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ANNEXIN II: A PLASMINOGEN-PLASMINOGEN ACTIVATOR CO-RECEPTOR

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1. ABSTRACT

Fibrinolysis is a precisely orchestrated process in which fibrin-containing thrombi are solubilized. Several receptors regulate this process by localizing proteolytic activity to the cell surface. One such receptor is annexin II, a calcium and phospholipid-binding protein. Annexin II serves as a profibrinolytic coreceptor for both plasminogen and tissue plasminogen activator on the surface of endothelial cells and facilitates the generation of plasmin. The dysregulation of fibrinolytic assembly on endothelial cells may lead to atherothrombotic disease. In addition to its role in fibrinolysis at the surface of endothelial cells, annexin II may play other potential cellular roles. For example, the overexpression of annexin II on the surface of leukemic cells and cell lines derived from acute promyelocytic leukemia correlates with both the clinical manifestation of bleeding and the *in vitro* ability of the leukemic cells to generate plasmin. The abundant presence of annexin II on the surface of other cell types including monocytic cell lines and different cancer cells may contribute to their invasive potential through extracellular matrix either by generation of plasmin or, by plasmin-mediated proteolytic activation of other metalloproteinases. This dissolution of extracellular matrix may also cause the release of potent matrix-bound angiogenic factors such as VEGF and FGF. On the other hand, by increasing the pool of plasmin, a precursor to an important anti-angiogenic factor, angiostatin, and by fragmentation of collagen XVIII (a precursor to the anti-angigenic factor, endostatin) by plasmin-activated metalloproteases, annexin II could play a pivotal physiological role in the pro- and anti-angiogenic switch mechanism.

2. INTRODUCTION

The fibrinolytic system is a cascade of serine protease activation events that culminate in the generation of plasmin from plasminogen (1). Plasminogen circulates in the plasma at micromolar concentration and can be activated either by urokinase-type plasminogen activator (u-PA), a product of renal epithelial cells, monocytes, macrophages, and many tumor cells, or tissue plasminogen activator (t-PA), produced by the endothelia in compartmentalized organ systems, as well as by some malignant cells. Plasmin cleaves cross-linked fibrin, which is a major provisional matrix and scaffold protein that forms following tissue injury. The plasminogen activation system is tightly controlled by a series of serine protease inhibitors, including plasminogen activator inhibitor-1 (PAI-1) and α_2-plasmin inhibitor. Several

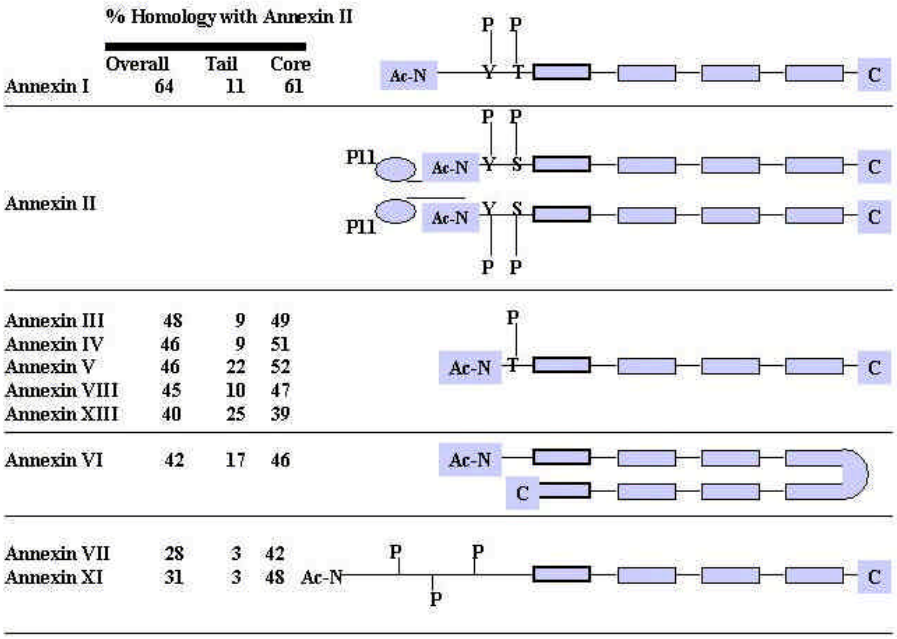

Figure 1. Annexin family of proteins. In the core domain annexins contain 4 or 8 conserved repeats of with 39-61% amino acid homology to annexin II. The amino terminal (tail) domains vary in length and in phosphorylation sites. Annexin II forms a heterotetramer with p11 as shown. Figure adapted from Hajjar & Krishnan (1999) with permission.

proposed cell surface receptors localize the reaction to the cell surface and thus increase the catalytic efficiency of the reaction while sequestering the reactants away from the circulating inhibitors. One such receptor is annexin II (2). Fibrin itself participates in a positive feedback loop by increasing the catalytic efficiency of t-PA-dependent plasmin generation by 500-fold.

3. ANNEXIN II

3.1. Overview of Annexins

Annexins are a family of widely distributed, phospholipid-binding peripheral membrane proteins (Figure 1). They are characterized by the canonical annexin fold which consists of the amino acid sequence G-X-G-T-(38)-(D/E) and which is responsible for the calcium-dependent binding of the annexins to membranes. Because of their wide tissue distribution and many potential functions, many questions about the annexins still remain unexplored (3-8).

There are at least 20 annexins characterized at the molecular level. Ten of these are distinct gene products in mammalian systems. The rest are found in the lower eukaryotes including *Drosophila melanogaster, Xenopus laevis, Dictyostelium discoideum, Hydra vulgaris, Caenorhabditis elegans,* and *Giardia lamblia*, and in many plants. The crystallographic analyses of annexins I (9), II (10), III (11), V (12), and XII (13) and partial structures of annexins I (9), and IV (14) have revealed a common tertiary structure with a concave core domain that consists of four subdomains each bearing five α-helices and a 70-amino-acid annexin repeat.

Despite their structural similarity, annexins have been assigned a wide range of functions. The alternative splicing in the tail region which results in differences in amino acid length from 11 to 199 among annexins II (15), III (16), VI (17, 18), VII(19, 20), and XI (21) may impart tissue specific functional properties.

3.2. Annexin II: Structure and function

An x-ray crystallographic study of annexin II tail peptide and its regulatory light chain, p11, suggests that annexin II exists as a heterotetramer with p11 (a.k.a. annexin light chain) in a tight hydrophobic complex (22,23). P11 is a member of the S100 EF-hand protein family, which is unique in having lost its calcium-binding properties (24) and is frozen in a conformation that other S-100 proteins take when bound to calcium. Other crystallographic interpretations postulate that the complex could even exist as a hetero-octamer which may potentially be the correct form as a membrane ion channel (10, 25).

Consistent with this analysis is the observation that the dissociation of this complex only occurs with both SDS- and heat-induced (boiling) denaturation. This almost inseparable partnership between the annexin II heavy and light chains is manifested by the corresponding abundant levels of the light chain protein expression in cells which overexpress the heavy chain molecule (26). The functional significance of this phenomenon is that the heavy chain molecule may protect the light chain from cellular protein degradation machinery and thus increase the functional half-life of the light chain molecule. One study examines the fate of p11 in a bronchial epithelial cell line in response to treatment with pharmacological concentrations of all-

trans-retinoic acid (ATRA) (27). The authors show that p11 degradation occurs in response to ATRA treatment; Menell et al.(28) have previously shown in leukemic cell lines that ATRA causes decreased transcription of annexin II heavy chain. One possible mechanism that can tie these two observations together is that annexin II could serve to protect p11 from the degradational machinery of the cells. Thus, ATRA, by decreasing the transcription of annexin II heavy chain, may also cause a decrease in the cellular pool of the light chain molecule (29). The downstream effect of ATRA could also be related to phosphorylation events. For example, the *in vitro* protein kinase C (PKC) phosphorylation of serine 25 is known to cause dissociation of annexin II heavy and light chains while that of Tyr23 phosphorylation by $pp60^{v\text{-}src}$ does not. One avenue to explore is whether the released annexin II light chain then behaves like other homologous S100 proteins such as S100B and S100A1 which may promote cell survival by engaging in the receptor glycation end products (RAGE) and nuclear factor kappa B (NF-κB) pathway (30).

The human annexin II gene is located on the long arm of chromosome 15 (15q21) (31), spans 40 kb, and contains 13 exons (32). The 5' untranslated leader sequence contains a canonical TATA box, three putative binding sites for the transcription factor SP-1, and a consensus sequence for the transcription factor AP-1. Retinoic acid negatively regulates AP-1-dependent gene expression (32). AP-1 directs the expression of a 1.2-kb annexin II mRNA in different cell systems (33-39, 28).

The protein consists of a short tail domain (Ser^{2}-Asn^{32}) and a hydrophilic core domain (Phe^{33}-$Asp^{339)}$ (40). The core domain exhibits 39-61% amino acid sequence identity with other members of the annexin family which are expressed in humans (I-VIII, XI, XIII) while the tail domain displays only 3-25% identity (Figure 1). The core domain possesses two annexin fold regions (41,42) and non-annexin type calcium binding sites defined by loops connecting helices D and E (43). The differences in the tail domain are thought to confer distinctive functions upon each annexin. The tail domain of annexin II bears phosphorylation sites for protein kinase C (Ser 25) (44, 45), $pp60^{v\text{-}src}$ kinase (Tyr 23) (46,47) and the PDGF receptor (Tyr 23)(47). As previously mentioned, serine 25 phosphorylation leads to dissociation of annexin II heavy and light chains (48), while tyrosine 23 phosphorylation does not alter the integrity of the complex.

One of the surprising findings of the recent human genome project was the relative paucity of expressed genes to account for the functioning of a complex organism (49). This finding suggests that many proteins, like annexin II, may act multi-functionally. For annexin II, post-translational modifications other than phosphorylation have not been well studied. Goulet et al. suggested that its ability to bind concanavalin-A suggests the presence of two biantennary mannosyl residues (50). On the other hand, studies from this laboratory using recombinant proteins derived from bacterial systems suggest that for annexin II, glycosylation may not be crucial for its function in mediating fibrinolysis (51). Although the main focus of this review is on the role of annexin II in fibrinolysis, a short summary of other interesting proposed extra- and intracellular functions are briefly enumerated below.

3.3. Versatile cellular roles of annexin II

Some global observations about annexin II heavy chain may provide clues to its function. Its expression is developmentally regulated and has been studied in the development of the avian limb (52), and heart (53), and in the rat and human fetal brain (54). Annexin II is abundantly expressed in the adult central nervous system, the cardiovascular system, the small intestine and the bone marrow endothelial cells and other cellular components (55). The functional versatility of annexin II is also marked by its presence in various locations both intra- and extracellularly. Intracellular annexin II has been implicated in cellular proliferation and differentiation. These processes may be linked to the ability of annexin II to be phosphorylated by a variety of key cellular kinases including protein kinase C (44, 45) and $pp60^{v\text{-}src}$ kinase (46, 47). Its tyrosine phosphorylation has been shown to follow activation of the insulin and PDGF receptors (56, 57). Its binding to the inner surface of the plasma membrane has been associated with reduced phospholipase A2 activity (58,59). The nuclear localization of annexin II in some immunohistochemical studies may reflect its presence in a primer recognition complex that stimulates the activity of DNA polymerase (60). Annexin II secreted in the bone marrow environment has been implicated in osteoclastogenesis (61). Since annexin II has been reported to bind 1,-25-vitamin D, the latter could have a link to this phenomenon (62). Annexin II has also been found to be an early glucose adduct product in the endothelial plasmalemma in a model of diabetes mellitus (25).

Intracellular annexin II-p11 tetramer has been implicated in the secretory pathway of adrenal chromaffin cells where it is found closely associated with chromaffin granules as they attach to the plasma membranes (63 -65). The colocalization of annexin II and caveolae (66) suggests that annexin II may aid in endo- and exocytic processes; this mechanism may relate to its translocation to the cell surface. On endothelial cells, annexin II is expressed as a peripheral membrane protein (50). Although it lacks a classical signal peptide, annexin II is constitutively translocated to the cell surface within 16 hours of its biosynthesis where it appears to constitute ~5% of the cellular protein pool.

Annexin II has been found on the surface of many cell types including endothelial (1), lymphoma (38), leukemic cells (28), and cell lines derived from macrophages (67). On the cell surface of glioma and endothelial cells, annexin II binds to tenascin C, an extracellular matrix component and aids in initiating the migration through the extracellular matrix (33, 68).

Annexin II expression is diminished in primary prostate cancer cells by a demethylation process (69) but it is unclear whether this contributes to a role in oncogenesis. Annexin II overexpression has been described in primary

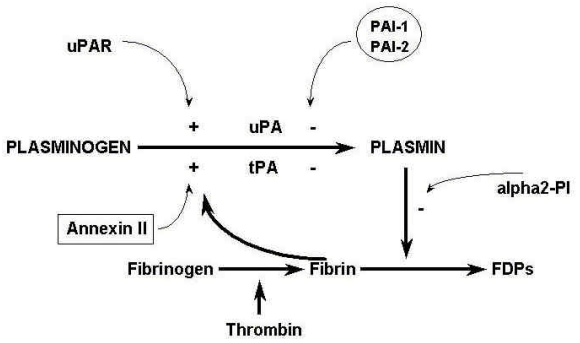

Figure 2. Fibrinolytic pathway. Plasminogen circulates at micromolar concentration in plasma and is activated to plasmin by urokinase or tissue plasminogen activator. Fibrin, which is formed by thrombin induced polymerization of fibrinogen, is degraded by plasmin to fibrin degradation products (FDPs). The inhibitory regulation is carried out by circulating plasminogen activator inhibitor 1 and 2 (PAI-1,-2) for uPA and tPA, while the circulating plasmin is inhibited by α_2-plasmin inhibitor (α_2-PI). Fibrin is a strong stimulator of tPA activity. Surface receptors including uPAR and annexin II are thought to enhance the catalytic efficiency of plasmin generation. Figure adapted from Hajjar & Krishnan (1999) with permission.

pancreatic cancer cells (26). In gastric cancer tissues, its overexpression correlates with poor prognosis (70). Extracellular annexin II has also been reported to mediate tumor cell interaction with hepatic sinusoidal endothelial cells (33), as well as endothelial and glioma cell adhesion to matrix by binding to tenascin C (33, 68). Annexin II also binds F-actin and may thus have a role in shape changes involved in movement of cells (71). Potential mechanisms whereby annexin II may confer a carcinogenic phenotype may be the following: First it may confer a survival advantage by increasing the cellular pool of p11 with subsequent NF-κB activation Second, annexin II may potentiate the secretion of survival factors which may act in an autocrine, paracrine and endocrine fashions. Third, annexin II may impart a metastatic potential by increasing the fibrinolysis through the extracellular matrix and by adhering to molecules which may help its passage through the endothelial barrier.

Annexin II also plays immunological roles. In the placenta, annexin II acts as an immunological barrier by inhibiting lymphocytic proliferation and by acting as an F_c receptor/ immunoglobulin transport molecule (72-75). Annexin II can also directly interact with pathogens by acting as a receptor for cytomegalovirus on the surface of endothelial cells (76,77). Autoimmune antibodies have been found in lung cancer patients (78) and are associated with increased serum levels of IL-6. T cells primed with dendritic cells presenting an annexin II peptide (residues 208-223) appear to recognize melanoma cells (79).

Despite these interesting observation, the focus of this review is on annexin II as a receptor for both tPA and plasminogen (80, 81), and its role in enhancing cell surface plasmin generation. Some basic studies are followed by a description of its role in the progression of atherosclerosis (82, 83), in the bleeding diathesis associated with acute promyelocytic leukemia (28), and in macrophage invasion though the extracellular matrix (68).

3.4. Annexin II and the plasmin/plasminogen activator system

3.4.1. Fibrinolysis

Fibrin is the major protein constituent of vascular thrombi. It forms in response to injury in both intravascular and extravascular settings (1). During wound healing, fibrin is degraded by plasmin. Plasmin is generated proteolytically from plasminogen by either of two serine proteases, tPA or uPA (Figure 2). The catalytic efficiency of tPA-dependent plasminogen activation is strongly amplified by fibrin which acts as a cofactor in plasmin generation. Modulation of this process is regulated by circulating plasminogen activator inhibitors types 1 and 2 (PAI-1, PAI-2). Plasmin itself is inhibited by α_2-plasmin inhibitor. Endothelial cells are thought to play a key role in intravascular plasmin generation because they synthesize and secrete both plasminogen activators, tPA and uPA, as well as their inhibitor, PAI-1 (84).

3.4.2. Annexin II-mediated assembly of plasminogen and t-PA

Cultured human umbilical vein endothelial cells (HUVEC) bind both plasminogen and t-PA in a concentration-dependent manner (K_d 310 nM and B_{max} 1,400,000 sites per cell, and K_d 18nM and B_{max} 815,000 sites per cell, respectively) (80,81). These binding interactions preserve catalytic efficiency and apparently protect plasmin and t-PA from their inhibitors, thus potentially modulating hemostatic and thrombotic events at the blood vessel surface. The isolated 36 kD t-PA/plasminogen coreceptor was identified by two internal peptides as analogous to annexin II (85). The full length cDNA, derived by PCR, was identical to annexin II heavy chain sequence (85) and the ligand-precipitated receptor immunoreacted specifically with a monoclonal antibody to annexin II. Transfection of recombinant annexin II in 293 cells with the full length cDNA conferred the ability to bind plasminogen (K_d 114 nM; B_{max} 347,000 sites per cell) and tPA (K_d 48 nM; B_{max} 380,000 sites per cell). In addition, HUVECs treated with phosphorothioated antisense oligonucleotides representing bases 4 through 24 of the annexin II cDNA bound plasminogen and tPA 40% and 50% less than untreated cells. This suggests that annexin II mediates a large proportion of the binding of plasminogen and tPA to the endothelial surface. Exogenous ^{125}I-labeled annexin II bound endothelial cell surface with high affinity (K_d 49 nM) in a calcium-dependent manner. This binding was further defined by inhibitory self-peptides. Peptides mimicking the classical "annexin repeat" (KGXGT) blocked its interaction with the endothelial cell. Annexin II mutated at the calcium binding site D161A (repeat2) was unique in its inability to prevent the wildtype annexin II from binding the endothelial cell membranes illustrating the crucial role of repeat 2 in calcium-dependent membrane binding of annexin II. Detailed *in vitro* studies by Waisman and

colleagues (86) suggest that plasmin generation itself takes place in the context of an annexinII-p11 heterotetrameric complex rather than by the annexin II heavy chain monomer alone (36,87). This is not a surprising result given the tight hydrophobic interaction previously described. However, the physiological role of annexin II heavy and light chains needs further exploration in *in vivo* models.

In vitro kinetic analyses revealed that annexin II confers a 60-fold increase in catalytic efficiency to the t-PA-dependent plasminogen activation (88). The lysine analog ε-aminocaproic acid or treatment of annexin II by carboxypeptidase B can abrogate the effect almost completely indicating the involvement of a carboxyl terminal lysine-dependent mechanism. This effect was specific for t-PA. It is as yet unexplored whether other parts of annexin II molecule may participate in uPA-mediated plasminogen activation. Falcone et al. showed that the migration of macrophages through the ECM is partly annexin II-dependent in a tPA-free system suggesting that uPA-dependent plasmin generation may also be affected by annexin II to a lesser extent than t-PA (68).

3.4.3. Annexin II binding domains for plasminogen and t-PA

Plasminogen binding to annexin II appears to require receptor modification or "activation" by a carboxyl terminal cleavage event (85). The K307T mutant annexin II transfected into 293 cells failed to bind plasminogen while a change of a lysine proximal to this site (K328I) bound plasminogen no differently than the wildtype.

The t-PA binding domain is in the tail-region of annexin II as shown by competitive binding assays using intact and core domain (Gly^{25} - Asp^{339}) annexin II (89). Furthermore, two overlapping peptides derived from the tail domain (residues 2-13, 8-19) but not a third peptide (14-25) blocked binding of annexin II to t-PA. A peptide representing residues 7-12 also blocked 95% of annexin II binding to t-PA (I_{50} 208 μM) and the crucial residue was determined to be cysteine 9 by other peptide studies. These data localized the binding site of t-PA to the tail domain.

3.4.4. Annexin II and atherogenesis

Annexin II may play a role in the progression of atherogenesis by modulating fibrinolysis. Two molecules which are linked to this process are Lp(a), a low density lipoprotein (LDL)-like particle that contains the plasminogen-like apolipoprotein (a) (82,90-92) and homocysteine (HC), a non-protein-forming, sulfur containing amino acid that stands at the crossroads of two metabolic pathways (83, 93).

In vitro studies showed that Lp(a) interferes with plasminogen binding to annexin II as well as plasmin generation at the cell surface. Lp(a) displaces plasminogen from the surface of HUVECs by interfering with the binding of annexin II to plasminogen. In direct binding studies, apo(a) was shown to be the component of Lp(a) that competes with annexin II. Lp(a) had no effect on t-PA-dependent plasminogen activation in the fluid phase but a 93% reduction occurred on the endothelial cell surface. Thus a potential link to progression in atherosclerosis and decreased cell surface fibrinolysis may exist as Lp(a) accumulates in the atherosclerotic placques.

Homocysteine accumulates in hereditary deficiencies of cystathionine β-synthase, methionine synthase or methyltetrahydrofolate reductase as well as in dietary deficient states of folate, B_6, or B_{12} . Hyperhomocysteinemia is associated with both atherosclerosis and thromboembolism. Some studies implicate the involvement of the t-PA mediated fibrinolytic system (94). One mechanistic explanation is that the binding of t-PA to both HUVECs and to annexin II can be inhibited by homocysteine (95). This inhibition is associated with decreased t-PA activity. Tryptic digests, mass spectrometric analysis and [^{35}S]-homocysteine labeled cultured endothelial cells data demonstrate that a disulfide-complex between homocysteine and annexin II (cysteine 9) can form. The I_{50} for inhibition of t-PA binding to annexin II is 11 μM which is close to the upper limit of normal plasma homocysteine level. These data implicate that the prothrombotic and atherosclerotic effects of homocysteine may be mediated in part by annexin II. An *in vivo* model will ultimately validate these mechanisms.

3.4.5. Annexin II overepression and bleeding diathesis in acute promyelocytic leukemia

Acute promyelocytic leukemia (APL), a subtype of myelogenous leukemia, is characterized by a chromosomal translocation t(15,17) which causes a juxtaposition of a putative transcription factor (*PML*) and a nuclear receptor for retinoic acid (RARα), resulting in a chimeric protein product PML-RARα. This product is thought to be responsible for the maturational arrest of the cells in the promyelocytic stage. Although several factors are probably involved in the bleeding diathesis observed frequently in this disease, recent evidence suggests that primary fibrinolysis may play a major role. Leukemic cells from APL patients express high levels of surface annexin II heavy chain (28). Although annexin II is located on chromosome 15, overexpression of annexin II is independent of the typical translocation found in this disease. The t-PA dependent plasmin generation was two times greater in t(15;17)-positive cells as compared to t(15;17)-negative cells and was associated with the level of annexin II surface expression. The mechanism still remains to be defined. One possibility is that the impaired retinoic acid receptor-dependent transcriptional regulation (Ap-1 and Sp-1 sites) may bind to the abnormal fusion protein and cause the overexpression of annexin II. Treatment with specific anti-annexin II monoclonal antibody eliminated this effect while a transfection of annexin II cDNA into t(15;17)-negative cells increased the plasmin generation. Treatment with the differentiating agent ATRA causes a decrease in the mRNA levels of annexin II within 24 hours followed by a decreased protein expression which coincided with a decrease in plasmin generation at 3-5 days. Thus, annexin II- mediated cell surface plasmin generation may play a major role in the bleeding diathesis associated with APL and may explain

the resolution of this symptom upon treatment with ATRA. *In vivo*, this process could be either tPA or uPA mediated as the primary leukemic cells can produce either type as the major product (96).

3.4.6. Annexin II plays a role in plasminogen-mediated matrix invasion by macrophages

Annexin II is also abundantly expressed in macrophage and monocyte-like cell lines. Using RAW264.7 macrophage cell line and THP-1 monocytic leukemic cell line, Falcone et al. demonstrated an abundant presence of annexin II on the surface of these cells by both flow cytometric analyses and EGTA elution, and the ability of the annexin II to bind plasminogen by a ligand blot study and antibody inhibition (65% decrease) of exogenous ^{125}I-lys-plasminogen binding (68). Furthermore, specific annexin-II antibodies inhibited the degradation of [^{3}H]-glucosamine-labeled extracellular matrices by RAW264.7 macrophages as well as the migration of THP-1 monocytes through matrigel coated porous membranes in response to monocyte chemotactic protein-1. Because an important mechanism by which macrophages regulate plasminogen activation is the differential expression of uPA and plasminogen activator inhibitor (97-100), anchoring of plasminogen to the cell surface by annexin II may be sufficient to increase the catalytic efficiency of uPA-mediated plasmin generation. Another possibility is that uPA may bind to the macrophage cell surface and proteolyze the cell surface-bound plasminogen. Perhaps other proteins may be involved at the cell surface in bringing uPA to the annexin II-plasminogen complex as binding of uPA to annexin II does not appear to occur (1).

4. PERSPECTIVE

Many interesting questions are raised by the proposed multi-functional properties of annexin II. *In vivo* models will ultimately validate the importance of various molecules already known by *in vitro* systems to play key or adjuvant roles in the fibrinolytic cascade. Surprisingly, mice deficient in various components of the fibrinolytic system, including plasminogen (101), tPA, uPA, PAI-1, and α-2-antiplasmin (102-104), show viable phenotypes; however, with exogenous radiolabeled clot injection studies, fibrinolytic defects are seen. Decreased metastases were observed in some of these knock out animals (105-106). Overall, these observations suggest that the fibrinolytic process is robust with redundant mechanisms, such that it is not easily perturbed by elimination of one or more components in the system. In the future, multiple combined knock-out models will further define the system as a whole and will address important questions such as how much each component contributes to fibrinolysis, embryogenesis, angiogenesis, and tumor metastasis. To add further complexity, the biochemical modifications such as the phosphorylation status of annexin II, its binding of plasminogen and tPA, as well as the catalytic effect of phosphorylation on the plasmin generation, also remain to be defined. Annexin II is an interesting molecule with multiple seemingly disparate roles. How these multiple roles may interrelate functionally will be an important challenge for the future.

5. ACKNOWLEDGMENTS

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