary or restenotic lesions might be considered as underperfused and, as the model of tumor progression, dependent on new vessel formation for continued growth. Indeed, this is sustained by the work of Moulton et al.[99] in which they report a profound reduction in the plaque area by an angiogenesis inhibitor strongly suggesting that plaque progresses at the same rate as vessel development. However, what the experiments do not show is how much angiogenesis contributes to the development of atherosclerosis. Consistent with the model of tumor growth one current opinion is that "angiogenesis is necessary but not sufficient" for the developing plaque thus representing a prerequisite for plaque growth. However, it should be considered that atherosclerosis results from an excessive inflammatory response of the endothelium and smooth muscle cells[92] that, by itself, may directly promote neovascularization. In such a case intraplaque neovascularization could simply be considered as an epiphenomenon. Likewise, bone marrow-derived circulating endothelial cell progenitors[19] might contribute to plaque neovascularization via the release of regulatory factors acting on their mobilization and homing.

New intraplaque vessel formation as an inflammatory response. For neovascularization to occur, a local stimulus may start a cascade of endothelial cell behaviors that results in the formation of new vessels[5,7]. Since a stimulus is essential for these angiogenic responses, the presence of angiogenic factors or cellular components that serves as a source of angiogenic factors in atherosclerotic plaque has been suspected. Indeed, plaque vessels are often found in areas rich in macrophages, T-cells and mast cells, cell types that can activate angiogenesis[92]. Their close proximity to inflammatory infiltrates and the expression of adhesion molecules, such as vascular adhesion molecule-1, intracellular adhesion molecule-1 and E-selectin on the endothelium of plaque vessels both suggest that these vessels may recruit inflammatory cells into lesions and initiate a positive-feedback mechanism[92]. Moreover, the observation that fragments of human atherosclerotic plaque, composed mainly of smooth muscle cells, when transplanted into rabbit corneal micropockets, were able to induce an angiogenic response, strongly supports the possibility that, despite inflammatory cells, smooth muscle cells can act as a paracrine source of angiogenic growth factors[100].

Role of angiogenesis in plaque instability. Rupture of coronary plaque is the most important mechanism underlying the sudden onset of acute coronary syndromes. The risk of plaque rupture may depend more on plaque composition than on plaque size. Plaques rich in soft extracellular lipids and macrophages, capable of releasing lytic enzymes, are possibly more vulnerable to rupture[101]. Attention has recently been refocused on the correlation between intraplaque neoformed vessel and lesion severity as well as on the release of vasoactive agents from intraplaque microvessels and coronary spasm[101]. Indeed, it has been recognized that rupture-related plaque progression due to luminal thrombosis and plaque hemorrhaging through fragile, newly formed vessels at the base of advanced plaques are also important mechanisms underlying acute coronary syndromes. In this regard a positive correlation between a high number of lesional microvessels and platelet-derived endothelial cell growth factor (PD-ECGF) immunoreactivity as well as between PD-ECGF immunoreativity and angina score has suggested a positive relationship among the expression of angiogenic factors, the degree of neovascularization, and lesion severity[102]. Similarly, the expression of FGFs, PDGF-AA and BB and their receptors has been correlated with lesion activity[103-105]. On the contrary studies on VEGF expression in smooth muscle cells of atherosclerotic coronary arteries have failed to demonstrate a correlation between VEGF immunoreactivity and the extent of vasa vasorum raising speculation about a possible endothelial cell repair effect of this growth factor[106]. Moreover, a correlation between neovascularization and plaque prone to rupture has also been suggested by the finding that, as activated foam cells, neoformed vessels are located in the vulnerable region of the plaque: the plaque shoulder[102]. In this regard, as above described, neoforming vessels can release matrix degrading enzymes and thus directly contribute to lesion weakening. Nevertheless, independently of which growth factor governs this process it is well established that neovascularization correlates with narrowing of the diseased vessel segment. Several hypotheses on the mechanisms involved in this process have been taken into consideration: a) intraplaque microvessels may by themselves contribute to luminal narrowing since they are space-occupying or because they can act as paracrine sources of growth factors able to recruit inflammatory cells; b) neovessel fragility could entail intramural bleeding with subsequent scarring; c) organizing neovascularization could follow thrombosis, secondary to the loss of cross-sectional and luminal vessel area.

Perspectives

Therapeutic modulation of angiogenesis represents an interesting frontier of cardiovascular medicine. Formation of new vessels on the ischemic heart or other tissues would be an important clue in the treatment of disorders for which medical intervention or surgical therapy turned out to be uneffective. Certainly several growth factors are capable of inducing significant angiogenesis, however, as recently stated by "an expert panel summary"[107], it is not clear whether one of these agents is better than another, or whether a combination of two or more factors may be preferable to a single agent. The natural process of angiogenesis is complex

involving a number of growth factors and depends upon the normal level and appropriately timed expression of them. The nascent endothelial tube requires VEGF for growth and survival, and endothelial cell-mural cell contact defines the end of VEGF dependence and the beginning of vessel remodeling and maturation. Given this complexity, therapies using a single angiogenic growth factor may produce incompletely functioning or unstable endothelial channels with defective arteriovenous and pericellular differentiation, characteristic of tumor angiogenesis. A combination of growth factors will be then preferable in future therapies directed to neovascularization of tissues with an adequate investment of the formed vessels. This is also sustained by the observation that the combination of both VEGF and bFGF is able to induce differentiation of endothelial progenitor cells into endothelial cells[19] providing a useful model for delivery of cellular or gene therapy to the site of neoangiogenesis[107].

Despite the potential therapeutic effect of these growth factors, one theoretical concern is that VEGF and bFGF may exacerbate plaque angiogenesis and thus may adversely affect progression of coronary artery disease through plaque expansion or rupture. In addition, these growth factors might stimulate progression of coronary stenosis by stimulating growth of fibroblast and medial smooth muscle cells. However, to date, no convincing data are available suggesting that, as in physiological conditions, the effects of released as well as given angiogenic growth factors can be carefully counterbalanced by the presence of inhibitors. Indeed, although prior attention concentrated on stimulators of angiogenesis, it has been recognized that angiogenesis is also negatively regulated by angiogenic inhibitors. One such putative inhibitor, homologous to the B-cell translocator gene (btg-1), was isolated from the porcine myocardium and was shown to suppress endothelial cells and smooth muscle cell proliferation and angiogenesis[108]. Therefore, it is conceivable to assume that in the near future the use of such inhibitors, so far limited to neoplastic angiogenesis, may provide an additional therapeutical strategy for cardiovascular diseases.

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