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Review

Annexins as disease modifiers

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Summary. The annexins are a family of calcium-dependent phospholipid binding proteins which are present in all eukaryotes. There are currently 12 identified human annexins all of which contain unique calcium binding sites, encoded in the highly conserved annexin repeat motifs within the C terminal core. In addition to the C terminal core the annexins contain a significantly more variable N terminal head. It is this domain which endows each annexin with unique functions in a diverse range of cellular processes including; endo- and exocytosis, cytoskeletal regulation and membrane conductance and organisation. Given their involvement in such a variety of processes it is not surprising that the annexins have also been implicated in a range of disease pathologies. Although there is no singular disease state directly attributed to a dysregulation in annexin function, several pathological conditions are suggested to be modified by the annexins. In this review we shall focus on the growing evidence for the role of the annexins in the progression of cancer, diabetes and the autoimmune disorder anti-phospholipid syndrome.

Key words: Cancer, Diabetes, Lipocortin, Calpactin, Synexin

Introduction

The annexins are a family of highly conserved proteins, present in protists through to humans. They are characterised by their ability to bind phospholipids, predominantly in a calcium dependent manner. There are 12 known human annexins, which first began to be identified in the late 1970s and 1980s when they were given disparate names dependent on their biochemical properties. These included lipocortin I (annexin A1) (Buday et al., 1989), calpactin I (annexin A2) (Glenney, 1986) and synexin (annexin A7) (Creutz et al., 1978). It was not until 1990 that the annexin nomenclature was formalised, following sequence homology analysis identifying these proteins as belonging to the same family. A common structure was observed, such that all proteins in this family comprise a C terminal core domain and an N terminal head domain. The C terminal core contains the highly conserved annexin repeats, of which all vertebrate annexins contain four with the exception of annexin A6 which contains eight (Crompton et al., 1988). These repeats encode the unique calcium binding sites of the annexins. The N terminal head is highly variable between different annexins and is thought to confer their individual properties which have evolved over time to allow the annexins to be involved in a diverse range of cellular processes. Ongoing studies on the annexins have elucidated their importance in normal cellular processes, such as the regulation of the actin cytoskeleton (Hayes et al., 2006), membrane trafficking (White et al., 2006) and the modification of ion channel activity (van de Graaf et al., 2003). However as this field of research has grown implications have also arisen for the role of the annexins in disease pathology, with particular emphasis on oncogenesis, diabetes and autoimmunity.

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Annexins and Cancer

Although annexins are ubiquitously expressed proteins, the patterns of expression of individual family members are known to change during normal cellular and tissue development. Numerous recent studies have shown the altered expression of annexins during neoplastic progression relative to normal tissue. These studies have utilised both proteomic and genomic approaches and in both cases the incidence of annexins noted as differentially regulated is high. If these were isolated and unverified accounts they could be dismissed as false positives, but the frequency of such findings and their corroboration in independent studies provides compelling evidence that the changing differentiation state of the tumour leads to changes in annexin expression. Or more intriguingly, that the changes in annexin expression are not so much a consequence of

tumour progression, but actually contribute in an active way to the transformed or metastatic phenotype. However, regardless of any possible roles for annexins in tumourigenesis, there is still a good case for the annexins as diagnostic and/or prognostic markers for cancer progression. This is due to their involvement in a wide range of cellular processes, including cell-cell junction maintenance, plasma membrane-cytoskeletal attachments, cell motility and endocytosis; processes that are all implicated in cancer pathology.

The study of annexins in cancer cell lines is vital to address the questions of mechanisms of action. However, they should not be viewed in isolation due to the sensitivity of annexin expression to stress, growth factors and cell confluency when maintained in culture. Therefore the most substantiated roles for the annexins are those where both cell lines and primary tissues have been investigated. Annexin A1 was first shown to be down-regulated in prostate cancer using the LNCaP cell line (Chetcuti et al., 2001). This was later corroborated by immunohistochemical analysis of patient samples (Kang et al., 2002). Though this study was dependent on the use of a single anti annexin A1 antibody to detect changes in protein levels, changes were also detected at the mRNA level and later independently confirmed by cDNA microarray analysis (Xin et al., 2003). In this way, the case for the dysregulation of annexins in cancer is building. For prostate cancer in particular several annexins, including annexin A1, A2, A4, A6, A7 and A11, have all been shown to be down-regulated (Srivastava et al., 2001; Xin et al., 2003). Of these annexins, annexin A7 has been highlighted as a potential tumour suppressor gene in prostate cancer (Srivastava et al., 2001). Tissue microarrays of prostate cancer samples showed an increased rate of loss of annexin A7 expression in metastatic tumours when compared to primary tumours. This has since been noted in lymph node metatases from both prostate and breast cancer (Srivastava et al., 2007). Furthermore a loss of heterozygosity has been observed in close proximity to the annexin A7 gene locus in a substantial proportion of primary prostate tumour cell samples (Srivastava et al., 2001).

Annexin A2, though shown to be down regulated in a prostate cancer tissue microarray (Xin et al., 2003), has more recently been shown to be up regulated in prostate cancer cell lines (Hastie et al., 2008). In an annexin A2-negative prostate cancer cell line over expression of annexin A2 enhanced the invasive nature of these cells. Furthermore in an annexin A2-positive prostate cancer cell line treatment of the cells with an anti annexin A2 antibody decreased their invasiveness (Hastie et al., 2008). This decrease also occurred upon treatment of the cells with interferon α (IFNα), which resulted in the down regulation of cell surface-associated annexin A2 and annexin A2-associated proteases. This study provides a potential means by which IFNα may help delay the spread of prostate cancer through regulating annexin A2 localisation and so cell invasion. The apparent discrepancy in the expression of annexin A2 and the correlation with prostate cancer between the Xin et al. (2003) and Hastie et al. (2008) studies may be explained by the specific stage of prostate cancer investigated. Xin et al. (2003) demonstrated a down regulation in annexin A2 expression between hormone refractive i.e. unresponsive to hormone therapy, and hormone naive i.e. still responsive to hormone therapy, prostate cancer samples. Hastie et al. (2008) focused on hormone responsive cell lines and demonstrated that the up regulation of annexin A2 enhanced the invasive nature of these cells. Therefore annexin A2 expression levels may reflect a difference in prostate cancer progression, with an up regulation in early stage cells that are still responsive to hormone treatment and a down regulation in late stage cells that are no longer responsive to hormone treatment.

Further evidence for the role of the annexins in the development and progression of tumours has been shown for annexin A1. Annexin A1 levels may be either up- or down–regulated in different cancers and have been shown via tissue microarray analysis to be down-regulated in breast cancer (Shen et al., 2006), contradicting earlier work carried out with a significantly smaller sample size (Ahn et al., 1997). Tissue microarray analysis suggests this down-regulation correlates with progression of the cancer. The up regulation of the receptor for epidermal growth factor (EGFR) is often associated with breast cancer (Bhargava et al., 2005). This may provide some insight into the role annexin A1 plays in the tumourogensis of breast cancer, as it is required for the endocytosis of the EGFR (White et al., 2006). Therefore the down-regulation of annexin A1 may contribute to tumour pathology by potentiating EGF signalling. In a similar manner the down-regulation of annexin A3 has been associated with the progression of prostate cancer (Kollermann et al., 2008). Conversely annexin A2, A4 and A11 up-regulation has been shown to correlate with progression of colorectal cancer (Duncan et al., 2008). These studies relied on single antibody immunohistochemical stainings and require further analysis at the genomic level. Nevertheless they benefit from the high throughput screening of a large number of pathological and control tissue samples, supporting their relative validity.

Following studies demonstrating correlations between annexin expression and the developmental stages of cancer, focus is now beginning to extend to the mechanisms by which the annexins may contribute to pathology. Immunohistochemical analysis of invasive ductal breast cancer tissue showed consistent expression of annexin A2 in ductal epithelia, which was absent in normal and hyperplastic ductal epithelia (Sharma et al., 2006). Further investigation in cancer cell lines suggested a link between the invasive nature of annexin A2-expressing cancer cells and their ability to produce plasmin, which can degrade extracellular matrix. Annexin A2 is thought to enhance this ability as it is a known co-receptor for tissue-type plasminogen activator.

In support of this, annexin A2 expression has been shown to be elevated in migrating versus stationery epithelial cells (Babbin et al., 2007). This is attributed to its ability to target Rho GTPases to the plasma membrane, resulting in subsequent affects on Rho dependent cytoskeletal reorganisations. This ties in with the known regulatory effects of annexin A2 on the actin cytoskeleton at the plasma membrane (Hayes et al., 2006). In a single study, a moderate decrease in annexin A1 expression was correlated with low grade, early stage bladder carcinoma (Xiao et al., 2007). The functional effects of decreased expression, decreased cell adhesion and increased cell motility, were related back to the actin binding properties of annexin A1. A more detailed investigation of the oncogenic signalling cascades in cancer cell lines has uncovered a role for annexin A6 in the regulation of the small GTPase Ras (Vila de et al., 2009). Annexin A6 was shown to be down-regulated in several cancer cell lines and its further depletion using siRNA increased Ras activity. Furthermore it is known to bind the Ras inactivator p120GAP (Davis et al., 1996) and through altering the localisation of p120GAP regulates Ras activity (Grewal et al., 2005). Annexin A6 may therefore be an important target in many cancers where Ras signalling is known to be dysregulated.

The ultimate aim of investigating the annexins in cancer would be to harness them as a therapeutic target, though their ubiquitous expression makes this a difficult task. Nevertheless the exploitation of annexins as potential drug targets is being investigated. Annexin A1 has been suggested as a possible target for the anti cancer activities of green tea polyphenols (Lu et al., 2007), which were shown to promote cell adhesion and decrease cell motility, alongside stimulating a moderate increase in annexin A1. Progress is also being made with regards to the steroidal lactone Withaferin A, which has been shown to significantly affect the cytoskeletal architecture of cancer cell lines through its specific targeting of annexin A2 (Falsey et al., 2006). This cytotoxic effect is attributed to the modification of the actin network, with which annexin A2 is known to interact (Hayes et al., 2006). Withaferin A is also a potent anti-angiogenic compound and has been utilised as a marker for angiogenesis (Yokota et al., 2006). This compliments observations in the annexin A2 knock out mouse, which demonstrates an inability to carry out pathological neoangiogenesis (Ling et al., 2004), a key event in tumour progression. Aside from the potential role of annexin A2 as a therapeutic target, it has been put forward alongside annexin A1 as a prognostic tool for the pathological response of breast cancer cells to neoadjuvant chemotherapy (Chuthapisith et al., 2009).

Annexins and Diabetes

The role for annexins in diabetes focuses on endothelial cell biology and the effects of hyperglycaemia, a manifestation of both type I and II diabetes, on these cells. Annexin A2 singularly stands out as the most convincing case for annexin involvement in diabetes. This is due to its role as a receptor for tissue plasminogen and tissue plasminogen activator (tPA), which act together to promote plasmin production on the surface of the vascular endothelium (Hajjar et al., 1994). A well known symptom of diabetes is hypercoagulation. Plasmin (activated plasminogen) dissolves blood clots via the process of fibrinolysis. The production of plasmin from plasminogen is reduced in primary endothelial cells cultured in high glucose and insulin and this effect is negated by adding recombinant annexin A2 (Ishii et al., 2001). Further studies carried out in a mouse model of type II diabetes have since shown the addition of recombinant annexin A2 also has protective effects on kidney dysfunction and is a potential new candidate for the treatment of diabetic nephropathy by targeting hypercoagulation in kidney glomeruli (Ishii et al., 2007).

The function of endogenous annexin A2 in these diabetic model systems is suggested to be impaired by glycation (Gugliucci and Ghitescu, 2002), although its recruitment to the plasma membrane of cultured endothelial cells is still enhanced in high glucose conditions (Lei et al., 2004). Lei et al. (2004) also showed increased plasmin activity in response to high glucose, which was partially inhibited by treatment with an anti annexin A2 antibody. Though the two studies appear incongruent it should be noted that Gugliucci and Ghitescu treated cells with both high glucose and insulin (Ghitescu et al., 2001), whereas Lei et al only used high glucose conditions (Lei et al., 2004). Insulin receptor activation is known to cause tyrosine phosphorylation of annexin A2 and subsequent annexin A2-dependent actin remodelling (Rescher et al., 2008). One could therefore speculate that high insulin conditions may induce other annexin A2-dependent changes and alter its ability to produce plasmin. Furthermore, the different phosphorylation states of annexin A2 in low versus high insulin conditions could result in different roles in type I (low insulin) and type II (high insulin) diabetes.

Annexin A2 and annexin A1 have been shown to be early glycation products in an experimental model for diabetes in rats. These proteins, alongside several other endothelial plasma membrane proteins involved in actin remodelling, are suggested to impair the fluid nature and mechanical properties of the endothelial membrane (Ghitescu et al., 2001). Annexin A1 is also thought to be involved in insulin secretion, though the precise function is unclear. This is due to its localisation to insulin granules in beta cells of rat pancreatic islets and increased localisation to these granules in response to ingestion of glucose (Ohnishi et al., 1995). In addition, annexin A1 was shown to be phosphorylated in isolated islets in response to high glucose, concomitant with a burst in insulin secretion (Ohnishi et al., 1995). More recently a proteomic screen was carried out to identify proteins differentially regulated in rat pancreatic islets, which were pharmacologically induced to increase insulin secretion at high glucose concentrations. Annexin A1 was again shown to be up-regulated in

response to increased insulin secretion (Jagerbrink et al., 2007), although whether this correlation contributes to efficient insulin secretion has yet to be answered.

Annexins and Inflammation

Annexin A1 is a key regulator in the resolution of inflammation in response to glucocorticoids and its role in this process is summarised in a recent review by Perretti and D'Acquisto (2009). In this review annexin A1 is highlighted as a pivotal player in both the innate and adaptive immune systems. In cells of the innate immune system including monocytes, macrophages and neutrophils, activation leads to the translocation of annexin A1 to the plasma membrane and its subsequent secretion. In T cells of the adaptive immune system annexin A1 is up-regulated upon activation. Glucocorticoids target both arms of the immune system and are proposed to exert their effects through stimulating annexin A1 secretion in the innate immune system and inhibiting annexin A1 expression in the adaptive immune system. Evidence for this activity in the innate immune system has come from mouse experimental models of inflammation where the application of annexin A1 results in anti-inflammatory effects, such as the detachment of neutrophils adhered to the walls of the microvasculature (Perretti et al., 1996). Several studies in the annexin A1 knock out mouse have yielded further support for its role in the innate immune system, for example neutrophils from these mice show increased microvascular transmigration (Chatterjee et al., 2005) and chemoattractive responses (Lim et al., 1998). In accordance with this the extracellular administration of annexin A1 peptides has been shown to disrupt cell adhesion and migration of leucocytes and promote detachment of neutrophils (Gavins et al., 2003). This is suggested to occur through its interaction with the formyl-peptide receptor (FPR or fMLP). The annexin A1 knockout mouse has also shed light on the role of annexin A1 in the adaptive immune system, as their T cells show a decreased signalling response in AKT and ERK pathways upon activation (D'Acquisto et al., 2007). The large body of studies carried out on annexin A1 and glucocorticoids firmly establishes annexin A1 as a potential target of glucocorticoid therapy.

Several other annexins have been implicated in the inflammatory response, in particular the pathological state of inflammation associated with autoimmune diseases such as anti-phospholipid syndrome (APS). APS is characterised by an increase in venous and arterial thrombosis and pregnancy-related complications such as miscarriage and pre eclampsia. Antibodies identified in the sera of patients with APS are predominantly reactive against $ß_2$-glycoprotein I ($ß_2$-GPI), a lipid binding protein, not a lipid itself as the term APS would suggest. $ß_2$-GPI is a blood plasma protein that binds to the surface of endothelial cells. Autoantibodies against annexin A2 have also been detected in the serum of APS patients (Cesarman-Maus et al., 2006; Salle et al., 2008). Annexin A2 acts as an endothelial cell surface receptor for tissue plasminogen and tissue plasminogen activator (Hajjar et al., 1994). It is now believed to have an additional role as a binding site for $ß_2$-GPI (Ma et al., 2000), as evidenced by experiments showing over-expression of annexin A2 increasing $ß_2$-GPI binding and in vitro binding assays. Furthermore, both anti $ß_2$-GPI and anti annexin A2 antibodies in the presence of $ß_2$-GPI have been shown to activate endothelial cells to the same degree (Zhang and McCrae, 2005). This activation was replicated using bivalent anti annexin A2 F(ab')2 fragments but inhibited in all three cases in the presence of monomeric anti annexin A2 Fab fragments, suggesting an annexin A2 clustering mechanism in the activation of endothelial cells. The function of monocytes in APS has also been investigated and it has been shown that anti $ß_2$-GPI antibodies stimulate the secretion of the pro-inflammatory cytokine tumour necrosis factor α and the procoagulant protein tissue factor (Sorice et al., 2007). The role of annexin A2 in these cells in unknown, however a single proteomic study has demonstrated the up-regulation of annexin A2 in monocytes of patients with APS and thrombosis (Lopez-Pedrera et al., 2008).

Annexin A5 has also been implicated in APS, as reviewed by Rand et al. (2008), where it is suggested to form an anti-coagulant barrier on the surface of cells due to its affinity for anionic phospholipids. One conceptual problem with this hypothesis is that cell surfaces are usually enriched in cationic phospholipids, such as phosphatidylcholine, although in the high calcium milieu of the extracellular environment it is possible that some cell surface binding of annexins may occur. Autoantibodies produced in APS are proposed to lead to the disruption of this annexin A5 array and so promote miscarriage in pregnant women (Rand et al., 2005). The role of annexins in other autoimmune diseases is less well understood and much is based on the detection of autoantibodies. For example autoantibodies against annexin A11 have been detected in APS, rheumatoid arthritis, systemic lupus erythematosus, Sjorgens syndrome and Raynauds disease (Misaki et al., 1994; Jorgensen et al., 2000). However the prevalence and functional relevance of these autoantibodies is unclear. The strongest link of annexin A11 to an autoimmune disease comes from a recent study highlighting a single nucleotide polymorphism (SNP) in the annexin A11 gene as the most highly associated susceptibility locus for sarcoidosis, in a German population. However it is not known whether it is the single SNP or the haplotype conferred by the surrounding SNPs that may result in a predisposition to sarcoidosis (Hofmann et al., 2008).

Concluding remarks

The role of the annexins in disease pathology is an emerging field, in which the mechanisms are not clearly defined, particularly with regard to oncogenesis. A clearer picture has however evolved for the role for

annexin A2 in diabetes and annexin A1 in inflammation. Currently no disease has been directly ascribed to the dysfunction of a particular annexin, though evidence is building for the roles of the annexins as disease modifiers, and in this context they may yet prove to be valid therapeutic targets.

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