invited review

Molecular analysis of blood vessel formation and disease

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Carmeliet, Peter, and Désiré Collen. Molecular analysis of blood vessel formation and disease. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2091–H2104, 1997.—Blood vessels affect the quality of life in many ways. They provide an essential nutritive function during growth and repair of tissues but, on the other hand, can become affected by disorders or trauma, resulting in bleeding, thrombosis, arterial stenosis, and atherosclerosis. Three molecular systems, the vascular endothelial growth factor (VEGF) system, the plasminogen system, and the coagulation system, have been implicated in the formation and pathobiology of blood vessels. This review focuses on the role of these systems in these processes. Recent gene-targeting studies have identified VEGF as a potent modulator of the formation of endothelial cell-lined channels. Somewhat unanticipated, the initiator of coagulation is not only involved in the control of hemostasis but also in the maturation of a muscular wall around the endothelium. With different murine models of cardiovascular disease, a pleiotropic role of the plasminogen system was elucidated in thrombosis, in arterial neointima formation after vascular wound healing and allograft transplantation, in atherosclerosis, and in the formation of atherosclerotic aneurysms. Surprisingly, tissue-type plasminogen activator is also involved in brain damage after ischemic or neurotoxic insults. The insights from these gene-targeting studies have formed the basis for designing gene therapy strategies for restenosis and thrombosis, which have been successfully tested in these knockout models.

targeted gene manipulation in animal models has largely contributed to a better understanding of the molecular mechanisms involved in the formation as well as the pathology of blood vessels. Several of these factors play distinct roles not only during embryonic development but, frequently, also during adult vascular pathology. The vessel wall consists of two major cell types, endothelial and smooth muscle cells, that closely interact with each other. The vascular endothelial growth factor (VEGF) system is essential for endothelial cell biology, and defective signaling results in abnormal formation of endothelial cell-lined channels. In contrast, the initiator of coagulation plays a role in smooth muscle cell function, and loss of its function during embryogenesis results in immature fragile vessels that form microaneurysms and rupture with secondary bleeding. The coagulation and plasminogen systems are essential for proper hemostasis because the former controls bleeding by formation of fibrin dots and the latter guarantees maintenance of vascular patency by removal of fibrin dots. When deregulated, both systems may contribute to thrombotic or hemorrhagic disorders. In addition, these systems have been implicated in tissue remodeling and cellular migration, essential mechanisms for the repair of blood vessels after acute injury (e.g., balloon angioplasty) or chronic inflammation (atherosclerosis). The present overview aims at integrating the pleiotropic roles of the VEGF, coagulation, and plasminogen systems in vascular biology, as deduced from targeted gene manipulation (gene inactivation or gene transfer) studies in the mouse.

THE VEGF SYSTEM

VEGF-A was purified as a growth factor able to increase vascular permeability and endothelial cell proliferation (45, 49; Fig. 1A). VEGF-A distinguishes itself from previously known angiogenic factors by a unique combination of properties: 1) it is secreted and exerts a direct effect on endothelial cells via interaction with cellular receptors Flk-1 and Flt-1 (45, 49); 2) it is produced by cells in close proximity to endothelial cells, suggesting paracrine regulation of blood vessel formation (10); 3) expression of VEGF-A is highly regulated by hypoxia, providing a physiological feedback mechanism to accommodate insufficient tissue oxygenation by promoting blood vessel formation (95); and 4) VEGF-A...
is a potent factor, because its over- or underexpression significantly affects blood vessel formation in vivo (52). VEGF-A is transcribed from a single gene but is alternatively processed to various isoforms. The shortest form (VEGF-A120) is diffusible in the surrounding extracellular milieu, whereas the longer isoforms (VEGF-A165, VEGF-A189, and VEGF-A206) contain basic amino acid residues that mediate increasing binding to heparin-rich extracellular matrix (45, 49). Although these various isoforms exhibit a tissue-specific pattern of expression, their differential (patho)physiological roles in vivo remain largely undetermined.

More recently, other VEGF-related factors have been identified (Fig. 1A): VEGF-B, which is expressed primarily in the heart, brain, muscle, and kidney (76), and VEGF-C, which appears to play a role in development of lymphatic vessels (61). Another homolog, placental growth factor (PIGF), was identified in the placenta and, to a lesser extent, in heart, lung, and thyroid (71). Three receptor tyrosine kinases, containing seven immunoglobulin domains, have thus far been identified that bind the VEGF family members with different specificity and affinity; VEGF receptor-1 (or Flt-1) binds VEGF-A and PIGF, VEGF receptor-2 (or Flk-1) binds VEGF-A and VEGF-C, and VEGF receptor-3 (or Flt-4) binds VEGF-C (41, 56, 72). Homo- or heterodimerization of these ligands may determine their biological specificity (44). Receptor activation, via intracellular signaling, results in a pleiotropic pattern of angiogenic activities including proliferation, apoptosis, migration, and permeability of endothelial cells and their production of matrix-degrading proteinases and tissue factor (TF; Refs. 45, 49).

VEGF expression has been implicated in vascular development during embryogenesis and postnatally during heart ischemia (5), atherosclerosis (64), diabetic retinopathy (50), tumorigenesis (79), arthritis, and wound healing (54). VEGF gene therapy has been considered to improve blood flow to ischemic limbs and hearts (100) and to reduce stenosis of blood vessels after arterial injury by promoting reendothelialization (4).

THE COAGULATION SYSTEM

Initiation of the plasma coagulation system is triggered by TF, which functions as a cellular receptor and cofactor for activation of the serine proteinase factor VII to factor VIIa (Refs. 40, 46, 55; Fig. 2A). This complex activates factor X directly or indirectly via activation of factor IX, resulting in the generation of thrombin, which mediates conversion of fibrinogen to fibrin. Factors X and IX are activated by the tissue factor pathway and the contact activation pathway, respectively. Thrombin, in turn, cleaves fibrinogen to fibrin.

Stabilization of the prethrombotic state is achieved by feedback inhibition of factor X activation via factor V activation by TFPI, the anticoagulant TFPI, plasminogen activator inhibitor-1 (PAI-1), and tissue factor pathway inhibitor (TFPI). PAI-1 is a serine protease inhibitor that inhibits the action of plasminogen activators, including urokinase and tissue plasminogen activator (t-PA).

Plasminogen is activated by t-PA and u-PA to generate plasmin, which in turn cleaves fibrinogen to fibrin.

The coagulation system is a complex network of protein-protein interactions that regulate blood clotting and hemostasis.
fibrin (40, 46, 55). Thrombin and factor Xa produce a positive-feedback stimulation of coagulation by activating factor VIII and factor V, which serve as membrane-bound receptors/cofactors for the proteolytic enzymes factor IXa and factor Xa, respectively (40, 46, 55).

In contrast, when thrombin is bound to its cellular receptor thrombomodulin, it functions as an anticoagulant by activating the protein C anticoagulant system (39, 47). Activated protein C, in the presence of its cofactor protein S, inactivates factor Va and factor VIIIa, thereby reducing thrombin generation (39, 47). Anticoagulation is further provided by antithrombin III, which binds to and inactivates thrombin, factor IXa, and factor Xa in a reaction that is greatly enhanced by heparin (78). Anticoagulation is further secured by TF pathway inhibitor, which inhibits factor Xa and, in a factor Xa-dependent manner, produces feedback inhibition of the factor VIIa/TF catalytic complex (11). A revised hypothesis of coagulation has been suggested in which factor VIIa/TF is responsible for the initiation of coagulation but, because of TF pathway inhibitor-mediated feedback inhibition, amplification of the procoagulant response through the actions of factor VIII, factor IX, and factor XI is required for sustained hemostasis (11). Deficiencies of anticoagulant factors or disturbed expression of procoagulant factors have been implicated in thrombosis during inflammation, sepsis, atherosclerosis, and cancer (7, 46), whereas deficiencies of procoagulant factors have been related to increased bleeding tendencies (9, 60). Evidence has been provided that the coagulation system may also be involved in other functions beyond hemostasis, including cellular migration and proliferation, immune response, angiogenesis, embryonic development, metastasis, and brain function (1, 37, 46). Its precise role and relevance in these processes in vivo remain, however, largely unknown.

THE PLASMINOGEN SYSTEM

The plasminogen system is composed of an inactive proenzyme, plasminogen, that can be converted to plasmin by either of two plasminogen activators (PA), tissue-type PA (t-PA) or urokinase-type PA (u-PA) (34, 105; Fig. 2B). This system is controlled at the level of PAs by PA inhibitors (PAIs), of which PAI-1 is believed to be physiologically the most important (65, 90, 107), and at the level of plasmin by α2-antiplasmin (34). Because of its fibrin specificity, t-PA is primarily involved in clot dissolution, although it has also been involved in ovulation, bone remodeling, and brain function (34, 105). Cellular receptors for t-PA and plasminogen have been identified that might localize plasmin proteolysis to the cell surface (57, 82). u-PA also binds a cellular receptor, the urokinase receptor (u-PAR), and has been implicated in pericellular proteolysis during cell migration and tissue remodeling in a variety of normal and pathological processes including angiogenesis, atherosclerosis, and restenosis (8, 104). u-PAR binds to vitronectin (106), whereas PAI-1 controls recognition of vitronectin by u-PAR or the αvβ3 integrin receptor, suggesting a role in coordinating cell adhesion and migration (97). It is presently unclear whether or under what conditions binding of u-PA to u-PAR is required in vivo. Plasmin is able to degrade fibrin and extracellular matrix proteins directly or indirectly via activation of other proteinases (such as the metalloproteinases; Refs. 30, 87). Plasmin can also activate or liberate growth factors from the extracellular matrix, including latent transforming growth factor-β (TGF-β), basic fibroblast growth factor, and vascular endothelial growth factor (49, 87). Cell-specific clearance of plasminogen activators or of complexes with their inhibitors by low-density lipoprotein receptor-related protein or gp330 may modulate pericellular plasmin proteolysis (2).

TARGETED MANIPULATION AND ADENOVIRUS-MEDIATED TRANSFER OF GENES IN MICE

Novel gene technologies have allowed the manipulation of the genetic balance of candidate molecules in mice in a controlled manner. Homologous or site-specific recombination in embryonic stem cells allows the study of the consequences of deficiencies, mutations, and conditional or tissue-specific expression of gene products in transgenic mice (15, 73). With a novel embryonic stem cell technology (aggregation of embryonic stem cells with tetraploid embryos), it has become possible to generate completely embryonic stem cell-derived embryos in a single step. In addition, the technology allows the bypass of conventional germ line transmission, to separate extra- from intraembryonic phenotypes and to study homozygous deficient phenotypes of genes that cause embryonic lethality when heterozygously deficient (18, 74). Viral gene transfer can also be used to manipulate the expression of genes, e.g., via implantation of retrovirally transduced cells or via adenovirus-mediated gene transfer in vivo (88). In fact, intravenous administration of a recombinant adenovirus results in expression of target genes to plasma levels >10 μg/ml. Such studies allow us not only to generate but also to rescue disease models and to evaluate possible gene-transfer therapies.

FORMATION OF BLOOD VESSELS AND HEMOSTASIS

Formation of endothelial cell-lined channels during embryogenesis and wound healing. After initial differentiation of stem cells into endothelial cells and their assembly into endothelial cell-lined channels (vasculogenesis), the embryonic vasculature further develops via sprouting of new channels from preexisting vessels (angiogenesis; Ref. 84, Fig. 1B). This latter process is recapitulated in adulthood during tissue neovascularization. The genetic mechanisms controlling these processes are, however, poorly defined. With the advent of the recently developed gene technology to generate mice with inactivation or mutation of target genes, remarkable progress has been made in the understanding of the molecular processes in blood vessel formation. The following discussion briefly summarizes recent findings on the role of the VEGF-PIGF system in
blood vessel development during embryogenesis and wound healing (Fig. 3).

Targeted inactivation of a single VEGF-A allele resulted in haploinsufficiency with resultant embryonic lethality caused by abnormal blood vessel development at a time of active vascular development (Refs. 18, 21; Fig. 3A). Deficiency of VEGF-A did not abort but significantly delayed endothelial cell differentiation, affected the formation of large vessels in the yolk sac (possibly via an effect on endothelial cell fusion or intussusception), impaired lumen formation of large vessels, compromised sprouting and branching of new vessels from preexisting vessels, and possibly induced abnormal vascular connections with the heart (18, 21). Because the embryonic lethality of heterozygous VEGF-A-deficient embryos precluded the analysis of homozygous VEGF-A deficiency by conventional transgenic technologies, a novel technology developed by Nagy and Rossant (74) was used to generate homozygous VEGF-A-deficient embryos via aggregation of mutant embryonic stem cells with tetraploid embryos. A significant correlation between the number of endothelial cells and their assembly into blood vessels and the number of VEGF-A alleles was observed, suggesting that VEGF-A is a potent and dominant modulator of blood vessel formation and implying a strict regulation of its expression (18, 21). In a parallel study, Ferrara et al. (51) demonstrated that growth and vascularization of embryonic stem cell-derived teratocarcinomas was impaired by deficiency of VEGF-A, suggesting that growth of these solid tumors was linked to VEGF-induced neovascularization. In collaboration with P. d’Amore (Harvard Univ., Boston, MA), we have extended these studies by generating mice that only express the VEGF-A120 isoform. Initial analysis indicates that mice expressing one wild-type VEGF-A allele (generating all VEGF isoforms) and one mutated VEGF-A120 allele are viable and apparently normal, suggesting that VEGF-A120 is able to rescue the defective embryonic vascular development in heterozygous VEGF-A-deficient embryos (unpublished observations). In contrast, homozygous VEGF-A120 mice appear to die shortly after birth, suggesting that the different VEGF-A isoforms play distinct physiological roles.

These studies have identified an important role for VEGF-A during embryonic vascular development. The question of whether VEGF-A plays a similar central role during neovascularization in adult (patho)physiology must await the generation of novel transgenic mice.

Fig. 3. Overview of VEGF targeting studies. Deficiency of VEGF-A results in haploinsufficient embryonic lethality because of delayed endothelial cell development and abnormal assembly, lumen formation, sprouting, and organization of vascular channels. Deficiency of PlGF results in normal embryonic development and fertility but impairs skin wound healing, possibly via abnormal formation of granulation tissue and neovascularization. Deficiency of Flk-1 results in embryonic lethality caused by abortive endothelial cell development and lack of blood vessels. This phenotype is different from that of VEGF-A deficiency, suggesting presence of other Flk-1 ligand(s). Deficiency of Flt-1 results in embryonic lethality because of abnormal organization of endothelial cells in vascular channels. There appear to be more endothelial cells.
with more sophisticated tissue-specific or conditional VEGF-A expression.

Targeting of the VEGF receptors Flt-1 and Flk-1 also resulted in embryonic lethality caused by abnormal vascular development (Refs. 53, 93; Fig. 3). In Flk-1-deficient embryos only early endothelial cell precursors were present, but they failed to differentiate and assemble into functional vascular channels (93), indicating an essential role for this receptor in the first steps of blood vessel development. In contrast, endothelial cells developed further in Flt-1-deficient embryos, but they failed to assemble into normal vascular channels (53). In fact, endothelial cells appeared more numerous and occluded the lumen, suggesting a possible inhibitory role of Flt-1 in endothelial cell proliferation or assembly (53). Interestingly, the observation that VEGF-A deficiency did not result in a phenotype similar to that of the Flk-1 deficiency suggests the presence of other ligands for Flk-1 or, alternatively, rescue by maternal VEGF-A.

In an initial analysis (in collaboration with G. Persico), we have found that homozygous PIGF-deficient mice develop properly, are fertile, and reveal no placental defects (unpublished observations; Fig. 3). This was not anticipated, in light of the presumed role of PIGF in establishing vascular connections in the placenta (71). However, healing of skin wounds was delayed in PIGF-deficient mice, possibly because of impaired formation of granulation tissue and neovascularization. VEGF is expressed by keratinocytes and infiltrating monocytes/macrophages in these wounds and improves wound healing by induction of neovascularization (as revealed by the impaired neovascularization and wound healing in ob/ob mice) (54). In addition, VEGF may increase the vascular permeability to plasma proteins such as fibronectin that form constituents of the healing wound matrix. The impaired wound-healing model in PIGF-deficient mice may provide a suitable model to analyze the role of VEGF and PIGF in these processes.

Hemostasis: vessel fragility vs. clot formation. Once the endothelial cells are assembled into vascular channels, they become surrounded by smooth muscle cells/pericytes that may affect maturation of the blood vessels not only by providing the fragile primitive blood vessels the structural support required to accommodate the increased blood pressure but also by controlling endothelial cell proliferation, vascular permeability, and tone (Ref. 75; Fig. 1B). Although a role for vascular smooth muscle cells/pericytes in the pathogenesis of vasculopathies during adulthood, including atherosclerosis, restenosis, and diabetic retinopathy, has been documented, their role during vascular development has been poorly characterized. Surprisingly, targeted gene inactivation of some coagulation factors has revealed their possible implication in blood vessel development. Because these coagulation factors are also expressed during restenosis or atherosclerosis, a better understanding of their role during embryogenesis may help us to understand their function during adult disease processes and to design appropriate therapeutic strategies.

TF is the primary cellular activator of the blood coagulation system, resulting in fibrin formation (Fig. 2A). Indirect evidence suggests, however, that TF may also be involved in nonhemostatic processes: 1) it is a member of the immunoglobulin superfamily and is expressed as an immediate-early gene during inflammation and immune challenge (46); (2) its intracellular domain mediates signaling during metastasis or cellular activation (86); 3) it may participate in tumor neovascularization, possibly via an effect on VEGF expression (36, 108); and 4) it is expressed in a variety of embryonic tissues including the visceral endoderm cells in the yolk sac that surround the endothelium and, at later stages, in the smooth muscle cells of larger blood vessels (70).

Targeted inactivation of the TF gene resulted in increased fragility of the endothelial cell-lined channels in the yolk sac, which are essential for transferring maternally derived nutrients from the yolk sac to the rapidly growing embryo (Refs. 20, 21; Fig. 4, A and B). At a time when the blood pressure increased during embryogenesis (9th day of gestation), the immature TF-deficient blood vessels ruptured, formed microaneurysms and “blood lakes,” and failed to sustain proper circulation between the yolk sac and embryo (Fig. 4, A and B). Secondarily, the embryo became wasted and died because of generalized necrosis. Only in advanced stages of deterioration did the immature blood vessels become leaky, resulting in bleeding into the extra-embryonic cavity. Similar observations were made when TF-deficient embryos were cultured in vitro, suggesting that the observed vascular defects in the yolk sac were not merely caused by a possible defect in fetomaternal exchange.

During normal embryogenesis, the endothelium in yolk sac vessels is surrounded by mesenchymal cells (smooth muscle cells/pericyte-like cells) that form a primitive “muscular” wall and provide structural support by their close physical association and increasing production of extracellular matrix proteins (Fig. 4B). Microscopic and ultrastructural analysis revealed that deficiency of TF resulted in a 75% reduction of the number of mesenchymal cells and a diminished amount of extracellular matrix (20). Immunohistochemical analysis further revealed a reduced level of smooth muscle a-actin staining in these cells, suggesting impaired differentiation or accumulation (20). In contrast, visceral endoderm and endothelial cells appeared normal. Because these primitive smooth muscle cells provide structural support for the endothelium, the vessels in the mutant embryos are too fragile and break open at a time during development when the blood pressure is increased because of more regular and vigorous heart contractions and increased blood cell viscosity (Fig. 4B). Inappropriate vascular fragility as a result of mesenchymal cell/pericyte defects also results in bleeding in platelet-derived growth factor-deficient embryos (Ref. 66 and C. Betsholtz, personal communication) and possibly also in the TGF-β-deficient embryos (43), although the precise cellular mechanism in the latter was not resolved. The important role of mesenchymal
cells/pericytes in vessel formation is further underscored by a recent report that angiopoietin-1-deficient embryos display defects in vessel maturation and branching because of impaired intussusception by periendothelial mesenchymal cells (99). Pericytes have been also implicated in adult diabetic retinopathy when pericyte “drop out” results in the formation and rupture of microaneurysms and blindness (75). Unresolved questions include how TF exerts this morphogenic action, i.e., via intracellular signaling as suggested previously (86), and/or whether fibrin formation occurs and is essential during early vascular
development, as suggested by others (Ref. 14, 101; Fig. 4C). In addition, it is unknown whether TF (only) interacts with its (only currently known) ligand, factor VII (Fig. 3A). Indeed, analysis of factor VII-deficient mice reveals that they develop normally until birth and die early postnatally because of massive hemorrhaging (E. Rosen, J. Chan, E. Iducogie, F. Clotman, G. Vlassuk, T. Lüther, L. R. Albright, L. Zhong, A. Lissens, L. Schoonjans, L. Moons, D. Collen, F. J. Castellino, and P. Carmeliet, unpublished observations). Whether this means that TF acts independently of factor VII or of its hemostatic properties or whether embryogenesis of factor VII-deficient embryos is rescued by placental transfer of maternal factor VII remains to be determined. Our preliminary analysis reveals, however, that intravenous injection in pregnant mice of human recombinant factor VIIa induces supraphysiological plasma levels in the mother but only background levels in the embryo (unpublished observations).

Other coagulation factors appear also to be involved in morphogenic processes during early embryogenesis, possibly also in blood vessel formation. Indeed, deficiency of factor V (38) and of the thrombin receptor (35) both resulted (in ~50% of homozygous deficient embryos) in abnormal yolk sac vascular development around a similar developmental stage as in TF-deficient embryos. The leakage of blood from the defective blood vessels in TF-deficient embryos (“vascular” bleeding) contrasts with the postnatal bleeding in mice deficient in factor VII, factor VIII (6) and fibrinogen (98) and in the surviving fraction of factor V (38) deficient mice, which occurs because of defective clot formation after trauma of normally developed blood vessels (“hemostatic” bleeding). Bleeding in the latter mice occurred shortly after birth (factor V, factor VII, and fibrinogen) or was associated with injury (factor VII). Bleeding in factor V- and factor VII-deficient neonates is more severe than in fibrinogen-deficient mice, possibly suggesting an essential role for thrombin in hemostasis beyond generation of fibrin. Thus it appears from these targeting studies that several coagulation factors (TF, factor V, and thrombin receptor) participate in morphogenic processes beyond control of hemostasis, whereas other coagulation factors (factor VIII, fibrinogen) play a predominant role in hemostasis via clot formation. This raises an interesting question as to whether TF and the thrombin receptor, which are expressed during restenosis and atherosclerosis, also play a similar (nonhemostatic) role in these processes. If so, this could open an attractive therapeutic avenue to selectively inhibit the hemostatic or morphogenic properties of these molecules.

**THROMBOSIS AND THROMBOLYSIS**

Fibrin deposits and pulmonary plasma clot lysis in transgenic mice. Deficient fibrinolytic activity, e.g., resulting from increased plasma PAI-1 levels or reduced plasma t-PA or plasminogen levels, might participate in the development of thrombotic events (3, 65, 90, 107). Fibrin surveillance in different knockout mice was analyzed in quiescent conditions and after challenge (Fig. 5). In unstressed conditions, u-PA-deficient mice developed occasional minor fibrin deposits in liver and intestines and excessive fibrin deposition in chronic, nonhealing skin ulcerations, whereas in t-PA-deficient mice, no spontaneous fibrin deposits were observed (16, 27). Mice with a single deficiency of plasminogen or a combined deficiency of t-PA and u-PA, however, revealed extensive intravascular and extravascular fibrin deposits in several organs (Refs. 13, 16, 27, and 81 and unpublished observations). Interestingly, mice with a combined deficiency of t-PA and u-PAR did not display such excessive fibrin deposits, suggesting that sufficient plasmin proteolysis can occur in the absence of u-PA binding to u-PAR (Fig. 5B; Refs. 12, 42). Loss of both PAs or of plasminogen severely affected general health and caused a multiorgan dysfunction syndrome characterized by dyspnea, anemia, sterility, cachexia, and premature death (Fig. 5A).

After traumatic or inflammatory challenge, mice with a single deficiency of t-PA or u-PA were significantly more susceptible to venous thrombosis, e.g., after local injection of proinflammatory endotoxin in the footpad (27; Fig. 5A). Significant fibrin and matrix deposition was present in plasminogen-deficient mice after skin wounds (85) or during experimental glomerulonephritis (63). Similar to plasminogen-deficient patients, plasminogen-deficient mice also suffered increased and prolonged arterial thrombosis, but only after injury (25). The requirement of injury for arterial thrombosis in plasminogen-deficient mice may relate to the fact that mice, in contrast to humans, do not normally develop vasculopathies such as atherosclerosis, which can provide highly thrombogenic surfaces, e.g., on ruptured plaques.

The increased thrombotic susceptibility of t-PA-deficient and of combined t-PA + u-PA- or plasminogen-deficient mice can be explained by their significantly reduced rate of spontaneous lysis of 125I-labeled fibrin pulmonary plasma clots (16, 27). On the contrary, PAI-1-deficient mice were virtually protected against development of venous thrombosis after injection of endotoxin, consistent with their ability to lyse these plasma clots at a significantly higher rate than wild-type mice (19, 29). The increased susceptibility of u-PA-deficient mice to thrombosis associated with inflammation or injury might be caused by their impaired macrophage function. Indeed, thioglycollate-stimulated macrophages (which are known to express cell-associated u-PA) isolated from u-PA-deficient mice lacked plasminogen-dependent breakdown of 125I-labeled fibrin (fibrinolysis) or of 3H-labeled subendothelial matrix (mostly collagen synthesis), whereas macrophages from t-PA- or PAI-1-deficient mice did not (16, 27).

Lipoprotein(a) contains the lipid and protein components of low-density lipoprotein plus apolipoprotein(a) (68). Extensive homology of apolipoprotein(a) to plasminogen has prompted the proposal that apolipopro-
Adenovirus-mediated transfer of t-PA or PAI-1. More recently, we have used adenovirus-mediated transfer of fibrinolytic system components in these knockout mice in an attempt to revert their phenotypes. Intravenous injection of adenoviruses expressing a recombinant PAI-1-resistant human t-PA (rt-PA) gene in t-PA-deficient mice increased plasma rt-PA levels 100- to 1,000-fold above normal and restored their impaired thrombolytic potential in a dose-related manner (28). Conversely, adenovirus-mediated transfer of recombinant human PAI-1 in PAI-1-deficient mice resulted in 100- to 1,000-fold increase in plasma PAI-1 levels above normal and efficiently reduced the increased thrombolytic potential of PAI-1-deficient mice (unpublished observations).

**NEOINTIMA FORMATION**

Vascular interventions for the treatment of atherosclerotic disease induce "restenosis" of the vessel within 3–6 mo in 30–50% of treated patients (33, 67). This may result from remodeling of the vessel wall (such as occurs predominantly after balloon angioplasty) and/or accumulation of cells and extracellular matrix in the intimal layer (such as occurs predominantly after intraluminal stent application). Proteinases may participate in proliferation and migration of smooth muscle cells.
endothelial cells and in matrix remodeling during this wound healing response (103). Two proteinase systems have been implicated, the plasminogen (or fibrinolytic) system and the metalloproteinase system, which in concert can degrade most extracellular matrix proteins. In contrast to the constitutive expression of t-PA by quiescent endothelial cells (34, 105) and of PAI-1 by uninjured vascular smooth muscle cells (90, 96), u-PA and t-PA activity in the vessel wall are significantly increased after injury, coincident with the time of smooth muscle cell proliferation and migration (32). This increase in plasmin proteolysis is counterbalanced by increased expression of PAI-1 in injured smooth muscle and endothelial cells and by its release from accumulating platelets (48).

u-PA-mediated plasmin proteolysis promotes arterial neointima formation. Two experimental models of arterial injury have been used, one based on application of an electric current (26) and the other based on an intraluminal guide wire (23, 24), to examine the molecular mechanisms of neointima formation in mice deficient in fibrinolytic system components. The electric current injury model differs from mechanical injury models in that it induces a more severe injury across the vessel wall, resulting in necrosis of all smooth muscle cells. This necessitates wound healing to initiate from the adjacent uninjured borders and to progress into the central necrotic region. Microscopic and morphometric analysis revealed that the rate and degree of neointima formation and the neointimal cell accumulation after injury were similar in wild-type, t-PA-deficient, and u-PAR-deficient arteries (Refs. 22 and 23 and unpublished data). However, neointima formation in PAI-1-deficient arteries occurred earlier after injury (24). In contrast, both the degree and the rate of arterial neointima formation in u-PA-, plasminogen-, and combined t-PA + u-PA-deficient arteries was significantly reduced until 6 wk after injury (22, 23, 25). Infiltration of the media by leukocytes was also significantly reduced in plasminogen-deficient mice (25). Similar genotypic differences were obtained after mechanical injury (23, 24), which more closely mimics the injury in patients.

Evaluation of the mechanisms responsible for these genotype-specific differences in neointima formation revealed that proliferation of medial and neointimal smooth muscle cells was only marginally different between the genotypes (22–25). Impaired migration of smooth muscle cells is a likely cause of reduced neointima formation in mice lacking u-PA-mediated plasmin proteolysis, because smooth muscle cells migrated over a shorter distance from the uninjured border into the central injured region in plasminogen-deficient than in wild-type arteries (22, 23). In addition, migration of cultured u-PA-deficient smooth muscle cells but not t-PA- or u-PAR-deficient smooth muscle cells in vitro was impaired after scrape wounding (22, 23). Although our results demonstrate that migration of smooth muscle cells requires plasmin proteolysis, it is possible that PAI-1 may also influence cellular migration by effecting cell adhesion through interaction with the αvβ3-integrin receptor (97). That u-PAR-deficient arteries developed a similar degree of neointima suggests that sufficient pericellular plasmin proteolysis can still occur in the absence of binding of u-PA to its cellular receptor. Somewhat surprisingly, no genotypic differences were obtained in reendothelialization (22–25), suggesting a cell-type specific requirement of plasmin proteolysis for cellular migration. Figure 6 schematically represents a hypothetical model of smooth muscle cell function and neointima formation in the absence of u-PA or u-PAR.

Inhibition of neointima formation by adenovirus-mediated PAI-1 gene transfer. The involvement of plasmin proteolysis in neointima formation was supported by intravenous injection in PAI-1-deficient mice of a replication-defective adenovirus that expresses human PAI-1, which resulted in >100- to 1,000-fold increased plasma PAI-1 levels and in a similar degree of inhibition of neointima formation as observed in u-PA-deficient mice (97). Proteinase inhibitors have been suggested as antirestenosis drugs. Our studies suggest that strategies aimed at reducing u-PA-mediated plasmin proteolysis may reduce intimal thickening. However, antifibrinolytic strategies should be aimed at inhibiting plasmin proteolysis and not at preventing the interaction of u-PA with its receptor.

Transplant atherosclerosis in plasminogen-deficient mice. More recently, we (in a collaboration with V. Shi and E. Haber, Harvard Univ., Boston, MA) have started to analyze the role of the plasminogen system in a mouse model of transplant arteriosclerosis that mimics in many ways the accelerated arteriosclerosis in coronary arteries of transplanted cardiac allografts in humans (94). In this model, host-derived leukocytes infiltrate beneath the endothelium and form a predominantly leukocyte-rich neointima within 15 days after transplantation, whereas at later times, smooth muscle cells derived from the donor graft accumulate in the neointima. Because previous targeting studies have shown that migration of leukocytes and smooth muscle cells is dependent on plasmin proteolysis (22–25, 80), carotid arteries from B10.A(2R) wild-type mice were transplanted into C57BL6:129 plasminogen-deficient mice. Initial analysis suggests that neointima formation within 45 days after transplantation is reduced in these mice, suggesting a significant role for plasmin proteolysis in this process. Whether cellular migration, proliferation, or matrix remodeling is affected remains to be determined.

ATHEROSCLEROSIS

Epidemiologic, genetic, and molecular evidence suggests that impaired fibrinolysis resulting from increased PAI-1 or reduced t-PA expression or from inhibition of plasminogen activation may contribute to the development and/or progression of atherosclerosis (59, 62, 91), presumably by promoting thrombosis or matrix deposition. Indeed, plasma levels of PAI-1 are elevated in patients with ischemic heart disease, angina pectoris, and recurrent myocardial infarction (58). Recent genetic analyses revealed a link between poly-

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morphisms in the PAI-1 promoter and the susceptibility of atherothrombosis (59). A possible role for increased plasmin proteolysis in atherosclerosis is, however, suggested by the enhanced expression of t-PA and u-PA in plaques (69, 89). Plasmin proteolysis might indeed participate in plaque neovascularization, induction of plaque rupture, or ulceration and formation of aneurysms (69, 89). A causative role of the plasminogen system in these processes has, however, not been conclusively demonstrated.

Atherosclerosis was studied in mice deficient in apolipoprotein E (apoE; Ref. 83) and in t-PA, u-PA, or PAI-1 and fed a cholesterol-rich diet for 5–25 wk (P. Carmeliet, L. Moons, R. Lijnen, J. Crawley, V. Lemaitre, P. Tipping, A. Drew, Y. Eeckhout, S. Shapiro, F. Lupu, and D. Collen, unpublished observations). No differences in the size or predilection site of plaques were observed between mice with a single deficiency of apoE or of apoE and u-PA or of apoE and t-PA. However, significant genotypic differences were observed in the destruction of the media with resultant erosion, transmedial ulceration, medial smooth muscle cell loss, dilatation of the vessel wall, and microaneurysm formation. At the ultrastructural level, elastin fibers were eroded, fragmented, and completely degraded, whereas collagen bundles and glycoprotein-rich matrix were disorganized and scattered. Although both apoE- and apoE + t-PA-deficient mice developed severe media destruction, apoE + u-PA-deficient mice were virtually completely protected. Plaque macrophages expressed abundant amounts of u-PA mRNA, antigen, and activity at the base of the plaque and in the media, similar to the atherosclerotic, aneurysmatic arteries in patients (69, 89). Macrophages crossed the elastin fibers but only after proteolytic digestion of the elastin, a process that was remarkably enhanced by u-PA. u-PA-generated plasmin may mediate degradation of glycoproteins, surrounding elastin fibers in the aortic wall, thereby exposing the highly insoluble elastin to elastases and facilitating elastolysis in vivo (31). In addition, plasmin promoted degra-

![Fig. 6. Receptor-independent u-PA promotes neointima formation. SMC is surrounded by an extracellular matrix (ECM) that must be proteolytically degraded to allow cellular migration. In wild-type (WT) SMC, u-PA is bound to u-PAR, mediating Plg activation and plasmin degradation of extracellular matrix such that cells can migrate. In u-PAR-deficient (u-PAR−/−) SMC, u-PA becomes localized to cell surface, possibly via interaction with other matrix molecules (denoted as “X”), allowing sufficient pericellular plasmin proteolysis for cells to migrate. u-PA might also accumulate to increased levels because of deficient u-PA-mediated clearance. In contrast, SMC that lack u-PA (u-PA−/−) have reduced pericellular plasmin proteolysis and fail to migrate efficiently, resulting in reduced neointima formation. Proposed impairment of SMC migration in mice lacking u-PA-mediated Plg activation is suggested by observations that proliferation of u-PA-deficient cells was similar to WT and that SMC migrated over a shorter distance in the Plg-deficient than in WT arteries. Reproduced with permission from Ref. 17.](https://ajpheart.physiology.org/)

![Fig. 7. Hypothetical role of u-PA in aneurysm formation. u-PA-mediated plasmin proteolysis might be involved in media destruction by degradation of extracellular matrix (including insoluble elastin) directly by degradation of fibrin and glycoproteins surrounding elastin fibrils and/or indirectly by activation of other matrix degrading proteases, including of elastolytic metalloproteinases (gelatinases and macrophage metalloelastase). By mediating infiltration in atherosclerotic vessel wall of cells that produce these matrix degrading proteases, u-PA may further amplify destruction of media.](https://ajpheart.physiology.org/)
tion of elastin and collagen via activation of matrix metalloproteinases (MMP) such as stromelysin-1 (MMP-3), gelatinase-B (MMP-9), collagenase-3 (MMP-13), and the macrophage metalloelastase (MMP-12) (unpublished observations and Refs. 30, 92). The expression of several of these metalloproteinases was induced in situ in advanced atherosclerotic plaques by macrophages that also expressed increased amounts of u-PA. Taken together, these results implicate an important role of u-PA in the structural integrity of the atherosclerotic vessel wall by triggering activation of metalloproteinases (Fig. 7).

In contrast, mice with a combined deficiency of apoE and PAI-1 developed normal fatty streak lesions but subsequently revealed a transient, delayed progression to fibroproliferative plaques. Whether the increased plasmin proteolytic balance in these mice might prevent matrix accumulation and, consequently, delay plaque progression, or whether more abundant plasmin increased activation of latent TGF-β with its pleiotropic role on smooth muscle cell function and matrix accumulation, remains to be determined. Taken together, these targeting studies identify a specific role for u-PA in the destruction of the media that may precede aneurysm formation and for PAI-1 in plaque progression, possibly by promoting matrix deposition.

CONCLUSIONS

Gene-targeting studies are useful to obtain novel insights into the role and relevance of a gene during normal or pathological biological processes in vivo. New insights into the role of VEGF/PIGF, the fibrinolytic system, and/or the coagulation system in the formation of a normal blood vessel and in its pathologic progression to disorders such as thrombosis, restenosis, and atherosclerosis have recently been obtained.

Several coagulation factors appear to play an unanticipated role in embryogenesis, presumably in the formation of the primitive muscular wall of blood vessels, indicating a role for these coagulation factors beyond mere control of hemostasis. Whether a molecule such as TF mediates these morphogenic properties via interaction with factor VII or, possibly, with a novel (yet unidentified) factor remains to be evaluated. This might open possible future therapeutic avenues for a specific differential inhibition of hemostasis or morphogenesis in processes such as restenosis or atherosclerosis.

Urokinase-mediated plasmin proteolysis appears to play a significant role in migration of smooth muscle cells during neointima formation after vascular injury and in the destruction of the media and aneurysm formation during atherosclerosis. Such insights have initiated studies aimed at preventing neointima formation. Whether inhibition of plasmin proteolysis is a feasible means to prevent aneurysm formation during atherosclerosis remains to be determined. Ongoing research will determine the role of the plasminogen system in other cardiovascular disorders such as myocardial infarction and transplant atherosclerosis.

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