THE BIOLOGY OF ANNEXIN A2: FROM VASCULAR FIBRINOLYSIS TO INNATE IMMUNITY

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ABSTRACT

Annexin A2 is a multicompartmental protein that orchestrates a spectrum of dynamic membrane-related events. At cell surfaces, A2 forms the $(A2 \bullet S100A10)_2$ complex which accelerates tissue plasminogen activator−dependent activation of the fibrinolytic protease, plasmin. Anti-A2 antibodies are associated with clinical thrombosis in antiphospholipid syndrome, whereas overexpression of A2 promotes hyperfibrinolytic bleeding in acute promyelocytic leukemia. A2 is upregulated in hypoxic tissues, and mice deficient in A2 are resistant to hypoxia-related retinal neovascularization in a model of diabetic retinopathy. Within the cell, A2 regulates membrane fusion processes involved in the secretion of pre-packaged, ultra-large molecules. In stimulated dendritic cells, A2 maintains lysosomal membrane integrity, thereby modulating inflammasome activation and cytokine secretion. Together, these findings suggest an emerging, multifaceted role for annexin A2 in human health and disease. The author's work has been inspired by numerous colleagues and mentors, and by the author's grandfather, and former ACCA member, Dr. J. Burns Amberson.

INTRODUCTION

The annexins constitute a family of more than 60 highly conserved, Ca^{2+}-regulated, phospholipid-binding proteins that have existed for more than 500 million years (1). Humans express 12 annexins (annexins A1-A11 and A13), and, among these, annexin A2 (A2) is arguably the most extensively investigated with respect to health and disease (2,3). Typical annexins consist of a 30- to 35-kilodalton "core" domain containing four alpha helical, Ca^{2+}-binding "annexin" repeats, and a more hydrophilic amino-terminal "tail" domain, which is essentially unique to each family member. Through their capacity for Ca^{2+}-de-

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pendent membrane binding, annexins add or "annex" proteins to membrane surfaces and also facilitate membrane fusion events. These properties allow the annexins to fulfill a wide variety of intra- and extracellular functions, and the term "annexinopathy" has come to reflect their newly recognized roles in human pathophysiology (4).

ANNEXIN A2 AND ITS PARTNER PROTEINS

In the last 20 years, it has become increasingly clear that the cell surface is a major site for protease assembly and activity (5,6). In the 1980s, however, the concept that human endothelial cells could assemble components of the fibrinolytic system was novel. Our research began with the observation that human endothelial cells reacted specifically with antibodies directed against plasminogen and its tissue activator (tissue plasminogen activator, tPA) (7,8). Ligand blotting of a plasma membrane fraction isolated from human endothelial cell homogenates revealed that both plasminogen and tPA interacted specifically with a 36-kilodalton protein expressed on the cell surface (9). The purified protein bound both plasminogen and tPA in a dose-dependent and high-affinity manner, and amino acid sequencing identified this cell surface protein as annexin A2 (10). We now know that A2 is synthesized by endothelial cells, monocytes, macrophages, dendritic cells, trophoblast cells, epithelial cells, and some tumor cells, and can exist either as a soluble monomer in the cytoplasm, or as a complex associated with cellular membranes (11,12).

The S100 family consists of low molecular weight (9- to 14-kilodalton) dimeric proteins that undergo structural shifts in response to changes in Ca^{++} concentration, and often interact with annexins (13). By forming a heterotetrameric complex with protein S100A10, A2 increases its sensitivity to Ca^{++} and its ability to bind to cellular membranes at resting intracellular Ca^{++} concentrations (14). S100A10 is unique among the family of S100 proteins in that it exists in a permanent "calcium-on" state, and does not require a Ca^{++}-induced conformational change to associate with annexin A2 (15). Crystallographic studies have revealed that, in the tetrameric $(A2{\bullet}S100A10)_2$ complex, two copies of S100A10 are linked non-covalently to create a molecular groove, which is occupied by the α-helical N-terminal 14 amino acids of A2. In endothelial cells, S100A10 is stabilized by this interaction with A2, which masks a polyubiquitination site that would otherwise destine unpartnered S100A10 for degradation within the proteasome (16). Three additional family members, S100A4, S100A6, and S100A11, have been reported to bind A2 *in*

vitro, but the potential physiologic consequences of these interactions are unknown (17).

THE ANNEXIN A2 COMPLEX ON THE CELL SURFACE

It is now well-established that the $(A2 \bullet S100A10)_2$ tetramer serves as an assembly site for two fibrinolytic proteins, plasminogen and tPA, on the endothelial cell surface (2,3) (Figure 1). This assembly promotes plasmin generation (18–21). Upon hydrolysis of its R^{560}-V^{561} peptide bond by tPA, the zymogen plasminogen is transformed into the principal fibrinolytic protease, plasmin (22–24). The catalytic efficiency of tPA-dependent plasminogen activation increases by 10- to 100-fold in the presence of A2; although significant, this increment is less dramatic than the 500-fold acceleration provided in the presence of fibrin (22). It is hypothesized that, whereas classical fibrinolysis serves to dissolve established intravascular thrombi, the A2-based system provides constitutive surveillance that allows for clearance of fibrin forming on the blood vessel surface in response to more subtle forms of vascular injury.

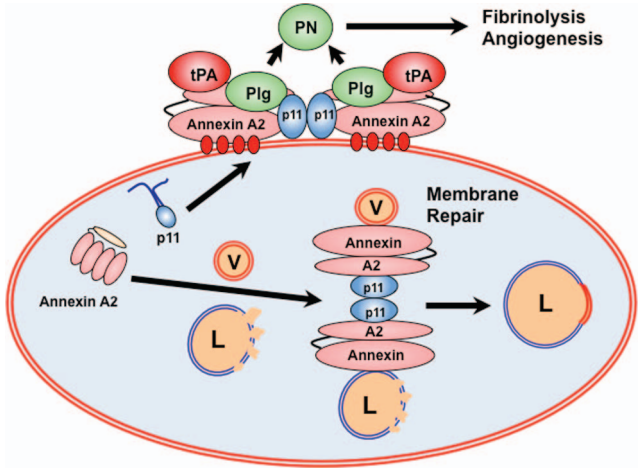

FIG. 1. Some biologic functions of annexin A2. At the cell surface, annexin A2 binds to protein S100A10 (p11). The heterotetrameric complex associates with the plasma membrane through calcium linkages (shown in red) with membrane phospholipid, and supports the assembly of plasminogen (Plg) and tissue plasminogen activator (tPA), leading to the efficient generation of the fibrinolytic serine protease, plasmin (PN). Plasmin enables fibrinolysis and angiogenesis. Within the cell, the $(\text{annexin A2-p11})_2$ complex participates in repair of injured lysosomal membranes (L) by "annexing" membrane from cytoplasmic vesicles (V).

Expression of the $(A2•S100A10)_2$ complex on the endothelial cell surface is a dynamic process. Translocation of the complex from the cytoplasm to the outer leaflet of the endothelial cell membrane appears to be a key regulatory step in vascular fibrinolysis, but the precise translocation mechanism is unknown (25,26). Increased cell surface expression of the heterotetramer occurs within minutes to hours of heat stress, receptor-mediated thrombin stimulation, or hypoxia (27−29), and requires both src kinase-mediated phosphorylation of Y^{23} and expression of S100A10 (28). In addition, the level of expression of the complex on the cell surface is regulated by intracellular protein kinase C, which phosphorylates S^{11} or S^{25} residues within the N-terminal tail domain of A2, thereby dissociating the $(A2•S100A10)_2$ complex, and preventing further translocation to the cell surface (30,31). Serine phosphorylation of A2 by protein kinase C (PKC) appears to be triggered by cell surface plasmin, which cleaves A2 and activates toll-like receptor 4. This negative feedback mechanism may allow plasmin to limit its own activation.

THE ANNEXIN A2 COMPLEX IN HEMOSTASIS

Several animal studies support the hypothesis that annexin A2 regulates hemostasis *in vivo*. First, *Anxa2*$^{-/-}$ mice, while displaying uncompromised development, fertility, and lifespan, accumulate fibrin in both intra- and extravascular locations within the lungs, spleen, small intestine, liver, and kidney (32) Second, experimental injury to the carotid artery leads to a significant increase in thrombotic occlusion in *Anxa2*$^{-/-}$ versus *Anxa2*$^{+/+}$ mice (32), and *S100a10*$^{-/-}$ mice also display increased vascular fibrin and reduced clearance of thrombi (33). Third, *Anxa2*$^{-/-}$ microvascular endothelial cells fail to support tPA-dependent plasmin generation (32). Fourth, in mice with diet-induced hyperhomocysteinemia, homocysteine derivatizes A2 and blocks its ability to bind tPA and generate plasmin, leading to fibrin accumulation and deficits in angiogenic potential (34,35). Fifth, A2 alone or in combination with tPA enhances vascular patency and reduces infarct size in several rodent models of stroke (36−40).

These results are reflected in recent observations in humans. In antiphospholipid syndrome and in a cohort of patients with cerebral venous thrombosis, high-titer anti-A2 autoantibodies are prevalent and correlate with major thrombosis (41−45), suggesting that cell surface A2 represents a prominent auto-antibody target associated with a thrombosis (46). In children with sickle cell disease, in addition, single nucleotide polymorphisms in the *ANXA2* gene are associated

with increased risk of stroke (47,48), whereas additional *ANXA2* SNPs have been associated with elevated risk of avascular necrosis of bone (osteonecrosis) (49).

In acute promyelocytic leukemia, which, conversely, is associated with life-threatening hemorrhage at the time of presentation (50), there is typically robust expression of A2 in leukemic blast cells (51). The resulting coagulopathy appears to reflect a combination of disseminated intravascular coagulation and hyperfibrinolysis, the latter evidenced by elevated fibrin degradation products, depletion of plasma fibrinogen, and consumption of alpha$_2$-antiplasmin (51). In cultured acute promyelocytic leukemia−like cells, elevated steady state levels of A2 mRNA returned to normal after treatment with the therapeutic differentiating agent all-*trans* retinoic acid (51). Follow-up studies have confirmed these findings and shown that S100A10 is also elevated in these cells (52,53).

ANNEXIN A2 IN PROLIFERATIVE RETINAL ANGIOPATHY

In several models of stimulated postnatal angiogenesis, *Anxa2*$^{-/-}$ mice have shown a diminished ability to form new blood vessels (32). In addition, wild-type mice with diet-induced hyperhomocysteinemia display impaired corneal neoangiogenesis due to covalent modification of annexin A2 by homocysteine; in this case, angiogenesis can be restored to normal with intravenous infusion of recombinant A2 (34). In the oxygen-induced retinopathy model, which mimics many aspects of human proliferative diabetic retinopathy (54), the typical vascular proliferative response is blunted by approximately 50% in *Anxa2*$^{-/-}$ mice (29). The data suggest that angiogenic impairment in the *Anxa2*$^{-/-}$ mouse may reflect reduced vascular fibrinolysis and fibrin accumulation around blood vessels.

INTRACELLULAR ANNEXIN A2

As a multicompartmental protein, annexin A2 is poised to fulfill a range of intracellular membrane-related functions, including organization of specialized membrane microdomains, recruitment of peripheral membrane proteins, and regulation of membrane fusion and repair events (11). Whereas heterotetrameric (A2•S100A10)$_2$ resides on the plasma membrane, monomeric annexin A2 is distributed throughout the cytoplasm, but may transition to intracellular membranes in response to signals, such as changes in Ca^{2+} concentration, pH, or membrane phospholipid composition, and the availability of ancillary

S100 proteins, such as S100A10. How these multiple activities may relate to human health and disease, however, is not yet clear.

Annexin A2 mediates a number of intracellular vesicular remodeling events. Within its N-terminal domain, annexin A2 possesses a single isoleucine-leucine pair motif (amino acids 6 and 7) that may function as an endosomal targeting sequence (55), thus allowing A2 to bind to endosomes and possibly mediate their fusion (56,57). In addition, A2 is required for the biogenesis of multivesicular bodies, and is also a constituent of exosomes that is frequently cited in proteomic studies (58,59). Through interactions with soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment receptor (SNARE) proteins, A2 participates in the regulated exocytosis of chromaffin granules (60,61), von Willebrand factor–containing Weibel-Palade bodies (62,63), lamellar body–containing surfactant (64,65), and collagen VI multimers (66) from chromaffin, endothelial, type II alveolar, and bronchial epithelial cells, respectively.

Through its ability to associate with lysosomal membranes (Figure 1), A2 dysfunction is implicated in the inflammatory response associated with aseptic arthritis, which occurs in 10% to 15% of patients undergoing the several million joint replacement procedures performed each year in the United States (67). In aseptic arthritis, wear debris particles are shed into the joint space upon articulation of prosthetic joint surfaces. These particles are endocytosed by inflammatory macrophages and dendritic cells, and can induce lysosomal and endosomal membrane damage, which is normally associated with recruitment of cytoplasmic annexin A2 to the lyso-endosomal membrane. In A2-deficient cells, lysosomal injury leads to leakage of lysosomal cathepsins into the cytosol. Through an as yet unknown mechanism, cytosolic cathepsins activate the nucleotide-binding, leucine-rich, pyrin-containing-3 (NLRP3) inflammasome, leading to secretion of interleukin-1 and accelerated inflammation (68).

SUMMARY

The annexin A2 system serves a growing spectrum of biologic functions both atop and beneath the plasma membrane. At the cell surface, the $(A2{\bullet}S100A10)_2$ heterotetrameric system localizes plasmin activity and promotes fibrinolysis, angiogenesis, and cell migration. Within intracellular compartments, A2 appears to facilitate membrane organization, fusion, and repair in an array of activities including endocytosis, exocytosis, and lysosomal membrane repair. The physiologic consequences of these activities are under active investigation, and the next several years of annexin A2 research should offer exciting insights into human health and disease.

TRIBUTE

In the work described herein, I have been inspired by numerous colleagues and mentors, including James Burns Amberson, Jr., MD (Figure 2). He was born in 1890 in Waynesboro, Pennsylvania, attended Lafayette College, and graduated from the Johns Hopkins University School of Medicine in 1917. In 1918, while working with Dr. E. W. Goodpasture in the pathology lab at Hopkins, Dr. Amberson experienced an episode of hemoptysis, and the diagnosis of pulmonary tuberculosis was made. He was admitted to Loomis Sanatorium in upstate New York, where, after a year of recuperation, he became a

FIG. 2. James Burns Amberson, Jr., MD. Reprinted from Richards, D. L. "Presentation of the Academy Plaque to James Burns Amberson, M.D." Bull NY Acad Med 1970;46:663–5. Courtesy of the New York Academy of Medicine.

staff physician, and ultimately Physician-in-Chief. In 1929, he accepted a faculty position at Columbia's College of Physicians and Surgeons, and began working at the Bellevue Chest Service. There, he devoted the rest of his career to the study and treatment of tuberculosis, serving as Physician-in-Charge.

Dr. Amberson was elected to the American Clinical and Climatological Association (ACCA) in 1922 at the age of 32, and served as its Vice President in 1940 (69). His first publication in the ACCA Transactions was entitled "Clinical Studies of the Healing of Tuberculosis: I. Absorption of Pulmonary Deposits," and demonstrated the importance of correlating serial clinical and radiologic findings in the treatment of tuberculosis (70). This paper revealed that pulmonary tuberculosis can heal by resolution, in addition to fibrosis and calcification, the more commonly recognized modes of healing. In his ACCA Memorial to J. Burns Amberson, Dr. George W. Wright described him as "a scholarly, gentle person. . .a teacher par excellence, a superb clinician, and a compassionate physician" (71). This is an apt description of someone well-known to me, as Dr. Amberson was, after all, my grandfather.

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DISCUSSION

Boxer, Ann Arbor: What is the mechanism by which annexin induces fusion? And my other question, does annexin II play a role in the periodic fever syndromes?

Hajjar, New York: So, Larry we don't know whether it plays a role in periodic fever syndromes. I think that might be a very interesting thing to investigate. There are actually a number of questions that have come to mind, and we have to prioritize them. But I think that would be very interesting. And your other question was about the mechanism by which it induces inflammation. What we think is that its normal function is to maintain the integrity of the lysosome. And when the lysosomal contents leak into the cytoplasm, that induces assembly of the inflammasome, and we get all these downstream events and secretion of cytokines.

Schuster, New York: Have you had a chance to try blocking antibodies in animal models of diabetic retinopathy. And secondly, not all endothelia are the same, so what is distribution of annexin across the arterial and then the venous endothelium?

Hajjar, New York: The distribution of annexin II; we find it in every endothelial cell that we have looked at throughout the body. So we think that it's fairly ubiquitously distributed. We have a panel of antibodies that we have developed with some collaborators and we are getting ready to test those in mice.

Hochberg, Baltimore: Is there any role for cyclo-oxygenase enzymes in the expression of annexin on the cell membrane and its intracellular location to the lysosomal membrane?

Hajjar, New York: The answer is we don't know.

Wenzel, Richmond: Have you looked at drugs that might block the inflammasome specifically and see what happens to the activity or to the levels of annexin in either sepsis or the periodic fever or something related?

Hajjar, New York: We have another model of inflammatory bowel disease that we have been working on, and I didn't think I could fit that into the 12 minutes. But essentially we have used two inhibitors of inflammasome activation in DSS-induced inflammatory bowel disease in the mouse, and in both cases the increased severity that we see in the knockout reverts to the level of disease we see in the wild type. So that would confirm your hypothesis.