[Frontiers in Bioscience 8, d286-293, January 1, 2003]

REGULATION OF ANGIOGENESIS BY THE HEMOSTATIC SYSTEM

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1. ABSTRACT

In recent years it has become apparent that the hemostatic system has a role to play in regulating angiogenesis. This is achieved by components of the coagulation and fibrinolytic systems regulating proteolysis in the milieu and context of extra cellular matrix and the fibrin clot. It is also an emerging paradigm that cryptic fragments, released by proteolysis from components of the coagulation and fibrinolytic systems, have a role to play in regulating angiogenesis. Presented herein is a brief overview of the regulation of angiogenesis by the hemostatic system.

2. INTRODUCTION

Angiogenesis is the growth of new blood vessels from pre-existing vasculature. The process can be divided into two main phases (1); an activation phase and a resolution phase. Each of these phases will comprise a variety of processes. The activation phase requires increased vascular permeability and extravascular fibrin deposition, vessel wall disassembly, degradation of the basement membrane, cell migration leading to ECM invasion, cell proliferation and tube formation. The resolution phase of angiogenesis requires that each of these processes be halted (cell migration and proliferation), reversed (basement membrane reconstitution), or brought to completion (recruitment of pericytes and smooth muscle cells facilitating vessel maturation). Extracellular proteolysis is a vital biochemical activity in all of these processes, and this proteolysis is a tightly regulated highly co-coordinated series of events.

It has been obvious since the late 1930's (2) that capillary sprouting results in fibrinolysis (the dissolution of fibrin clots) at the tips of these sprouts. As capillary sprouts mature, so the fibrin is replaced by other components of ECM. Thus, fibrin provides a temporary matrix scaffold along which endothelial cells can migrate and has a fundamental role to play in angiogenesis. Fibrinogen, the precursor of fibrin, is a large glycoprotein found in the blood plasma of all vertebrates.

Thrombin is the main enzyme involved in generating insoluble fibrin from its soluble precursor fibrinogen. The central enzyme in fibrin proteolysis is plasmin. The activity of thrombin and plasmin are regulated by a variety of activator molecules (many of them proteinases) and inhibitor molecules. These activator and

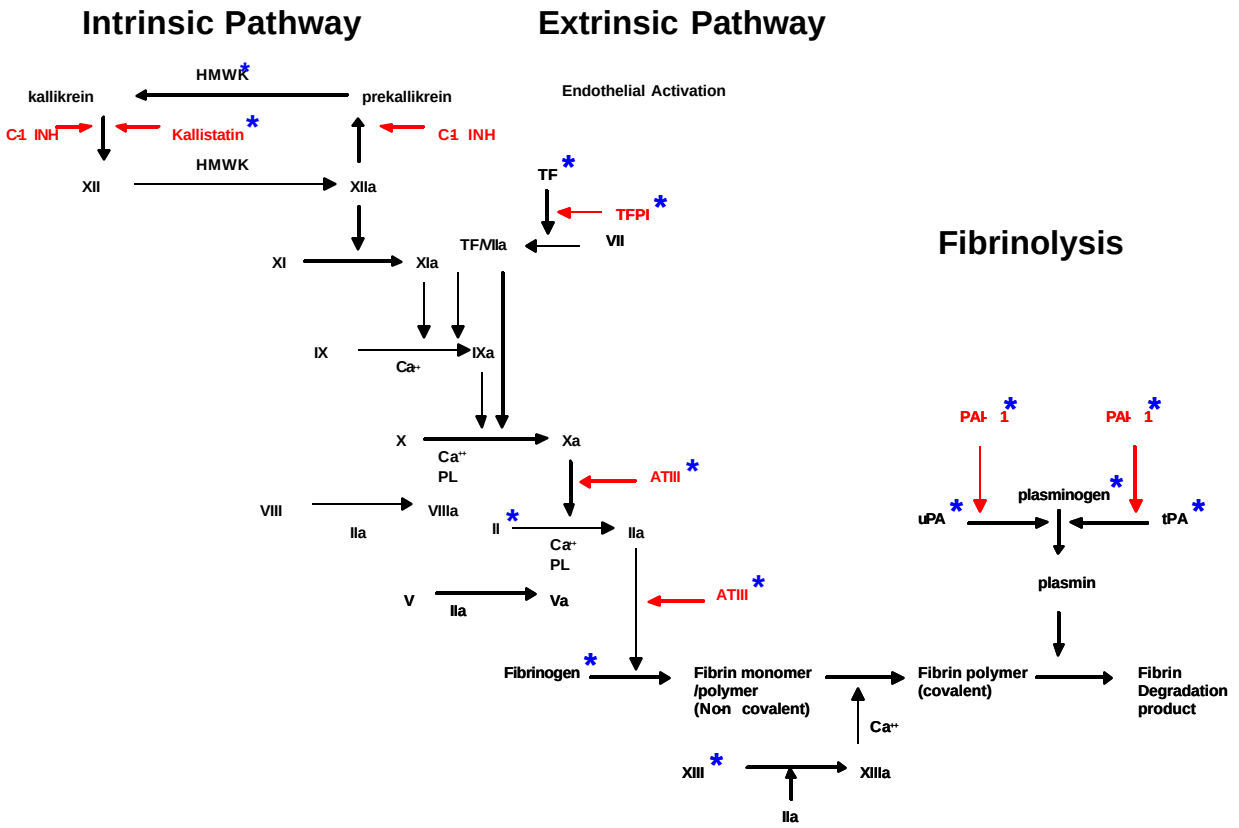

Figure 1. Simplified schematic of coagulation/fibrinolysis Proteins in black are involved in the cascade, proteins in red are inhibitors, proteins with an asterisk have reported anti endothelial function, either as whole proteins, or as parent molecules for cryptic fragments. PL , anionic phospholipids. For details see review.

inhibitor molecules are part of the larger hemostatic system which regulates coagulation and fibrinolysis. Further, as many components of the hemostatic system are proteinases that can become involved in ECM remodeling (or in the activation of ever more proteinases responsible for ECM remodeling) it is obvious that the hemostatic system has a central role to play in the regulation of angiogenesis. Certain components of the hemostatic system have a direct role to play in the regulation of angiogenesis. Other proteins appear to need proteolytic induction of a conformational change, exposing cryptic peptides and novel surfaces, before they can play a role in the regulation of angiogenesis.

3. COAGULATION: INTRINSIC PATHWAY

The coagulation cascade (see Figure 1) is initiated by either the Intrinsic (Contact Factor) Pathway, or the Extrinsic (Tissue Factor) pathway. Both pathways rely on key regulatory molecules for control of the cascade. The main pathway for initiation of the coagulation cascade is the Extrinsic pathway, The Intrinsic pathway serves to augment or amplify the coagulation cascade. The Intrinsic pathway is activated by when a complex of High Molecular Weight Kininogen (HK) and prekallikrein interact with a negatively charged surface in close proximity to Factor XII. This results in the activation of factor XII, which in turn generates kallikrein from prekallikrein. Cleavage of HK by plasma kallikrein results in the generation of a two-chain form of HK (HKa). The light chain of HKa contains two domains, D5 and D6. One of these domains, D5 binds to anionic surfaces, including heparin and phospholipids as well as zinc. For a review of the biochemistry of HK see Colman (3) HKa, domain 5 and peptides derived from domain 5 are all capable of inducing apoptosis in proliferating endothelial cells.

3.1. HKa

Two chain HKa is reported to bind to endothelial cells via the urokinase receptor (uPAR) (4) and this interaction is one that has been proposed as the basis for the anti-endothelial effect of HKa. However, although there is no doubt that HKa is anti-endothelial, there is some controversy as to the actual mechanism. Experiments involving domain 5 alone, or peptides generated from internal sequences within domain 5 suggest that the cleavage of HK by kallikrein may result in conformational changes that expose previously cryptic domains responsible for initiating a pro-apoptotic cascade within populations of proliferating endothelial cells. Recent experiments demonstrate that domain 5 derived from human kininogen can bind to endothelial cells via cell-surface associated tropomyosin (5). Thus, domain 5 of human kininogen may affect the cell cytoskeleton directly. Biomechanical

influences mediated through the cytoskeleton play a critical role in vital processes such as cell cycle entry and apoptosis (6) Another hypothesis is that HKa binds to vitronectin, blocking the interaction of cells with this ECM component via key integrins (7) Other laboratories (8) have argued that since HKa can induce apoptosis in proliferating endothelial cells, regardless of the matrix the cells are plated on, the mechanism may not be integrin specific. Further, since neither integrin ligands nor antibodies directed against ligand sub-units can inhibit the binding of HKa to endothelial cells (8), it is unlikely that the pro-apoptotic effects of HKa are mediated solely by integrins.

3.2. Kallistatin

A recently discovered serpin with specificity for kallikrein (kallistatin) has been shown to have a role in angiogenesis (9) Serpins are a structural superfamily termed of inhibitors that regulate the activity of serine proteinases, by forming an essentially irreversible complex, which is rapidly cleared from plasma. Kallistatin, in common with other serpins, possesses a reactive site loop (RSL) which extrudes from the molecule, and functions as a 'bait' for a target serine proteinase. Specificity is determined by primary sequence within the loop. The proteinase binds this loop and the serpin undergoes a conformational change, trapping the proteinase. The resultant tight complex is cleared by a receptor mediated mechanism. Kallistatin inhibited the proliferation, migration and adhesion of endothelial cells in vitro, and also inhibited angiogenesis in a rodent model of hindlimb ischemia. Thus the protein components involved in the initiation of the Intrinsic pathway are also intimately involved in the regulation of angiogenesis.

4. COAGULATION: EXTRINSIC PATHWAY

The extrinsic pathway that leads to rapid generation of fibrin is initiated by exposure of tissue factor to blood.

4.1. Tissue Factor

Tissue factor is a membrane bound protein that is found where it may play a protective role, initiating coagulation upon blood vessel damage. Upon exposure to blood, tissue factor activates factor VII to factor VIIa (with the aid of calcium and phospholipids), and this complex in turn activates factor IX and X. Tissue factor is found on the surfaces of tumor cells (10) as well as tumor associated macrophages and tumor associated endothelium (11). In fact some would argue that Tissue factor expression is a hallmark of cancer progression (12). The importance of tissue factor in tumor angiogenesis is highlighted by the observation that dramatic reductions in experimental tumor angiogenesis can be induced by inhibiting TF procoagulant activity via the use of specific antibodies (13). Furthermore, experiments involving the transfection of VEGF low-producing tumor cells with full length clones of TF restore VEGF production in these cells, whereas transfection of these same cells with a clone of TF lacking the cytoplasmic serine residues does not restore VEGF production. These results suggest that the cytoplasmic tail of TF is in some way required for full expression of VEGF (14). Thus TF has a role to play in tumor angiogenesis as well as coagulation.

4.2. Tissue Factor Pathway Inhibitor

Tissue Factor Pathway Inhibitor (TFPI) is a multi-domain protein with three kunitz-type proteinase inhibitor domains. Recent reports suggest that TFPI can inhibit the proliferation of bFGF driven endothelial cells (15). A truncated form of TFPI, containing the first two domains only, has very little anti-proliferative activity, which suggests that the carboxyl-terminal domain of TFPI is responsible for this activity. Full length TFPI binds to the very low-density lipoprotein receptor (VLDL receptor). Binding of this receptor by Receptor Associated Protein can abrogate the anti-proliferative effect of TFPI. A peptide fragment of TFPI, located near one of the Kunitz-type domains has similar anti-proliferative properties to the intact TFPI (16). This fragment can inhibit both bFGF and VEGF stimulated proliferation with an IC_{50} value of 5 microM. Doses of up to 250 microM failed to inhibit the proliferation of Lewis Lung carcinoma or Eoma endothelioma cells. This peptide is reported to bind to VLDL receptor with an approximate K_D of 1 microM. Blocking the VLDL receptor with an antibody abrogated the anti-proliferative activity of the peptide. Perhaps most interestingly, this peptide is capable of inhibiting the growth of metastatic tumors in a dose dependent manner, with a 50% inhibition of growth rate at 500 microM peptide.

5. COMMON PATHWAY

Both the extrinsic and intrinsic pathways result in the activation of Factor X which results in the generation of thrombin from prothrombin. Thrombin is the serine proteinase responsible for generating insoluble fibrin from fibrinogen. The resultant fibrin forms complexes with adjacent fibrin and fibrinogen molecules resulting in the spontaneous polymerisation of the fibrin clot, which is stabilized by the action of a transglutaminase called Factor XIII.

5.1. Thrombin

Thrombin cleaves fibrinopeptides A and B converting fibrinogen to fibrin, and allowing for the assembly of protofibrils, which leads to fine clot formation. Prothrombin, the inactive precursor of thrombin, is a multi domain protein, comprising two kringle domains and a serine proteinase domain. An internal fragment of prothrombin was recently isolated from the serum of LPS treated rabbits. This fragment inhibits the proliferation of endothelial cells. The peptide fragment comprised 110 residues and was shown to be kringle 2 of prothrombin. Both the purified inhibitor, derived from rabbit serum, and prothrombin kringle 2 domain, released from purified rabbit prothrombin by factor Xa cleavage, inhibited the proliferation of endothelial cells, and inhibited angiogenesis in the CAM assay (17). An extension of this work showed that factor Xa cleavage of human prothrombin produces active thrombin, and two anti-endothelial fragments. The two anti-endothelial fragments comprise kringle 1 and kringle 2 domains of human

prothrombin. These two single kringle domains inhibit the proliferation of endothelial cells and angiogenesis, as measured in the chorioallantoic membrane assay (18).

5.2. Factor XIII

Factor XIIIa (the activated form of factor XIII) can mediate adhesion of endothelial cells and inhibits capillary tube formation in fibrin clots (19). This activity was not dependent on the transglutaminase activity. By using antibodies, it was determined that the adhesion was mediated through $alpha_v$ $beta_3$ integrin and $beta_1$ containing integrins. Further, the activity was dependent on the presence of Mg^{++} or Mn^{++}. An implication of these data is that high concentrations of factor XIII at the site of a wound may impede revascularization at that site. Thus angiogenesis may be inhibited in a fibrin clot until fibrinolytic enzymes or passive diffusion clears the clot of coagulation factors.

5.3. Antithrombin

Recent data has indicated that a cleaved form of antithrombin III (AT-III) also has potent anti-endothelial activity (20). Neutrophil elastase is capable of binding the RSL of AT-III, but instead of being trapped it cleaves AT-III within the RSL. The elastase does not form a complex with this cleaved AT-III, instead the RSL inserts itself into the A beta -sheet, becoming the 3rd strand in a 6 stranded sheet. AT-III in this 'relaxed' conformation will inhibit the proliferation and migration of endothelial cells, as well as angiogenesis in the CAM assay. Antiangiogenic ATIII can also inhibit the rate of growth of implanted tumors in mice (21,22).

6. FIBRINOYLSIS

6.1. Fibrinogen

Fibrinogen is composed of a dimer of three non-identical polypeptide chains (Aa, Bb and g) held together by 29 intra- and inter-chain disulphide bridges. The N-terminii of the Aa, Bb, and g chains from the two halves of the molecule come together to form a small globular domain at the centre, termed the "disulphide knot". The C-termini of each of the three chains end in globular domains. The regions between the Bb and the g globular domains in each half molecule are predicted to be mainly a-helical and together with the Aa chain form triple stranded coiled coils. The extended single chain portion of the Aa chain following the coiled-coil region is flexible (23).

Fibrinogen can be fragmented by proteolytic action into several well defined regions, including the E fragment, comprising the central disulphide knot region and two D fragments. Each D fragment consists of the C-terminal Bb and the g globular domains, and part of the triple coiled-coil, which make up about a third of the half molecule.

It has been shown that internal fragments of fibrinogen can act as anti-angiogenic molecules. The central fragment of fibrin, the E fragment, was recently shown to be anti-endothelial for human microvascular cells (24). This activity has been shown to reside, in part, in a region at the amino-terminus of the alpha chain of fragment E (25). A 24-mer peptide, named alphastatin, derived from this region has been shown to be anti-migratory, as well as inhibiting the formation of tubes by microvascular cells. Mice, implanted with a colon carcinoma *s.c*, were treated with *i.p* injections of alphastatin. Therapy was able to reduce the rate of tumor growth markedly over the course of 14 days. Furthermore, the treated tumors exhibited larger areas of intravascular thrombosis and necrosis. Alphastatin appeared to induce little or no necrosis in healthy vascularised tissue such as liver and lung. The structure of the fibrin matrix can also have an effect on endothelial cell responses. When thrombin cleaves fibrinogen to fibrin, it does so by removing two fibrinopeptides from the amino terminal ends of the alpha and beta chains of fibrinogen. This allows the fibrin monomer to assemble into protofibrils, as the exposed sites fit into 'holes' present on the carboxy-terminal domains of the alpha and beta chains of fibrin. This produces the familiar 'half-staggered' assembly of protofibrils. The exposure of residues 15-42 on the beta chain also stimulates capillary tube formation. Exogenous peptide, can also stimulate tube formation and residues 15-42 will competitively abolish tube formation in an in vitro assay. Further studies showed that vascular endothelial (VE)- cadhedrin, present on the surface of endothelial cells, acts as a receptor for this exposed region on the fibrin beta chain (26). Fibrin also contains RGD sequences on the a chain, which mediate binding of endothelial cells via the integrin $alpha_v beta_3$.

6.2. Plasminogen/Plasminogen Activator system

Plasmin is the proteinase responsible for degrading fibrin into fibrin degradation products. Plasmin is generated from an inactive precursor (plasminogen) by the action of urinary-type plasminogen activator (uPA) or tissue type plasminogen activator (tPA). The integrity of the the fibrin clot can affect endothelial cell responses either as described above, or by sequestering growth factors such as bFGF. Thus any enzyme which expedites removal of fibrin will modulate angiogenesis.

6.2.1. Tissue-type Plasminogen Activator (tPA)

Little is known about the role tPA plays in angiogenesis, other than it's role in generating plasmin which allows for the proteolysis of fibrin. Recent reports are hinting that cryptic proliferative and anti-proliferative domains may be contained within the parent tPA molecule. Tissue type plasminogen activator contains two kringle domains (similar to prothrombin) as well as a finger domain and an EGF domain. A monoclonal antibody directed against kringle 2 of tPA can induce the proliferation of endothelial cells in culture, implying that endogenously secreted tPA has some role in inhibiting proliferation of endothelial cells. Further investigation revealed that recombinant kringle 2 or proteolytically derived kringle 2 were capable of inhibiting endothelial cell growth. To complicate the issue, antibody experiments suggested that the finger domain and the EGF domain may in fact be proliferative for endothelial cells (27).

6.2.2. Urinary-type plasminogen activator (uPA)

Urinary type plasminogen activator (or urokinase) also plays a role in modulating angiogenesis.

One facet of this is its ability to activate plasmin, resulting in the degradation of fibrin. Quiescent endothelial cells do not appear to express uPA (1), which is a soluble proteinase secreted into the milieu. Thus only endothelial cells that are actively angiogenic, having been stimulated by growth factors, secrete uPA. This secretion may have a role as a negative feedback loop, whereby angiogenic endothelial cells, stimulated by the fibrin matrix and the growth factors sequestered by fibrin to migrate and proliferate, express uPA, which generates plasmin capable of degrading the very matrix providing stimuli to the endothelial cells. However uPA has a more complex role to play in angiogenesis. The uPA receptor (uPAR) is a GPI-anchored membrane protein capable of binding active uPA (28). The interaction is crucial for tumor angiogenesis. Tumor cells can bind exogenous uPA via uPAR, and this interaction provides a proteolytic "front" for migrating tumor or endothelial cells. Peptides or small molecules that bind to uPAR, thereby blocking the interaction of uPAR with uPA, are antiangiogenic in several tumor models in mice. Furthermore, uPA is capable of initiating an extracellular cascade of proteolysis that involves the activation of other proteinases such as plasminogen and matrix metalloproteases. These proteolytic cascades process and release various growth and differentiation factors that are sequestered on the cell surface or within the ECM, which contribute to the evolution of a migratory or invasive cell phenotype. uPA is also able to modulate signaling and cell adhesion through its specific cell surface receptor, uPAR. Recent data suggest that the nonproteolytic activities of the uPA system are coupled to adhesion, migration and signaling through various integrins (29).

6.2.3. PAI-I

Plasminogen Activator Inhibitor-I (PAI-I) is the serpin responsible for regulating the activity of uPA. As such it also has a role to play in angiogenesis. Tumor associated angiogenesis was inhibited in a prostate cancer model when the cancer cells were stably transfected with PAI-I (30). However, PAI-I has an equivocal role in angiogenesis (31), as in some studies the conclusion is that PAI-I is essential for angiogenesis. Mice deficient in PAI-1 expression had poor local invasion and tumor vascularization of transplanted malignant keratinocytes. If this PAI-1 deficiency was corrected by gene therapy utilizing an adenoviral vector expressing human PAI-1, invasion and associated angiogenesis were restored. Thus, host-produced PAI is essential for cancer cell invasion and angiogenesis (32). This equivocation may be explained as follows; PAI-I, by inhibiting uPA activation of plasmin and the activity of plasmin directly, will delay degradation of the fibrin clot and other ECM components, thus allowing for a sustained angiogenic milieu. In this scenario, PAI-I is pro-angiogenic. Alternatively, inhibition of uPA will inhibit uPA mediated migration of endothelial cells through ECM, and in this mode of action PAI-I may be antiangiogenic. Recent experiments using mutants of PAI-I have suggested that some of the antangiogenic activity may reside in vitronectin binding domains (33,34). Binding of PAI-I to vitronectin (or indeed binding of specific antibodies to vitronectin) will inhibit the binding of endothelial cells to vitronectin and result in an antiangiogenic response in CAM assays.

6.2.4. Angiostatin

Plasmin itself is the parent molecule of angiostatin. Angiostatin was first discovered in mice bearing a variant of Lewis lung carcinoma that was able to inhibit the growth of secondary tumors in an in vivo mouse model. Urine and serum from these mice were able to inhibit the proliferation of endothelial cells in an in vitro assay, and it was hypothesized that the primary tumor was producing an inhibitor of angiogenesis (35). Such a model explains why secondary metastases cannot grow in the presence of certain primary tumors. Subsequently an inhibitor of endothelial cell proliferation was purified from these animals. This molecule was able to induce dormancy or primary tumors when administered systemically to tumor bearing mice. This molecule was termed angiostatin and proved to be an internal fragment of plasminogen (35). Plasminogen is the precursor of plasmin, the serine proteinase responsible for lysing the fibrin clot. It is a multi-domain protein comprising 5 kringle domains, and a serine proteinase domain. Angiostatin spans kringles 1-3 at a minimum (35), with some variants spanning kringles 1-4 1/2 and even kringle 1-5 (36).

Angiostatin can inhibit the proliferation and migration of endothelial cells, as well as angiogenesis in the chick chorioallantoic membrane (CAM) assay. Further, angiostatin has been shown to be efficacious in inducing tumor dormancy in a mouse model by reducing the vascular density with the tumor body. Such dormancy can be induced in mouse and human tumours implanted in immune deficient mice (37).

Angiostatin has been shown to bind ATP synthase located on the surface of endothelial cells, which may lead to apoptosis under hypoxic conditions (38). Further, angiostatin can act as a non-competitive inhibitor of extra cellular matrix (ECM)-enhanced plasminogen activation by tPA suggesting a mechanism whereby angiostatin-mediated regulation of plasmin formation may influence cellular migration and invasion (39). A recently discovered novel protein that binds angiostatin is angiomotin (40). Angiomotin is a 72kDa protein, expressed in the endothelial cells of capillaries as well as the larger vessels of the human placenta. The protein is normally localized to the leading edge of migrating cells, and increased expression of angiomotin results in increased migration of endothelial cells. Angiomotin-mediated migration is blocked by angiostatin, and angiostatin is internalized by angiomotin. Angiomotin lacks a signal sequence found in membrane bound receptors. However, this appears to be a common feature of membrane associated proteins than bind angiostatin. Angiomotin does contain motifs that may be potential binding sites of Src homology 3 domains.

Recent data has suggested that angiostatin can bind to the integrin $alpha_v beta_3$ and that this integrin may be the predominant receptor for angiostatin in certain endothelial cells (41). Plasmin can also bind to this integrin (42) via kringle domains, and this induces migration of endothelial cells. Proteolytic activity of plasmin is required for the induction of migration. This provides a potential

mechanism for angiostatin's ability to inhibit migration in these cells, whereby proteolytically inactive angiostatin can block the binding of plasmin to integrin receptors.

It has been shown that several proteinases can process angiostatin from systemic plasminogen, including macrophage metalloelastase (43), metalloproteinases (mmp's) -3, -7, and –9 (44, 45) and plasmin itself in the presence of a free sulphydryl donor such as cysteine (46, 47). Angiostatin is also autoproteolytically generated in non neoplastic situations on the surface of macrophages by a mechanism whereby microplasmin is released systemically, leaving angiostatin bound to the cell surface (48). The released microplasmin is rapidly inhibited by $alpha_2$antiplasmin suggesting that the generation of angiostatin and the inactivation of plasmin may be temporally and spatially linked events.

7. CONCLUSION

Thus the entire cascade leading to coagulation and fibrinolysis contains proteins that regulate angiogenesis. In some cases this is simply by leading to the deposition or degradation of fibrin, which is inherently angiogenic due to its ability to sequester growth factors and guide the migration of endothelial cells. Other proteins appear to have direct effects on endothelial cells, either in their native form, or after proteolysis leads to the exposure of cryptic domains with pro- or antiangiogenic activity. It is of particular interest that many of the cryptic fragments are or can be generated by proteinases that already have a function in generating the active form of a given protein. It appears that many proteins have a more active role than we have discovered thus far. For example, plasminogen is activated to plasmin, which can be inactivated by further proteolysis such as the proteolytic removal of microplasmin from the cell surface. This reaction leads to the rapid inhibition of microplasmin by $alpha_2$ antiplasmin, and results in the generation of angiostatin as a by product.

The coordination of these events is evident from an interesting study whereby the angiogenic activity of wound extracts was shown peak 3 days after wounding, followed by a maximal vascularity of these wounds by day 5 or 6 (49). Maximal angiogenic activity appeared to be associated with proteolysis events. Thus a healing wound offers a clear example of the gross coordination of these events. Given the rapid discovery of antiangiogenic fragments of proteins within the coagulation cascade in recent years we should expect to find more fragments and domains in the near future, which will not only allow us to expand our arsenal of antiangiogenic drugs, but also allow us to understand much more about the process of wound healing and the role that coagulation and fibrinolysis play in angiogenesis.

8. ACKNOWLEDGEMENTS

I would like to thank Fernando Doñate for critical reading of the manuscript and suggestions to help clarify the figure.

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Key Words: Angiogenesis, Coagulation, Fibrinolysis, Endothelium, Review

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