Mechanisms of arteriogenesis

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The study of arteriogenesis, the development of an arterial circulation circumventing the occlusion of a large artery from pre-existent or de novo small arterioles, has overcome three much-debated hurdles during the last 100 years. First, the existence of precursor structures was anatomically proven; second, the precursor structures’ function under normal and pathological conditions was demonstrated; and third, it was shown that biological or pharmacological agents can influence collateral artery development. However, there is one hurdle that this field has yet to overcome: the treatment of human patients with new arteriogenic agents.

Commenting on the importance of arteriogenesis, William F. M. Fulton, a pioneer in the field of the human coronary collateral circulation, once said, “If some means could emerge of encouraging the rate at which normal coronary arterial anastomoses enlarge to form the wide channels for collateral blood flow, then a considerable potential advantage would be gained in the management of ischemic heart disease [1].” Similar words could easily have been applied to peripheral, cerebral and renal circulation. After several decades of research, “some means” are in reach, but not in the form of a pill. However, some progress has been made towards a comprehensive understanding of the mechanisms responsible for arteriogenesis.

Definitions

Arteriogenesis, formerly regarded as a variant of angiogenesis, is a relatively new term that was introduced to distinguish it from other mechanisms of vascular growth, such as angiogenesis and vasculogenesis [2]. Angiogenesis describes the formation of new capillaries by sprouting and intussusception from pre-existent capillaries, and vasculogenesis is the embryonic development of blood vessels from angioblasts [3–5]. Arteriogenesis describes the formation of mature arteries from pre-existent interconnecting arterioles after an arterial occlusion. It shares some features with angiogenesis, but the pathways leading to it are different, as are the final results: arteriogenesis is potentially able to fully replace an occluded artery whereas angiogenesis cannot. Under special circumstances, arteriogenesis may lead to the recovery of markedly reduced blood flow. Increasing the number of capillaries within the ischemic region cannot increase blood flow when its limiting structure lies upstream. Another
fundamental difference between the two types of vascular growth is angiogenesis’ dependency on tissue hypoxia/ischemia, which leads to the activation of the transcription factor HIF; in contrast, arteriogenesis occurs in an environment of normoxia [6]. A collateral vessel, resulting from the arteriogenic process, always conducts arterial blood flow and cannot, by definition, become hypoxic. Collateral vessels in the vascular periphery are surrounded by normoxic tissue even after acute femoral artery occlusion [6].

Collateral Vessels: de-novo or Pre-existent?

Controversy still exists as to whether mechanisms, such as the recruitment of smooth muscle by capillaries or the attraction of smooth muscle progenitor cells, which do not enlarge the pre-existent arteriolar networks increase perfusion of ischemic tissue. Supporters of pre-existent arteriolar networks argue that they can be demonstrated by a variety of techniques and that they are present in the peripheral circulation of most mammals, including humans. Since these well-defined vessels enlarge, the term arteriogenesis was coined for them. During the early stages of collateral growth, many more vessels are angiographically demonstrable in the vascular periphery, especially in the rabbit and rat. These may have grown de-novo by the mechanisms cited above. However, it remains possible that this vessel surplus reflects the fact that smaller arterioles only become detectable after growth. Present angiographic techniques can resolve vessels only to approximately 30 μm. Another argument favoring pre-existent arterioles relates to the mouse (C57BL/6); it has only six normally demonstrable interconnecting arterioles in the peripheral circulation, and this number remains invariant after growth following femoral occlusion. In rat, relatively large collateral can be detected with the naked eye after contrast injection even without arterial occlusion. It is this pre-existent vessel that enlarges upon femoral artery occlusion and carries most of the collateral blood flow [7]. Also, Fulton showed that pre-existent collaterals can be detected in the normal human heart and that those that enlarge after coronary occlusion occupy the same topography [8]. The canine heart relies completely on the pre-existent arteriolar network where the shortest connections enlarge by growth. There is no evidence for angiogenesis either within or outside the ischemic region.

In contrast, observations from the heart of the domestic pig challenge the importance of a pre-existent arteriolar network. The heart of the domestic pig, unlike that of the dog, neither possesses an epicardial network of arteriolar anastomoses nor develops arterial collateral vessels after slowly progressing coronary stenosis. Rather it develops subendocardial giant capillary networks devoid of smooth muscle. In addition, angiogenesis is activated within the ischemic zone thereby reducing the anatomically defined minimal resistance [9,10]. This means that the porcine heart relies almost completely on angiogenesis as a defense mechanism following coronary artery occlusion provided the speed of stenosis is very slow. The low intravascular pressure in the porcine collateral circulation lowers the perfusion pressure gradient, especially with high diastolic ventricular pressures (diastolic crunch), making the myocardium highly susceptible to ischemia. In the vascular periphery, the pig relies completely on pre-existent arteriolar connections (i.e. the response to femoral artery occlusion is strictly arteriogenic). So this is not an either-or question as both mechanisms may be present and active, though the quantitative contribution of pre-existent arteriolar vessels is significantly larger.

Physical Forces as Primary Stimuli

It has been known for a long time that flow determines arterial size and that pressure determines wall thickness (i.e. that form follows function). Thoma [11], Schretzenmayr [12], Rodbard [13], Langille [14], Holtz [15], Tronc [16] and Ben Driss [17] made the observation that arterial size depends on flow during development, that adult arteries respond with structural changes to changes in blood flow, and that lumen is controlled by “an immediate physiological adjustment in vascular tone induced by the change in flow, and a delayed anatomical change that occurs when the change in flow persists [18–20].” Since these studies, many have identified a host of molecules whose endothelial production is also mediated by fluid shear stress (FSS) [18–20]. Recent studies by the laboratories of Tedgui [21, 22], Busse [23], Dejana [24] and from our own group [25] have shed light on the mechanisms of transduction of the mechanical stimulus into a growth response. FSS is proportional to blood flow velocity and inversely related to the cube of the collateral vessel radius. Hence, increased blood flow directly results in increased FSS. Since growth increases the collateral vessel radius, FSS falls quickly; this may be the reason why arteriogenesis stops prematurely and restores only 35%–40% of the maximal conductance of the replaced artery [26,27]. It appears logical to increase FSS in order to improve the defective collateral vessel growth. We recently developed a model where a state of high chronic FSS was generated by shunting most of the collateral flow into the...
accompanying vein thereby maintaining high collateral flow rates and high FSS levels. Under these conditions, collateral conductance increased 4-fold compared with the contralateral side, which was only occluded but not shunted. Under these conditions, the maximal collateral conductance was already 100% at 7 d and had, after 4 weeks, surpassed (2-fold) normal maximal conductance of the arterial bed before occlusion [28]. Wall tension defined by pressure and radius [29] and by pulsatile and cyclic stretch [23] were also discussed as activators of the endothelium and, implicitly, as stimuli of vascular growth. However, immediately after arterial occlusion, distal pressure falls, making wall stresses unlikely growth stimuli. Likewise, pulsatile stretch of the arterial wall is low at low pressure while the role of stretch-induced EDHF in vascular growth is unknown. Maximal arteriogenesis occurs in situations at maximal flow (like in AV-shunts) when pressure pulsations are minimal.

The Role of the Endothelium

Shortly after coronary occlusion, the endothelial cells of collateral vessels appeared swollen and longitudinal bulges, as opposed to the completely flat inner surface of small normal coronary arteries, appeared. The cells are randomly oriented and do not longer line up in the direction of blood flow. The activation of the endothelium was first reported by Schaper et al who observed DNA synthesis with thymidine labeling in canine coronary collateral arterioles subjected to progressive stenosis of the left circumflex coronary artery [30,31]. Similar results were reported later with BrdU labeling or the cell cycle-specific antibody Ki67 [32,33]. These activated endothelial cells contain numerous cellular organelles, particularly free ribosomes in the cytoplasm [34−36]. Activated endothelium also shows increased endothelial NO synthetase (eNOS), monocyte chemoattractant protein (MCP-1), TGF-β, and the adhesion molecules ICAM-1 and VCAM [36]. Consequently, increased permeability of the endothelium, as indicated by the leakage of plasma proteins, erythrocytes and platelets into the vascular wall and the adherence of monocytes to the endothelium, were observed [34]. Activated endothelium also changes the open probability of the calcium-dependent chloride channels, and chloride channel inhibitors interfere with arteriogenesis [25,37]. A large number of molecules involved in cell proliferation and migration were found to be up-regulated. These include MMP-2, 9, t-PA, u-PA, FAK, integrin α5β1 and integrin αvβ3 [38−40]. Most recently bone marrow tyrosine kinase (Bmx; also called endothelial/epithelial tyrosine kinase [41]), an FAK-activation molecule whose downstream effectors involve cell migration, was reported to be highly induced in the endothelium during ischemia-mediated arteriogenesis [41]. It is known that the evolutionary conserved Notch signaling pathway is involved in vascular development [25,42−44]. Limbourget al recently showed that Notch ligand Delta-like 1 (Dll1) expression in endothelium is strongly induced, and Notch signaling is activated and ephrin-B2 is up-regulated during arteriogenesis [45]. However, in Dll1 mutant mice after hind limb ischemia, arterial collateral growth was abrogated and recovery of blood flow was severely impaired, suggesting that Dll1 is essential for postnatal arteriogenesis [25]. Ingber’s Tensegrity model [46] states that the deformation of the endothelial cell by fluid shear stress, resulting in a different distribution of force in the cytoskeleton, initiates gene transcription and is an important part of the endothelial activation. However, the cytoskeletal marker vimentin disappears completely from activated and proliferating endothelium [47].

Smooth Muscle Cells

Smooth muscle carries most of the burden of arterioles’ transformation into collateral vessels. Vascular smooth muscle cells (VSMCs) undergo the most drastic changes and increase their tissue mass depending on the species; for example, it increases 3-fold in mice, 10-fold in rabbits, 20-fold in canines and even more in humans [25]. The remarkable plasticity of the SMCs, particularly their ability to change phenotype from the contractile to the synthetic, in response to stimuli from environmental cues [48−51] makes this possible. VSMC proliferation, phenotypic changes and the occurrence of a neointima are typical for arteriogenesis [29,32,34,39]. Coronary collaterals show two zones of growth: a very active neointima consisting mostly of synthetic-type SMC and a media with somewhat less active SMC [25]. Although the filamentous α-smooth muscle actin had almost completely disappeared in the synthetic phenotype, depolymerized actin is still present in abundance. Desmin, one of the principal intermediate filament proteins in VSMC, is a marker for contractile SMC and a media with somewhat less active SMC [25]. Although the filamentous α-smooth muscle actin had almost completely disappeared in the synthetic phenotype, depolymerized actin is still present in abundance. Desmin, one of the principal intermediate filament proteins in VSMC, is a marker for contractile SMC, but it disappears during active growth of the synthetic SMC. Similar data were reported by Buus et al in flow-related remodeling of rat mesenteric resistance arteries where there was reduced expression of desmin in VSMC [25]. Therefore, the absence of desmin in the SMC of the neointima makes it a marker for the active phase of arteriogenesis [52].

Vinculin, a cytoskeleton-associated protein that links the extracellular matrix and the intracellular milieu via integrins,
almost completely disappears from both layers, possibly enabling the mobilization of SMC. Furthermore, proteolytic MMP-2 and MMP-9 are very active in both layers, but their inhibitor TIMP-1 is only expressed in the tunica media. Mobilization of SMC is facilitated by lysis of extracellular matrix proteins. PAI-1 is overexpressed in the neointima as well as in the media and does not entirely normalize during vessel maturation.

The regression of the neointima in the canine coronary system takes several months. However, in some mature collateral vessels, the neointima grows excessively by proliferating SMC until the vessel is completely obliterated. This process is called “pruning” and results in a reduction of the high number of small collateral vessels in favor of a few large ones [25].

The degradation of BM, IEL and EEL by proteolysis mobilizes SMC. This is also facilitated by other proteins, such as the non-receptor tyrosine kinase FAK, integrins αβ1 and αβ3, and Erk1/2. FAK is a cytoplasmic protein-tyrosine kinase that localizes to focal contacts and adhesions. It triggers intracellular signals promoting cell migration [53] by reacting to extracellular matrix-integrin and growth factor stimulation. The involvement of FAK in blood vessel morphogenesis, SMC proliferation, phenotype change, migration and intimal hyperplasia was documented in several recent articles [54–59]. Mechanical strain, growth factor stimulation and shear stress in collateral vessels lead to autophosphorylation of FAK/ERK and to migration and proliferation of VSMC [28,60,61].

FAK localizes where cells interact with the extracellular matrix through integrin receptors. αβ3, which was shown to mediate SMC accumulation in the neointima in ligated carotid arteries in mice [62] was also strongly overexpressed in the neointima of collateral vessels [35]. Integrin clustering upon binding to extracellular matrix components, such as fibronectin, results in activation of FAK [63].

Integrin αβ1 is mainly a fibronectin-receptor. It mediates most of fibronectin’s biological activities. Integrin αβ1, stimulated by soluble or anchored fibronectin, promotes cellular locomotion [64]. Integrin αβ1 and fibronectin [32] are markedly increased in growing collateral vessels and may play an important role in SMC mobility and signal transduction [39].

**Smooth muscle lineage**

The remarkable phenotypic plasticity of vascular smooth muscle and its ability to re-enter the cell cycle raise the question of lineage, especially when morphological markers are no longer helpful. During embryonic development, SMC arise in multiple regions from different precursor populations; specifically, the large vessels in the vascular periphery are derived from mesenchymal neural crest cells, whereas the coronary arteries are of non-neural crest origin [65]. It is of note that coronary collaterals differ from those in the vascular periphery because of their tendency to form a neointima with the ability to later regress or to continue proliferating finally leading to occlusion (i.e. pruning). The clinical counterpart for this is re-stenosis after stent placement. During development all muscle types share some common markers, some of which are shed in later stages and some of which are retained, such as calponin and SM22 in vascular smooth muscle [65,66]. These markers do not disappear during collateral vessel growth, which means that invading cells have not trans-differentiated. Some of the embryonal common markers, such as CARP and abra in both cardiac and in smooth muscle of collateral vessels, are re-expressed under stressful conditions. SMC of collateral vessels begin the S-phase of the cell cycle when still fully differentiated and change phenotype only shortly before M-phase, which supports the argument that collateral vessels grow by proliferation of cells in situ and do not require cells of a different lineage. Connexin 37, an endothelial marker of the embryonal arterial system that is down-regulated after birth, is re-expressed in smooth muscle of growing collateral vessels, suggesting a re-capitulation of the developmental pattern of gene expression as the basis of arteriogenesis [67,68].

**Extracellular Proteolysis and Antiproteolysis**

Proteolysis of the internal and external elastic lamina and of the basement membranes [69–72] is necessary to overcome the structural barriers to growth. Early in collateral vessel growth, the process is up-regulated and activates MMP-2, MMP-9 and urokinase-type plasminogen activator (u-PA). As a consequence, the IEL, the EEL and the BM become fragmented [39,47]. The controlled destruction of the vascular scaffold paves the way for the expansion and outward growth of collateral vessels. Moreover, apoptosis of SMC may facilitate the renewal of the vascular wall [32]. Since laminin and collagen IV promote the differentiation and inhibit proliferation of SMC, the degradation of the BM may also facilitate the shift of SMC phenotypes. Elastin is essential for arterial morphogenesis [73,74]. Degradation of elastic fibers may contribute to the proliferation and migration of VSMC. This has been supported by our experiments with...
heterozygous elastin knockout mice that showed an accelerated recovery of blood flow after femoral artery occlusion (Schaper et al, unpublished data).

**Bone Marrow-derived Cells**

Between 1 d and 3 d after complete coronary occlusion following a few weeks of progressive narrowing, Schaper et al observed massive adhesion of monocytes to the endothelial lining of coronary collaterals [35]. The endothelial cells had lost spatial orientation as well as osmotic control; this coincided with monocyte adhesion. Days later, monocytes as well as lymphocytes, mast cells and leucocytes invaded the perivascular space. To test whether these inflammatory reactions were important to the arteriogenic process, anti-inflammatory treatment was applied; this treatment indicated that collateral vessel growth indeed depends on an inflammatory environment [29]. Polverini et al also [75] showed that monocytes have angiogenic properties. Decades later, it was shown that activated endothelium produced MCP-1 and resulted in adhesion molecules appearing on the endothelial surface [35]. The causal relationship was ascertained by showing that targeted disruption of the MCP-1 gene and its cognate receptor, CCR-2, markedly impairs arteriogenesis in an ischemic mouse hind limb experiment [25,76,77]. Neutralizing antibodies against ICAM-1 as well as an infusion of free ICAM-1 that neutralized the Mac-1 receptor on circulating monocytes both markedly impaired the arteriogenic process. Deletion of monocytes by chemical bone marrow suppression or by liposomes loaded with phosphonates that killed monocytes/macrophages also inhibited collateral vessel formation [25]. After femoral artery occlusion during the post-chemotherapy rebound and a transfusion of an excess of monocytes, the monocyte population increased and accelerated arteriogenesis. Furthermore, animals with hereditary monocytopenia (i.e. op/op mice and osteopetrotic rats) showed only a stunted collateral response to femoral artery occlusion. These experiments clearly established the causal relationship between monocytes and the arteriogenic process [78].

Other bone marrow-derived cells, in particular lymphocytes of the NK type and CD-4 and CD-8 cells, also play a role [79,80]. Their importance became known when the marked difference in response to femoral artery occlusion between mouse strains was observed. “Black” mice (C75BL/6) tolerated the occlusion much better than “white” mice (BALB/c). They showed a slightly higher residual blood flow immediately after femoral occlusion, they exhibited almost no toe necroses and their blood flow recovery was complete after only one week. In contrast, self-amputated toes were frequent in the white mice and their blood flow recovery was slow and incomplete. The basic difference between these strains is their immune systems. Black mice are specially bred for studies concerning the T-cell receptors and show an abundance of NK cells. When antibodies or genetic manipulation eliminate these cells, black mice lose their advantage. However, the genetic trait is dominant and the F1 generation of black/white crosses still enjoys the ischemia resistance. Hematopoietic stem cells (rather than differentiated mononuclear white cells) were reported to be metastatic and, when attaching to collateral vessels, transform into endothelial and smooth muscle cells. Detailed laser confocal studies showed that BM-derived cells from GFP-transplanted bone marrow neither incorporated into the wall of collateral arteries nor exhibited the endothelial or smooth muscle phenotype [81]. Since BM-derived cells secrete a wide array of cytokines, such as bFGF and VEGF, it is suggested that they contribute to collateral remodeling through paracrine mechanisms [27]. Recently the role of BM-derived mesenchymal stem cells in arteriogenesis was investigated in rat and swine acute myocardial infarction [82–84]. Though the arteriogenic data were controversial, these experiments confirmed improved heart function. Martens et al showed induction of vascular network formation and arteriogenesis [82], but Yang et al did not detect growth of collateral arteries [84]. Whether these results varied due to species differences therefore remains an open question.

**Activation of the adventitia**

In normal small arterioles, the adventitia is a small rim composed primarily of a few fibroblasts, collagen, elastin fibers and other ECM materials, which form the barrier between the adventitia and the media. In growing collateral vessels the adventitia undergoes drastic changes. An early study showed that an acute inflammatory reaction was present in the adventitia of collateral vessels in canine heart [85]. Later experiments in rabbit and rat hind limbs confirmed these findings by showing an accumulation of macrophages in the adventitia. Adhesion molecules, like VCAM, may account for this inflammatory process since they were dramatically up-regulated in the adventitia [86]. As a consequence, macrophages and activated fibroblasts produce growth factors and MMPs [87,88], contributing to the enlargement of collateral vessels. At present, the role of mast cells that are also present in much higher than normal numbers around growing collateral vessels is unclear [32]. Targeted disruption of the mast cell growth

factor in mice increased the recovery of the ischemic hind limb after femoral artery occlusion, measured by TOF magnetic resonance imaging, indicating a restraining influence of mast cells on vascular growth [25].

**Collateral vessels in various vascular provinces**

In the coronary system, monocytes adhere initially to the endothelial surface and invade the intima. The perivascular space later becomes populated with white cells, which provide space for the expanding collateral vessels by destroying and removing tissue that is in the way. In the vascular periphery, only a few monocytes adhere to the shear stressed endothelium, and leaky post-capillary venules may have made the marked invasion of the perivascular space possible. This may be due to the expression of VEGF, which primarily increases permeability. Another difference is that the neointima is not as well developed in the vascular periphery, especially in the mouse hind leg.

**The Enigma of NO**

The role of NO in arteriogenesis remains unclear because collateral artery growth is totally dependent on mitosis, and NO is a known anti-mitogen [25]. Even when taking into account that NO stimulates VEGF release, a plausible explanation of NO’s role has not emerged because VEGF is not a known mitogen for SMC, the dominant cell type in arteriogenesis. The enigmatic role of NO is highlighted by the fact that mice with targeted disruption of the eNOS gene have had retarded recovery of blood flow following femoral artery occlusion, which is indicative at first sight of defective arteriogenesis. However, morphometric analysis of collateral vessels has shown them to be of normal caliber and number, and the application of NO donors has restored blood flow to levels identical to those of wild-type mice [25,89]. This indicates that the growth of collateral vessels was not impaired [25], and that retarded recovery was only apparent, dominated by vasoconstriction. Femoral artery occlusion in transgenic eNOS overexpressing mice showed normal recovery of blood flow after femoral occlusion and the time course of recovery, and its final extent did not differ from that of wild-type mice [89]. These experiments would allow only one explanation, namely that eNOS-produced NO plays no role in arteriogenesis. However, the effects of maximally FSS-stimulated collateral artery growth in rabbits were totally reversed by the application of the NOS inhibitor L-NAME [28]. In addition, application of L-NAME also impaired exercise improved collateral-dependent blood flow in rats [90,91]. This was difficult to reconcile with the mouse experiments. Since L-NAME is rather non-specific and also inhibits also iNOS, we hypothesize that L-NAME’s strong inhibitory effects inactivated the monocytes/macrophages (carriers of iNOS) essential to collateral vessel growth. Therefore, we conclude that NO plays a crucial role in arteriogenesis as a tool of the monocytes/macrophages whose function is dependent on the activity of iNOS. NO donors, other than eNOS, may have aided in collateral growth in the eNOS-targeted mice.

**Is neural regulation a contributor to arteriogenesis?**

An increasing body of evidence suggests that during development nerve fibers and blood vessels share common signaling cues, including Netrins, Slits, Ephrins, Neuropilins and Semaphorins [92–94]. Mukouyama et al reported that arteries, but not veins, are specifically aligned with peripheral nerves in embryonic mouse limb skin. Elimination of peripheral sensory nerves, or Schwann cells, by Neurogenin1/Neurogenin2 double homozygous knockouts results in defective arteriogenesis. Disorganization of the peripheral nerves by a mutation in the Semaphorin3A gene maintains the alignment of arteries with misrouted axons, indicating that nerves direct vascular remodeling patterns and arteriogenesis [95].

It is well known that the nerves that modulate blood vessel function release many kinds of neuropeptides, including secretoneurin, substance P, neuropeptide Y and calcitonin gene-related peptide. These neuropeptides have the ability to stimulate endothelial cell, fibroblast and SMC migration and proliferation in vitro [96–99]. These neuropeptides have also been shown to be involved in mediating the inflammatory process by enhancing the expression of adhesion molecules, such as VECAM [100,101]. They increase arteriolar density and branching when overexpressed in the heart [102], and attract monocytes, eosinophils and endothelial cells [103,104]. Recently these neuropeptides were reported to exhibit direct angiogenic properties stimulating neo-vascularization and inducing postnatal vasculogenesis [105,106], and to restore ischemic muscle blood flow and performance [107]. Taken together, these results suggest that neural regulation may be involved in collateral vessel growth.

**The Role of Growth Factors**

The occurrence of mitosis in both the endothelial and smooth muscle cells of collateral vessels indicates the presence of mitogens. However, none of the well-known growth factors like VEGF, FGF, PDGF etc, could be unanimously identified as being involved as a “master”
factor. VEGF-A protein is up-regulated in shear-stressed endothelium, and may enable endothelial mitosis and increased vascular permeability leading to regularly observed perivascular edema [81,108]. Although the VEGF receptor 2 is identifiable on the endothelial surface and could hence be responsible for endothelial mitosis and inactivation of this receptor inhibited arteriogenesis in rats [109], it is present as a protein complex sensing shear stress [24]. However, its up-regulation takes place when most of the mitotic activity is already over. The FGF receptor-1 is present in the collateral tissue, but arteriogenesis proceeds undisturbed in mice with targeted disruption of the FGF-1 and FGF-2 genes and in double knockouts [110]. Overexpression of FGF-2 in skeletal muscle does not accelerate arteriogenesis [25]. In contrast, PSA, a chemical agent that interferes with FGF-receptor binding, inhibits arteriogenesis in the rabbit hind limb model [111].

All mentioned growth factors accelerate collateral artery growth when exogenously applied, but blood flow recovery never reaches the levels obtained with high shear stress. Arteriogenic mitogens may be the actin-binding proteins Abra and Thymosin beta 4 [112]. Although these are intracellular proteins without hints from their sequence for secretion, they have mitogenic potency for SMC when applied to cells in culture. They share the absence of secretion motifs with FGF-1 and FGF-2. Furthermore, it is not clear to what kind of receptor they may bind to.

Therefore, the mitogens responsible for the natural process of arteriogenesis remain undiscovered.

**Arteriogenic pathways and signal transduction**

Several signaling cascades have to converge to form a new artery to defend against tissue ischemia caused by arterial obstruction (Fig. 1). At least two pathways originate at the flow/shear stressed endothelium: (1) the attraction, adhesion and invasion of bone marrow-derived cells that are needed for structural remodeling and (2) the pathway for endothelial and smooth muscle cell proliferation. The primary signal in response to shear stress is NO followed by

![Fig. 1 This image portrays the initiation of signals by deformation of the endothelial cells by increased fluid shear stress](image_url)

NO is rapidly produced and released, and induces VEGF which increases permeability leading to loss of osmotic control and cellular edema aided by Calcium activated cation channels. A protein complex, Tsimba complex, consisting of PECAM, E-cadherin and VEGFR, is upregulated some time later and monitors FSS [24]. About 6 h after onset of stimulation, the endothelium produces MCP-1 leading to the attraction of bone marrow-derived mononuclear cells that attach and penetrate the intimal layer. The underlying smooth muscle layer prepares for mitosis after changing its phenotype by inhibiting actin polymerization. Genes/proteins responsible for this are Abra, Thymosin beta 4, Cofilin and Desmin. SMC proliferation is preceded by activation of the mitogen activated protein kinases (MAPK) and by activation of the transcription factors Carp, asb5 and Egr-1. The Rho pathway is active and can be blocked by the tool drug Fasudil. Connexin37, a marker for the embryonal arterial system, is strongly re-expressed in the proliferating smooth muscle cells, suggesting a re-capitulation of the embryonal arterial development as the underlying pattern of collateral vessel development. The growth factor(s) responsible for SMC proliferation most probably bind to the FGF-Receptor-1, but several different other ligands of the large FGF family may also bind to it.
by expression of a protein complex consisting of E-cadherin, VEGFR2 and PECAM plus several transcription factors, such as CARP and klf2, whose precise function is not well known. NO leads to the production of VEGF that, together with calcium-activated ion channels, interferes with osmotic regulation of the endothelium. This leads to the synthetic phenotype of endothelial cells and the production of chemokines, such as MCP-1. The smooth muscle of the media likewise undergoes a phenotype change and enters the cell cycle. In preparation for mitosis, actin-binding proteins, such as abra and thymosin β4, play an important role, and the Rho pathway becomes activated. Arteriogenesis is inhibited by fasudil, a Rho-pathway inhibitor. Studies with cultured VSMC exposed to FGF-2 or PDGF showed activation of signaling cascades that were similarly observed in collateral vessel tissue that had developed after arterial occlusion under the influence of shear forces (Fig. 2). This comprised in particular the Ras/MEK/ERK pathway, the down-regulation of desmin, the polymerization of actin, up-regulation of the transcription factors CARP, SRE and EGR-1 [25]. The similarity between the in vivo and in vitro responses suggests the presence of similar growth factors, perhaps with the exception of PDGF because protein kinase B is down-regulated under conditions of high fluid shear stress. In contrast, neither targeted disruption nor transgenic overexpression of FGF-1 and FGF-2 influence arteriogenesis probably because other members of the FGF family of growth factors are able to substitute. We have anecdotal evidence that the triple knockout of FGF-5, 6 and 7 markedly inhibits arteriogenesis (the animals were a kind gift of Dr. E. Bober, Max-Planck-Institute, Bad Nauheim). It is of note that EGR-1 is up-regulated in the endothelium by shear stress via NO and in cultured SMC by FGF-2 and PDGF-AB [110]. This emphasizes the fact that we lack a clear understanding of how the shear stress signal is transmitted from the endothelium to the smooth muscle layer in the absence of cell-to-cell junctions, with the barrier of the internal elastic lamina and with the short reach of the highly reactive radical NO.

The importance of the MEK/ERK pathway is highlighted by the inhibition of smooth muscle mitosis after treatment

**Fig. 2** The cartoon describes the signaling pathways of vascular smooth muscle cells in culture under the stimulating influence of the growth factors FGF-2 or PDGF-AB. The signal elicited by FGF-2 resembles the situation in vivo with the activation of Ras, Raf, MEK and MAPK. Since Akt is downregulated in vivo under increased fluid shear stress, we assume that PDGF does not play a significant role. The observation that MEK1/2 becomes hyper-phosphorylated under UO126 suggests that MEK constitutes an important on/off switch for SMC proliferation (Figure adapted from Vogel et al [25].) It is of note that this pathway can be inhibited by NO as well as by NOS inhibitors.
with the MEK antagonists UO126 and PD98059. These antagonists induce hyper-phosphorylation at serine 217/221, which might act as an on/off switch depending on context (Fig. 2) Pharmacological inhibition of NOS blocks the FSS-cascade regardless of the isoform. Blockade of bone marrow-derived cells also markedly inhibits arteriogenesis.

Summary

Under favorable conditions (e.g. slowly progressing coronary occlusion), arteriogenesis is able to salvage tissue through the rapid growth of collateral vessels. In the vascular periphery, even acute occlusions are tolerated and full function may return within days or weeks due to collateral development. Understanding of the molecular mechanisms has progressed in recent years, but one important step is still not well understood: how forces acting on the endothelium are transmitted to the smooth muscle tunica media. Under the influence of pressure gradients that occur after arterial occlusions, blood flow in pre-existent arteriolar connections is increased and, with it fluid shear stress transforms these vascular structures into arteries that are potentially able to replace the occluded vessel. The tissue surrounding growing collateral vessels does not become ischemic, and arteriogenesis does not depend on tissue oxygenation. High fluid shear stress activates ion channels, releases NO and starts at least two signaling pathways: one that attracts bone marrow-derived cells for remodeling and another that causes endothelial and smooth muscle cells to enter the cell cycle, leading to proliferation. The transformation of a small arteriole into a large blood flow transporter proceeds by controlled destruction of the old structure by digestion of the elastic scaffold and apoptosis of non-mitotic SMC. As producers of mitogens and proteases, invading monocytes and lymphocytes are crucial for the transformation. The role of angiogenic growth factors remains unsolved, and arteriogenesis differs in this aspect and in several others from angiogenesis. Signaling pathways for arteriogenesis involve the MAPKinasases, the Rho-pathway and NO-dependent pathways. The latter two may be worth studying to design stimulators of arteriogenesis.

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