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Visions & Reflections (Minireview)

Annexin A5: shifting from a diagnostic towards a therapeutic realm

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Abstract. The surge in apoptosis research and the discovery of the phosphatidylserine binding properties of annexin A5 have propelled a tremendous interest in cell death detection technologies. In the past years, annexin A5 has evolved from an efficient assay for detection of apoptotic cells *in vitro* to an *in vivo* molecular imaging technology with potential clinical use. A second key discovery, the specific internalization properties of annexin A5, has opened the opportunity to use annexin A5 for therapeutic applications. Annexin A5-mediated internalization creates a novel therapeutic platform for targeted drug delivery and cell entry to treat various diseases, including cancer and cardiovascular disease.

Keywords. Annexin A5, phosphatidylserine, apoptosis, diagnosis, therapy.

Annexin A5 belongs to a multigene family of phospholipid binding proteins, the annexins [1]. Annexin A5 was discovered as an anticoagulant protein [2] with antithrombotic activity *in vivo* [3]. Its anticoagulant activity arises from high-affinity binding to negatively charged phospholipids such as phosphatidylserine (PS) [4] and subsequent multimerization on the lipid surface by homotypic interactions [5,6]. To date, several other biological properties of annexin A5 have been described, including Ca^{2+}-channel activities [7,8], phospholipase A2 regulation [9,10], inhibition of phagocytosis of apoptotic cells by both activated and unactivated macrophages [11–13] and immune modulation [14,15]. In spite of available data, annexin A5's physiological function remains to be determined. The annexin A5 knockout mouse has thus far regrettably not shed light on this issue [16]. It is generally accepted, however, that its physiological significance is closely associated with its ability to bind to PS.

In 1992, Fadok et al. reported that apoptotic cells express PS on their cell membrane as an 'eat me' signal towards phagocytes [17]. Combining her finding with the then known physiochemical properties of annexin A5 led to the birth of the annexin A5 affinity assay to measure apoptosis [18,19]. Thereafter, the annexin A5 affinity assay developed from an *in vitro* assay to an *in vivo* diagnostic application currently under investigation in patients [20].

The success of the annexin A5 affinity assay is in part based on the fact that annexin A5 can be conjugated to a wide range of reporter compounds, including fluorophores and radioisotopes, without significantly impairing its PS binding and consequently its apoptotic cell-binding properties. The quality of conjugation has been further improved by the generation of annexin A5 variants. The variants contain a cysteine residue in the N-terminal tail either as part of an extension of the tail [21] or within the tail by replace-

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ment of glutamine at position 2 [22]. The cysteine variants allow easy site-directed conjugation chemistry, yielding a complex of known stoichiometry with full biological activity since the tail is located at the surface opposite to the surface containing the phospholipid binding sites [23].

Van den Eijnde and co-workers were the first to show that annexin A5 can visualize apoptosis *in vivo* in an animal model [24]. This study nicely demonstrated that within the complex environment of the whole body annexin A5 binds to dying cells and not to living cells with two exceptions: the myoblast and the megakaryoblast [24]. Others have extended the list of *in vivo* applications of the annexin A5 affinity protocol in animal models [25–27]. Technetium labeled annexin A5 was used to detect apoptosis non-invasively in patients suffering from acute myocardial infarction [28], intracardiac tumor [29] and heart transplant rejection [30]. Recently, annexin A5 was shown to be a valuable marker to distinguish between stable and unstable atherosclerotic plaques in patients due to the binding of annexin A5 to a number of key characteristics of plaque instability, such as apoptosis, macrophage infiltration and red blood cells delivered by intraplaque hemorrhage [31]. In addition to the assessment of the biology of diseased lesions, the annexin A5 affinity protocol shows promise in monitoring treatment efficacy in cancer patients [32–34].

The development of annexin A5 as a molecular imaging probe has started to pave mostly unintentionally an avenue towards the therapeutic arena. A biomarker for molecular imaging implicitly has the potential to be a target for drug-delivery strategies. PS is one such biomarker and annexin A5 is one such targeting vector. The cons of annexin A5 as a targeting vector are still unknown but the tantalizing pros arising from the Molecular Imaging experience are that such experiments are simply waiting to be performed. Annexin A5 has been coupled to thrombolytic enzymes, and the chimera shows promise in localizing thrombolytic activity at intravascular thrombi [35]. Recently, it was reported that annexin A5 opens a portal of cell entry on the PS-expressing cell. Annexin A5 not only binds to PS but also forms a two-dimensional network on the surface that causes its internalization concomitantly with covalently attached compounds. This opens possibilities for targeting pharmacological compounds to the intracellular compartments of PS-expressing cells. PS exposure has not only been observed in apoptotic, primary and secondary necrotic cells but also in living cells. For example, activated murine bone marrow B cells [36], fusing myoblasts [37] and fusing cytotrophoblasts [38] expose PS temporarily at their cell surface without committing to execute apoptosis. On these cells, surface expressed PS obviously does not trigger phagocytes to activate engulfment. Appelt et al. suggested that the PS density is too low to emit the "eat me" signal [39]. Annexin A5 also appears to bind to low-density PS-expressing cells that do not trigger engulfment by phagocytes. Several studies indicate that annexin A5 is also internalized by living cells such as tumor cells [40] and myoblasts [37] *in vitro* and stressed neurons [41] *in vivo.* Recently, caspase-independent pathways leading to PS expression were proposed, providing mechanistic explanations for transient PS expression by living cells [42]. Hence, annexin A5 can also bind living cells such as tumor cells and enter the living cell once bound to it.

The cell-entry function is crucial for therapeutic efficacy if the targeted drug should act intracellularly, as is the case for example with chemotherapeutics. One palpable example of annexin A5 as a targeting vector in oncology is the construction of annexin A5-targeted liposomes encapsulating chemotherapeutic drugs to treat cancer. The current limitations of chemotherapeutics are dictated by severe side effects. For instance, the use of doxorubicin is limited by its severe cardiotoxic side effects, which may result in life-threatening dysfunction of the left ventricle of the heart. Encapsulation of doxorubicin in liposomes has resulted in better side-effect and safety profiles: however, the tradeoff is reduction of therapeutic efficacy due to limited bioavailability of encapsulated doxorubicin. Annexin A5 and its seek and cell-entry functions could improve the therapeutic efficacy of liposome-encapsulated chemotherapeutics. Liposomes carrying drugs are usually around 100 nm in diameter. Coupling to these large structures should compromise neither annexin A5's PS-binding activity nor its cell-entry function. The engineered cysteine variants of annexin A5 should preserve these functions if coupled to 100-nm liposomes. Recent experiments in our laboratory have demonstrated that the cysteine variant of annexin A5 retains both functions after covalent coupling to 100 nm liposomes.

Annexin A5 appears even more fascinating as a therapeutic targeting vector in light of the fact that the biomarker PS is not only present in cardiovascular and neoplastic diseases but also in autoimmune and neurodegenerative diseases.

Concluding remarks

Taken together, the recently discovered internalization properties of annexin A5 opened the opportunity to shift from diagnostic towards therapeutic application. This may result in a combination of both

applications in one procedure wherein diagnosis and therapy concur. The unmasking of PS exposure in diseased lesions of patients using annexin A5-imaging technology may help to select patients for annexin-mediated delivery strategies. Molecular imaging coupled to drug-targeted therapy represents a novel feature for controlled drug release, allowing one to localize abnormalities, estimate local drug concentrations and follow in time the efficacy of treatment. The dual use of annexin A5 to help in the treatment of cancer and cardiovascular disease is now more realistic then ever.

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