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REVIEW

The advantageous role of annexin A1 in cardiovascular disease

Renske de Jong[a,b], Giovanna Leoni[a,b], Maik Drechsler[a,b,c], and Oliver Soehnlein[a,b,c]

[a]Institute for Cardiovascular Prevention, Ludwig-Maximilians University, Munich, Germany; [b]Department of Pathology, Academic Medical Center, Amsterdam University, Amsterdam, the Netherlands; [c]DZHK (German Centre for Cardiovascular Research), partner site Munich Heart Alliance, Munich, Germany

ABSTRACT

The inflammatory response protects the human body against infection and injury. However, uncontrolled and unresolved inflammation can lead to tissue damage and chronic inflammatory diseases. Therefore, active resolution of inflammation is essential to restore tissue homeostasis. This review focuses on the pro-resolving molecule annexin A1 (ANXA1) and its derived peptides. Mechanisms instructed by ANXA1 are multidisciplinary and affect leukocytes as well as endothelial cells and tissue resident cells like macrophages and mast cells. ANXA1 has an outstanding role in limiting leukocyte recruitment and different aspects of ANXA1 as modulator of the leukocyte adhesion cascade are discussed here. Additionally, this review details the therapeutic relevance of ANXA1 and its derived peptides in cardiovascular diseases since atherosclerosis stands out as a chronic inflammatory disease with impaired resolution and continuous leukocyte recruitment.

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Introduction

Atherosclerosis is a common cause of cardiovascular diseases and resulting in a considerable socioeconomic burden to western societies.[1] The disease is triggered by hypercholesterolemia causing the retention of lipoproteins in the vessel walls, which initiates an inflammatory response. As a consequence, atherosclerotic plaques develop at specific sites of the arterial tree where the blood flow is disturbed. Continued hypercholesterolemia and inflammation aggravate atherosclerotic plaque progression over time and this inevitably results in luminal narrowing or thrombi that obstruct or block the blood flow. This can lead to life-threatening events such as myocardial infarction and stroke (reviewed in ref. 2).

Current therapies are mainly focused on reducing the level of plasma cholesterol by HMG-CoA reductase inhibitors, also known as statins, often administered in combination with Ezetemibe which reduces the absorption of plasma cholesterol by the small intestine. Moreover, an inhibitor of proprotein convertase subtilisin/kexin type 9 (PCSK9) has recently been proven to be beneficial in reducing plasma cholesterol (reviewed in ref. 3). Conventionally, PCSK9 binds the receptor of low-density lipoprotein (LDL) which is subsequently internalized and broken down. By blocking PCSK9 the LDL-receptor is kept positioned on the cells surface and is given the opportunity to scavenge and remove excessive cholesterol from the plasma. Presently, cholesterol-lowering is the most effective way to treat cardiovascular diseases and this approach has reduced age-adjusted mortality (reviewed in ref. 4). However, atherosclerotic vascular diseases remain a chronic health concern.[1] This is partly because not all patients tolerate statins properly, and in addition, some patients suffer from cardiovascular events in the absence of hypercholesterolemia. Therefore, a different view of atherosclerosis as an inflammatory disease has emerged and targeting inflammation has become a promising direction to improve and complement current approaches (reviewed in ref. 5).

Inflammation is intimately involved in all stages of atherosclerosis.[6] Initially, tissue resident macrophages and mast cells sense the presence of the noxious insult (e.g. oxidized low-density lipoprotein) by their pattern recognition receptors. Subsequently, those effector cells start to release pro-inflammatory cytokines, chemokines, and vasoactive mediators to instigate and amplify the immune response. Hereby, endothelial cells are activated and vascular permeability is enhanced resulting in immune cell recruitment. Newly recruited neutrophils augment the immune response with the release of granule proteins (e.g., human cathelicidin LL-37 or mouse cathelicidin-related antimicrobial peptide (CRAMP),

CONTACT Renske de Jong Renske.Dejong@med.uni-muenchen.de; Oliver Soehnlein oliver.soehnlein@gmail.com Institute for Cardiovascular Prevention Ludwig-Maximilians-University Munich Pettenkoferstr. 9 80336 Munich, Germany.

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azurocidin, cathepsin G, and α-defensins), which aggravate endothelial dysfunction and additional immune cell entry.[7-9] Circulating monocytes attracted into the vascular wall transform into lipid-laden foam cells that accumulate in the atherosclerotic plaque.

Atherosclerosis is recognized as a chronic inflammatory disease in which the resolution phase is overwhelmed and fails to succeed to annihilate the inflammation (reviewed in ref. 10). A controlled resolution process is essential to prevent excessive or chronic inflammation (reviewed in ref. 11). The process of resolution includes the termination of inflammatory cell recruitment, the removal of effector cells by the induction of apoptosis and a contained clearance of apoptotic cells by macrophages; a process called efferocytosis. Furthermore, to support tissue regeneration macrophages are polarized toward an anti-inflammatory phenotype. Promoting the resolution of inflammation might be a suitable therapeutic approach to reduce atherosclerotic plaque development and prevention from secondary cardiovascular events.

Annexin A1 (ANXA1), a 37 kDa pro-resolving protein, is part of the annexin superfamily of Ca^{2+} dependent phospholipid binding proteins. Annexins share a common structure involving 2 distinct regions: an annexin core and an amino (N)-terminus. The annexin core region is greatly conserved between subfamilies, but all proteins have a unique N-terminal sequence exploring specific functions (reviewed in ref. 12). ANXA1 is a molecule promoting the termination of inflammation engaging various important pro-resolution properties and could therefore be an attractive protein to treat several inflammatory diseases as well as cardiovascular complications.

In this review we discuss cells expressing ANXA1 and the different pathways of ANXA1 externalization and secretion. Thereafter, the formyl-peptide receptors (FPRs), which recognize ANXA1 and its derived peptides, will be introduced. The main focus of this review, however, is the role of ANXA1 in the resolution of inflammation and more specifically its effects on leukocyte recruitment and its potential in preventing and curing cardiovascular diseases.

The expression and externalization of ANXA1

Nearly four decades ago ANXA1 was first described as a steroid-induced inhibitor of phospholipase A_2 and prostaglandin biosynthesis.[13] Subsequently, various studies pointed at ANXA1 as a second messenger of glucocorticoids (reviewed in ref. 14). Upon administration of hydrocortisone circulating leukocytes from healthy volunteers, for example, showed an increased ANXA1 expression level.[15] Presently, other stimuli are recognized to induce ANXA1 expression. For instance, interleukin (IL)6 upregulated ANXA1 in human adenocarcinomic alveolar epithelial cells.[16]

ANXA1 is expressed by multiple cell types including leukocytes, endothelial cells and mast cells.[17-19] Endogenous ANXA1, functioning as a scaffolding protein, is imperative in membrane organization and trafficking.[12] More specifically, ANXA1 has been shown to ameliorate inward vesiculation in multivesicular endosomes and to act as a functional linker between actin filaments and phagosomes in the presence of Ca^{2+} during phagocytosis.[20,21] Importantly, ANXA1 exerts anti-inflammatory properties outside the cell and hence the protein needs to be externalized to the cell membrane or secreted into the extracellular fluids. Exogenous ANXA1 is cleaved by proteolytic enzymes, including human proteinase and neutrophil elastase leading to the release and presence of ANXA1 and ANXA1-derived peptides such as Ac2–26.[22,23] Indeed, ANXA1 and ANXA1-derived peptides are detectable in extracellular fluids such as human plasma and serum under inflammatory conditions.[24-26] Interestingly, ANXA1 has no hydrophobic signal sequences and can therefore not be secreted *via* the classical route through the endoplasmic reticulum and Golgi apparatus.[25] As a result, several alternative pathways have been revealed to be responsible for ANXA1 externalization and secretion.

The adenosintriphosphat (ATP)-binding cassette transporters are a large group of transporters with varied roles that include the externalization of proteins. The ATP binding cassette A1 transporter was revealed to be involved in the secretion of ANXA1 derived from various cell types under inflammatory conditions.[27,28] Moreover, ANXA1 mobilization has been shown to be dependent on serine-27 phosphorylation.[29]

The P2X purinoceptor 7 receptor (P2X7R) is an innate immune receptor detecting extracellular ATP. The activation of P2X7R on pro-inflammatory M1 polarized macrophages was shown to induce the assembly of the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome resulting in the release of pro-inflammatory cytokines. However, on alternatively activated, anti-inflammatory M2 macrophages, ANXA1 was released upon P2X7R stimulation independent of NLRP3 inflammasome. By this means, ANXA1 release was not dependent on *de novo* gene transcription since ANXA1 was already detectable after 5 min upon receptor stimulation with ATP. Evidence suggests the translocation of phosphatidylserine (PS) to the outer plasma membrane leaflet upon P2X7R activation. Hereby, ANXA1 bound to plasma membrane phospholipids in the presence of Ca^{2+} and therefore was accompanying phosphatidylserine by its transfer.[30]

In neutrophils, ANXA1 was predominantly found within gelatinase granules and in the cytoplasm.[31] Circulating neutrophils showed a higher expression of intracellular ANXA1 compare with transmigrated neutrophils upon acute inflammation indicating a loss of ANXA1 during neutrophil cell adhesion and transmigration.[32,33] *In vitro*, human neutrophils interacted with the endothelium, and more specifically with Intercellular Adhesion Molecule 1 (ICAM-1) and Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1). This interaction provoked the externalization of ANXA1 toward the outer leaflet of the plasma membrane.[31,32] Moreover, other factors have been shown to provoke ANXA1 mobilization and externalization in neutrophils.[34] Examples include, pro-resolving lipid lipoxin A4 (LXA4) and pro-inflammatory ligand *N*-FormyL-Met-Leu-Phe (fMLF), which induced cytosolic (but not granular) ANXA1 to mobilize to the cell surface. Again, ANXA1 mobilization and externalization was observed to be dependent on phosphorylation of the protein. Following, externalized ANXA1 can exert several anti-inflammatory properties *via* binding Formyl Peptide Receptors (FPRs) when extracellular concentrations of Ca^{2+} induce a conformational change, resulting in an active form of the protein.[35]

Additionally, ANXA1 is found to be present in extracellular vesicles which can be subclassified into exosomes (40 to 60 nm) and microparticles (100 to 1000 nm). Endogenous ANXA1 was shown to be released as a component of exosomes derived from intestinal human epithelial cells and those activated pathways imperative in wound repair.[36] Besides, ANXA1 was found to be present in human neutrophil-derived microparticles obtained from activated neutrophils, which interacted with an endothelial monolayer.[37,38] *In vivo*, those neutrophil-derived microparticles showed to enter the cartilage, maintained its integrity, and therefore protected against tissue remodeling in inflammatory arthritis.[39]

Formyl-peptide receptors recognize ANXA1 and Ac2–26

ANXA1 and its derived peptide Ac2–26 bind FPRs, which are G-protein-coupled receptors expressed by several cell types, but are highly present on leukocytes. There are 3 known human FPRs named FPR1, FPR2 and FPR3 and fpr orthologous are found in mice and rats (reviewed in ref. 40). Full length ANXA1 binds specifically to FPR2. ANXA1 peptides bind with lower affinity to FPR2 and have a lack of receptor specificity because they bind with similar effectiveness to FPR1.[41,42]

FPRs were initially identified to bind highly chemotactic N-formyl peptides, originated from invading pathogens or derived from disrupted mitochondria[43,44] Hence, FPRs, and in particular FPR1, play an important role in both host defense against bacterial infection and in the clearance of damaged cells during sterile inflammations.[45,46] It is now acknowledged that N-formyl peptides are not the only ligands known to bind FPRs and numerous pro- and anti-inflammatory ligands are identified to induce cell activation *via* FPRs. Important pro-inflammatory ligands for FPR2 are fMLF, serum amyloid A (SAA), Aβ42 and cathelicidin (LL37 in humans, CRAMP in mice). On the other hand, LXA4, resolvin D1, ANXA1 and Ac2–26 induce anti-inflammatory signaling *via* FPR2.

Considerable hypotheses are given and examined to explain the divergent role of FPR2 in inflammation and resolution since both pro- and anti-inflammatory ligands regulate inflammatory and resolving circuits *via* the same receptor. First, ligand recognition was shown to be dependent on different binding sites.[47] Second, FPR2 has been shown to form homodimers and heterodimers with other FPRs dependent on its interaction with a specific ligand and thereby provoking different signaling.[48] For instance, ANXA1, but not SAA, was found to activate FPR2 homodimers triggering intracellular changes culminating in the release of anti-inflammatory cytokines such as IL-10. Furthermore, Ac2–26 evoked FPR2/FPR1 heterodimerization resulting in the activation of pro-apoptotic signaling pathways.[48]

ANXA1 turns off leukocyte recruitment

Leukocyte recruitment forms an important constitute to the immune response caused by pathogens as well as sterile provocations. Leukocytes move from the circulation through the endothelium toward the inflammatory insult in a controlled sequence, simply divided in rolling, adhesion, endothelial transmigration, and chemotactic migration (reviewed in ref. 49, 50). To control chronic or excessive leukocyte recruitment, endogenous anti-inflammatory pro-resolving mediators such as lipid mediators (LXA4, resolvins, maresins and protectins) and peptides/proteins (growth differentiation factor (GDF)-15, developmental endothelial locus (Del)-1, melanocortins, galectins, ANXA1 and Ac2–26) released by various cell types or from synthetic orgin interfere with different steps of the leukocyte adhesion cascade and therefore block leukocyte recruitment (reviewed in ref. 51). For instance, GDF-15 blocked neutrophil integrin activation and therefore neutrophil recruitment after myocardial infarction.[52] Otherwise Del-1 prevented the interaction between lymphocyte function-associated antigen-1 and ICAM-1 and consequently leukocyte adhesion to the endothelium.[53] Similarly, ANXA1 and its derived peptide Ac2–26 were recognized as important modulators of leukocyte recruitment (see Fig. 1).

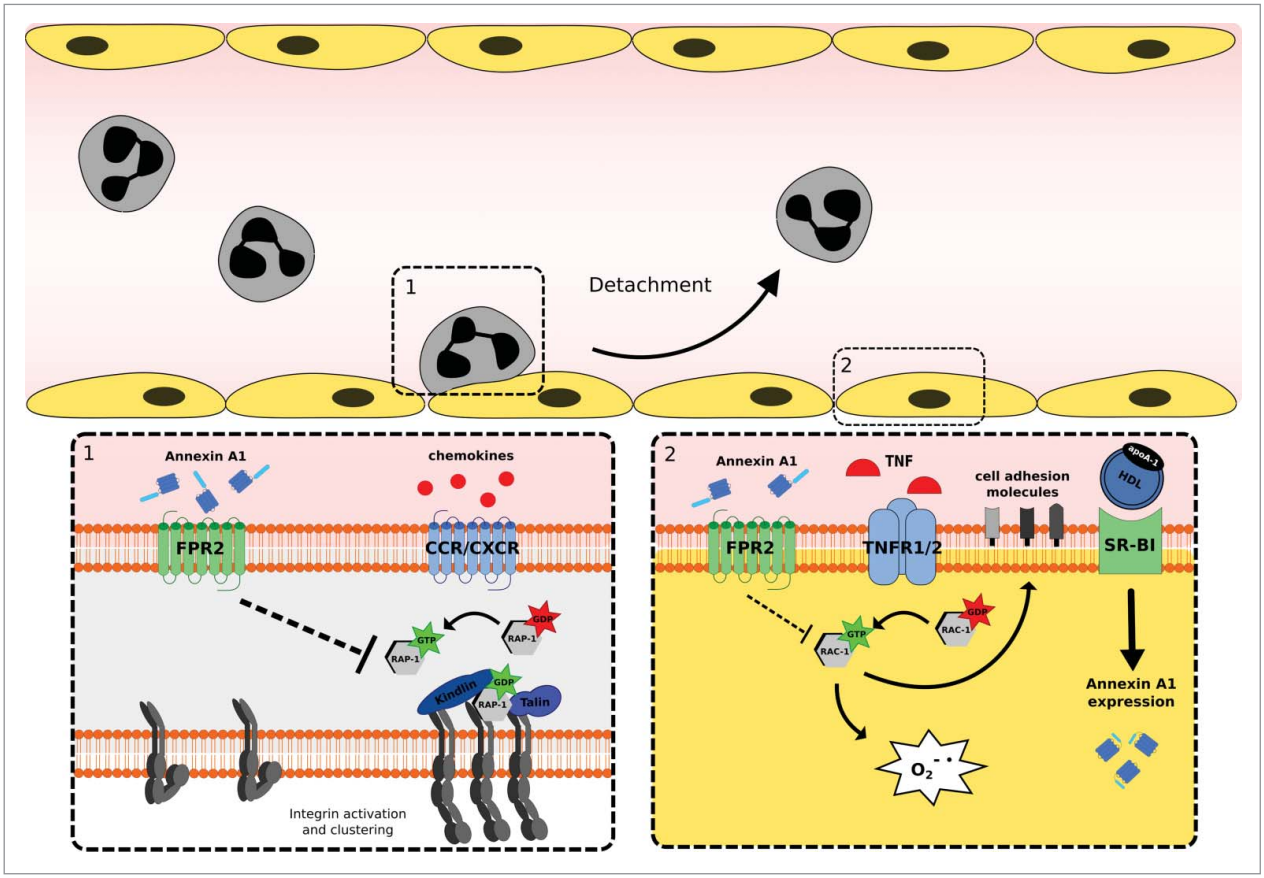

Figure 1. Annexin A1 (ANXA1) moderates leukocyte recruitment by instructing leukocytes and endothelial cells to prevent excessive leukocyte adhesion to the vascular wall. (1) ANXA1 interferes with chemokine induced Rap1 activation and consequently with integrin activation and clustering *via* FPR2. (2) HDL increases endogenous ANXA1 expression in TNF-α activated endothelial cells *via* SR-B1. Exogenous ANXA1 prohibits TNF-induced Rac1 activation and therefore superoxide production and cell adhesion molecule expression *via* binding FPR2. Reduced expression of adhesion molecules on the cell surface of endothelial cells limits leukocyte adhesion and recruitment. Abbreviations: FPR2, formyl peptide receptor 2; CCR/CXCR, CC-chemokine receptor/CXC-chemokine receptor; HDL, high density lipoprotein; apoA-1, apolipoprotein A1; SR-B1, scavenger receptor class B type 1; TNF-α, tumor necrosis factor α.

Several studies using animal models of inflammation have demonstrated that the administration of ANXA1 or Ac2–26 moderates neutrophil recruitment.[54-58] Early studies showed that both ANXA1 and Ac2–26 inhibited adhesion of human neutrophils to activated endothelial cells under static[54,59] and flow conditions[60,61] *in vitro* and thus impairing neutrophil recruitment. Use of an ANXA1 blocking antibody increased the ability of neutrophils to transmigrate through an endothelial monolayer indicating that ANXA1 is able to impair neutrophil transmigration.[32,62] The ability of inhibiting neutrophil adhesion and emigration by ANXA1 and its derived peptide Ac2–26 was further supported by intravital microscopy analysis of the mesenteric postcapillary venules, which showed no change in rolling, but reduced adhesion and emigration upon inflammation.[63,64] Interestingly, ANXA1 and Ac2–26 detached adherent neutrophils within 2 minutes after administration during ongoing inflammation proving a tremendous effect of those proteins.[50] Besides, Ac2–26 has been shown to inhibit leukocyte recruitment to the carotid artery under high cholesterol conditions, indicating that the anti-inflammatory actions of ANXA1 are not dependent on the type of blood vessel.[65] Likely, administration of ANXA1 or Ac2–26 mimics the effect of endogenous ANXA1 that is externalized upon neutrophil adhesion to the endothelium. Since most studies focused on the effects of ANXA1 on neutrophils, it is important to remark that also monocyte recruitment is inhibited by ANXA1 or its derived peptides.[56,65,66] Possibly, these findings can be extrapolated to other leukocyte subsets.

In line with these findings ANXA1-deficient mice subjected to various inflammatory stimuli exhibited a pronounced inflammation compare with control mice indicating a protective role of ANXA1.[67,70] Additionally, dexamethasone was shown to be less effective in ANXA1 deficient mice[67] or when mice where immunized against ANXA1[56,69,71] showing the importance of ANXA1 in the anti-inflammatory and immunosuppressive effects of glucocorticoids. Again, evaluation of the cremasteric

microcirculation by intravital microscopy revealed ANXA1 as a modulator of the leukocyte adhesion cascade since ANXA1 null mice demonstrated increased transendothelial migration.[68] Furthermore those mice showed an enhanced adhesion of leukocytes to the carotid artery under high fat diet conditions.[65]

ANXA1 and Ac2–26 inhibit leukocyte-endothelial interaction by binding FPR2 reducing leukocyte adhesion and detachment.[65,72-76] Conforming with previously described research, *fpr2*-deficient mice suffered from a more severe inflammatory response indicated by enhanced cell adhesion and emigration into the inflamed mesenteric microcirculation and aggravated inflammation in models for paw edema and arthritis.[77] Strikingly, endogenous ANXA1 was released as a component of extracellular vesicles such as microparticles, which were also shown to inhibit neutrophil recruitment.[38] *In vitro* those microparticles inhibited the adhesion of naïve neutrophils to human endothelial cells and this effect was abrogated in the presence of a FPR2 blocking antibody.[38] Moreover, intravenous administration of ANXA1-containing microparticles inhibited leukocyte recruitment in a mouse model of air pouch inflammation. Curiously, this effect was not observed when microparticles were obtained from ANXA1 null mice.[38]

On a molecular level, leukocyte adhesion is orchestrated by neutrophil intrinsic molecules (e.g., L-selectin, β_1 and β_2 integrins) interacting with endothelial cell intrinsic molecules (e.g., e-selectin, vascular cell adhesion molecule (VCAM)-1 and ICAM-1). L-selectin is an adhesion/homing receptor, which recognizes sialylated carbohydrate groups and is mainly involved in tethering and rolling. Several studies showed that administration of glucocorticoids induces L-selectin shedding on neutrophils in humans.[78] In addition, glucocorticoid-induced L-selectin shedding was mediated by ANXA1.[79,80] Integrins interact with cell adhesion molecules and thereby the overall strength of cellular adhesiveness is governed by the intrinsic affinity of the individual receptor-ligand bonds and their valency. The affinity of integrins to interact with their partner depends on its activation status. Integrin valency is determined by the basal expression of the receptor and its geometric arrangement. Several studies have shown that ANXA1 and Ac2–26 interfere with basal $\alpha_m ß_2$ integrin expression on leukocytes under inflammatory challenges.[64,67,68,81] Additionally, it has been observed that Ac2–26 prevents chemokine-induced $ß_1$ and $ß_2$ integrin activation and clustering *via* Rap1, a member of the Ras family of GTPases, which has an important role in the regulation of cell migration and adhesion. In line, Ac2–26 effects were absent in leukocytes isolated from *fpr2*-deficient mice[65] (Fig. 1).

Most studies demonstrate a neutrophil intrinsic effect of ANXA1 and its role in leukocyte adhesion and migration toward the inflammatory insult. However, few studies indicate endothelial cells, the second important player in leukocyte recruitment, as a target of ANXA1 or its derived peptides.[19,82] At first, Ac2–26 was shown to prohibit tumor necrosis factor α (TNFα)-induced superoxide production; ICAM- 1 and VCAM-1 expression in Human Mammary Epithelial Cells. Thus, Ac2–26 blocked TNFα activation *via* Rac1 (studied with N17rac1, dominant negative rac1) through binding FPR2. Whether those interesting findings are pathophysiologic relevant has not been confirmed so far[82] (Fig. 1).

Accordingly, high density lipoprotein (HDL) increased TNF-α activated aortic endothelial ANXA1 expression *in vivo* and *in vitro*, suggesting an important role of this protein in this cell type.[19] Subsequently, HDL-induced ANXA1 expression prevented human monocytic (THP-1) cell adhesion to activated endothelial cells. HDL and more specifically apolipoprotein AI, enhanced ANXA1 expression by binding scavenger receptor B1 and inducing extracellular signal–regulated kinase, p38 mitogen-activated protein kinase (P38MAPK), serine/threonine kinase Akt and protein kinase C (PKC) signaling and subsequently reduced endothelial activation indicated by an abridged expression of ICAM-1, VCAM-1, E-selectin, CC-chemokine ligand (CCL)2 and IL-8[19] (Fig. 1).

Furthermore, platelets play a pivotal role in leukocyte recruitment and can interact with the endothelium or with the leukocyte itself (reviewed in ref. 83). Platelet-endothelial interactions are facilitated by the adhesion receptors P-selectin, glycoprotein (GP)Ib-IX-V, GPVI, GPIIb-IIIa and CD40L expressed by platelets and P-selectin, E-selectin, ICAM-1, VCAM-1 and the blood glycoprotein von Willebrand factor by endothelial cells. Platelets synthesize, store and release inflammatory cytokines as well as chemokines, and thereby activate the endothelium and promote vascular permeability. Besides, platelets directly recruit leukocytes by depositing chemokines (e.g., CCL5) on the endothelium to attract monocytes.[84] Platelet-leukocyte interactions are foremost mediated by P-selectin expressed by platelets interacting with P-selectin glycoprotein ligand-1 on the surface of leukocytes. This interaction primes the leukocyte and promotes integrin activation. Interestingly enough, platelet recruitment is likewise supported by circulating leukocytes indicating the existence of a tightly controlled network including platelet-leukocyte crosstalk. Early studies indicated the presence of ANXA1 in human platelets in the cytosolic fraction.[85,86] However, in platelets ANXA1 did not appear to be released upon stimulation.[71] In a mouse model of cerebral inflammation, ANXA1 was shown to prevent neutrophil-platelet aggregation and therefore limited neutrophil adhesion and

recruitment. Interestingly, ANXA1 induced this effect by binding murine fpr2 on neutrophils and not platelets, since neutrophil-platelet aggregation was not altered in mice with *fpr2*-deficient platelets, but was enhanced in mice with *fpr2*-deficient neutrophils.[72]

Additionally, tissue resident effector cells including mast cells and macrophages play an important role in leukocyte recruitment by the release of inflammatory cytokines (e.g., TNFα and IL1β) and chemokines (e.g., chemokine (C-X-C motif) ligand 10 and CCL11), which attract leukocytes and might facilitate their adhesion to the endothelium (reviewed in ref. 87, 88). In contrast, those effector cells release anti-inflammatory mediators (e.g., IL10 and transforming growth factor β) during the resolution phase to terminate the inflammation. Mast cells express ANXA1 abundantly and there is compelling evidence supporting the notion that mast cells regulate leukocyte recruitment by the release of inflammatory mediators.[18] In a rat model of pleurisy ANXA1 mimetic peptide Ac2–26 prohibited the release of inflammatory histamine and CCL11 in pleural effluents. Besides, IL-13-evoked CCL11 release was inhibited by Ac2–26 in rat mesothelial cells and full length ANXA1 was shown to inhibit histamine and prostaglandin D2 release from activated human and mouse mast cells.[89,90] Simultaneously, macrophages are important effector cells involved in fine-tuning the immune response. In macrophages, ANXA1 or its derived peptide Ac2–26, enhanced IL10 production and secretion. Moreover, ANXA1 or related peptides inhibited nitric oxide release from macrophages.[91] Furthermore, a combination of LXA4 and ANXA1 induced macrophages polarization toward a more anti-inflammatory macrophage phenotype that secreted IL10, a mechanism working *via* FPR2.[92]

Hence, ANXA1 acts as an anti-inflammatory protein, which is important in fine-tuning the leukocyte recruitment and inflammatory response to protect from aggressive and chronic inflammation.

ANXA1 in cardiovascular disease

ANXA1 in atherosclerosis

Atherosclerosis is characterized by atheroma build up inside the arterial wall, a process mainly triggered by LDL, which is susceptible to oxidative modification by reactive oxygen species (reviewed in ref. 88). In addition, atherosclerosis is characterized by the adherence of circulating leukocytes to vascular endothelial cells and subsequently the migration of those cells to the sub-endothelial space (reviewed in ref. 93). In the sub-endothelial space macrophages, derived from newly recruited monocytes or local proliferation, remove modified lipoproteins to restore tissue functions. However, by a persistent leakage of lipoproteins, this resolving mechanism is overwhelmed and macrophages turn into lipid-laden inflammatory foam cell. Hence, the leukocyte recruitment cascade has shown to be imperative in the development of atherosclerosis and atherosclerosis-related diseases (reviewed in ref. 94, 95). Since ANXA1 has been indicated as an important regulator of leukocyte recruitment by modulating distinct steps of the leukocyte adhesion cascade, it might be a suitable candidate to limit inflammation in atherosclerotic plaque formation and its derived cardiovascular diseases. Besides, ANXA1 has been recognized as a modulator of apoptosis, efferocytosis and macrophage polarization, which are all important facets that are malfunctioning in atherosclerosis.

Efferocytosis of apoptotic cells by macrophages polarizes macrophages toward an anti-inflammatory M2 phenotype. ANXA1 has been shown to promote neutrophil apoptosis by targeting constitutive apoptotic pathways[96] or counteracting survival signals from other inflammatory mediators.[48,97] The working mechanism of ANXA1 in efferocytosis is multi-functional. ANXA1 was shown to function as a bridging molecule and co-localizes with PS on apoptotic cells to interact with scavenging macrophages.[98] Moreover, the protein was released by neutrophils to attract the macrophage[99,100] and it has been observed to be externalized by macrophages to facilitate engulfment of apoptotic cells in an autocrine/paracrine fashion.[101] Additionally, ANXA1 polarized macrophages toward an anti-inflammatory phenotype,[92] all of which are qualities with potential importance to reduce damage and improve resolution in atherosclerosis or related cardiovascular insults.

In human coronary atherosclerotic plaques ANXA1 was found to localize in macrophages and endothelial cells in the tunica intima.[65,102] Likewise, ANXA1 was observed to be highly expressed in areas containing apoptotic cells ($TUNEL^+$) indicating that the high expression of ANXA1 by macrophages reflects its importance the phagocytosis of apoptotic cells.[102] Two other studies detected ANXA1 expression in plaques obtained from patients with carotid stenosis undergoing carotid endarterectomy. A higher expression of ANXA1 was found in carotid plaques from asymptomatic patients compare with symptomatic patients implying a protective role of ANXA1 in atherosclerosis.[103,104]

Table 1 summarizes studies on ANXA1 or its derived peptides performed in animal models of cardiovascular diseases. In line with previous findings ANXA1 and Ac2–26 protect from atherogenesis and atheroprogression in mice.[61,65,105] Administration of Ac2–26 demonstrated a protective effect in a model of atherogenesis modulating an early stage of plaque development.[65] In

Table 1. Pharmacokinetics and therapeutic potential of Annexin A1 and its derived peptides in cardiovascular disease models.

Drug	Disease model	Administration	Pharmacokinetics	Outcome	Ref.
Atherosclerosis					
Col IV–Ac2–26 NPs	Advanced atherosclerosis	NPs contain 10 μg of Ac2–26 per injection (mouse) 1x/week, 5 weeks (starting after 12 weeks HFD)	Linear release in 96 hours (*in vitro*)	Lesion size ↓ Plaque stability ↑ Oxidative stress ↓ Necrotic area ↓ ICAM-1↓ Lesional IL10 ↑	105
Ac2–26	Atherogenesis	50 μg/injection (mouse) 3x/week, 4 weeks	Not studied	Lesion size ↓ Leukocyte recruitment ↓ Integrin activation ↓	65
h-annexin A1	Atherogenesis Advanced atherosclerosis	1 mg/kg (mouse) 3x/week, 6 weeks 3x/week, 6 weeks (starting after 6 weeks HFD)	Peaks after 50 min in the blood circulation $T^1/_2 = 6$ hours	No effect Lesion size ↓ Necrotic core ↓	61
Myocardial infarction					
Ac2–26	I/R	5 or 50 μg/injection (rat) At the start of reperfusion	Not studied	Infarct area ↓ Myocardial MPO ↓ Myocardial TNF-α ↓ Myocardial CCL3 ↓ Leukocyte adhesion ↓	113
Ac2–26 h-annexin A1	I/R	0.5 or 1 mg/kg (rat) 0, 30 and 60 min after reperfusion 25 mg/kg (rat)	Not studied	Infarct area ↓ Myocardial MPO ↓ Myocardial IL-1β ↓ Infarct area ↓	111
Ac2–26	I/R	1 mg/kg (mouse) At the start of reperfusion	Not studied	Infarct area ↓ Neutrophil count ↓ Myocardial CXCL1 ↓	112
AnxA12–50 or CR-AnxA12–50	I/R	5 μg/injection (mouse) 0 and 60 min after reperfusion	Not studied	Infarct size ↓ Plasma troponin levels ↓ Plasma CCL5 levels ↓ Plasma IL1β levels ↓ Incidence of death (after 24 hr) ↓	74
Stroke					
Annexin A1 fragment (NH_2-terminal 1–188 aa)	I/R	1.2 μg/injection (rat) 10 min after start ischemia	Not studied	Infarct size ↓ Cerebral edema ↓	118
h-annexin A1 Ac2–26	I/R	1 μg/injection (mouse) Repetitive injection after 0, 6 and 18 hours 100 μg/injection (mouse) (Repetitive) injection after 0, 6 and/or 18 hours	Not studied	Infarct volume ↓ Neurological score ↓ Leukocyte adhesion ↓ MPO activity ↓ Infarct volume (best results obtained with an injection after 1 and 18 hours) ↓ Neurological score ↓ Leukocyte adhesion ↓	119
Ac2–26	I/R	2.5 μg/kg (mouse) At the start of the reperfusion	Not studied	Leukocyte adhesion ↓	75
Ac2–26	I/R	100 μg/injection (mouse) At the start of cerebral reperfusion	Not studied	Infarct volume ↓ Neurological score ↓ Neutrophil-platelet aggregation ↓ Leukocyte adherence ↓ Platelet adherence ↓	72

Abbreviations: Col, collagen; NPs, nanoparticles; HFD, high fat diet; IL, interleukin; ICAM-1, intracellular adhesion molecule-1; Ac2–26, *N*-terminal fragment of Annexin A1; h-, human-; I/R, ischemia/reperfusion; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; CR-AnxA12–50, cleavage resistant annexin $A1_{2-50}$; MPO, Myeloperoxidase.

this model, Ac2–26 reduced the accumulation of leukocytes by inhibiting neutrophil and monocytes adhesion to the inflamed carotid artery. As described previously, Ac2–26 blocked chemokine induced leukocyte adhesion *via* FPR2 and Rap1.[65]

Similar results were shown in a model of advanced atherosclerosis[105] where *Apoe*$^{-/-}$ mice were subjected to a high cholesterol diet for a period of 12 weeks and treated with collagen IV (Col IV)-targeted nanoparticles (NPs) containing Ac2–26 (Col IV-Ac2–26 NPs) in the last 5 weeks.[105] Col IV-Ac2–26 NPs were able to increase cap thickness of atherosclerotic plaques and reduced collagenase production, 2 indicators of atherosclerotic plaque progression. Resembling effects were observed when mice where treated with full length ANXA1.[61]

Taken together, ANXA1 might represent an innovative strategy to counteract continuous leukocyte recruitment and macrophage activity during atheroprogression.

ANXA1 in myocardial infarction

Myocardial infarction occurs when the blood flow to the heart is abrogated, mostly instigated by a rupture of an atherosclerotic plaque in coronary arteries, causing damage to the heart muscle. Leukocyte infiltration after myocardial infarction has recently become an important focus of research (reviewed in refs. 94, 106) and can be divided in distinct waves. At the start of the ischemia, neutrophil infiltration is initiated and peaks after 24 hours. Its primary role is to amplify the inflammatory response. Neutrophil infiltration is followed by a wave of monocytes of which there are different subsets present in the circulation with functionally different properties (reviewed in ref. 107). The Ly6C^{high} monocytes (human equivalent of CD14^{+}CD16^{-} monocytes) are rapidly recruited to sites of inflammation and give rise to monocyte-derived dendritic cells and macrophages and are of importance to scavenge dead cells and debris. Pro-inflammatory ly-6C^{high} monocytes appear after 24 hours in the myocardium and their presence peaks at day 3.[108] Those monocytes are of high importance in the restoration after myocardial damage, since the depletion of monocytes compromises the hearts to heal.[109] Ly6C^{low} monocytes (human equivalent of CD14^{low}CD16^{+} monocytes) or also called patrolling monocytes are associating with the vascular endothelium and coordinate repair. On day 7 anti-inflammatory ly-6C^{low}macrophages dominate and are as well important in the resolution of inflammation and tissue repair.[108] Reperfusion therapy (e.g., by vasodilatative drug or thrombolytic drugs) is applied to improve the blood supply to the heart; however the restoration of oxygen and nutrients supply to the ischemic area also result in inflammation and tissue damage, called reperfusion injury (reviewed in ref. 110).

The ANXA1 derived peptide Ac2–26 has been described to be protective in mouse and rat models of myocardial infarction, however solely in models of acute damage after reperfusion.[111-113] Studies with a larger time frame looking at the effects of ANXA1 in the myocardial repair have not been reported. After an ischemia period of 25 min followed by a reperfusion period of 1–2 hours ANXA1 mimetic peptide Ac2–26 reduced the infarct area. Furthermore, inflammatory cytokine (e.g., TNFα and IL-1ß) and myeloperoxidase (as a marker of neutrophil recruitment) expression was reduced after Ac2–26 treatment.[111-113]

Myocardial infarction is an acute life-threatening disorder and therefore timing of treatment is indispensable. Treatment potential was indicated by injecting Ac2–26 0, 30 and 60 min after the start of the reperfusion period to mimic the clinical situation of patients which likely cannot be treated directly upon restoration of the blood flow.[111] The most prominent effect of Ac2–26 was found when the protein was administered 30 min after the start of the reperfusion. One potential problem in using ANXA1 or Ac2–26 in clinics might be the cleavage and inactivation of those proteins by proteases since externalized and exogenous ANXA1 is cleaved by human proteinase and neutrophil elastase.[22,23] Cleavage resistant Annexin A1$_{2–50}$ (CR-AnxA1$_{2–50}$) was designed to overcome this problem.[74] Tested in a mouse model of myocardial reperfusion injury, CR-AnxA1$_{2–50}$ reduced infarct area 2 hours after reperfusion. Additionally, CR-AnxA1$_{2–50}$ increased the survival rate 24 hours post-reperfusion of mice exposed to ischemia and reperfusion injury.

Ex vivo studies using isolated but perfused hearts from rat and mouse demonstrated a protective effect of Ac2–26 after ischemia and reperfusion.[114] In this model, Ac2–26 administration from the onset of reperfusion restored cardiac function *via* FPR1 activation. *In vitro*, Ac2–26 potently prevented from ischemic injury induced by metabolic inhibition in cardiomyocytes, an action dependent on PKC, P38/MAPK and ATP-dependent potassium channels K_{ATP} in cardiomyocytes *in vitro*.[115]

Patients suffering from myocardial infarction are typically exposed to angioplasty to push open blocked arteries. Next to it, medications are used to induce thrombolysis. Equally, patients are commonly treated with aspirin, known as an anti-platelet drug.[2] For this aspect, a combined treatment of LXA4 and ANXA1 could be beneficial and evidence to support this hypothesis was obtained in a murine air pouch model where an additive effect of a combined treatment with aspirin-triggered lipoxins and glucocorticoids-induced ANXA1 was observed.[116] Potentially, both ANXA1 and LXA4 interacted with FPR2 and act in concert to

downregulate neutrophil recruitment and overcome functional redundancies. However, how far an additive compound can improve ANXA1 therapy in cardiovascular disease models has not been examined.

ANXA1 in stroke

Cerebral ischemia, also called stroke, occurs when the blood supply to the brain is obstructed. This happens when atherosclerosis blocks or narrows the lumen of blood vessels to certain parts of the brain (reviewed in ref. 117). Ischemia activates tissue resident cells (mainly microglia) and promotes the release of inflammatory mediators. As consequence leukocytes and T cells are recruited to the inflamed area.

Four different studies investigated the effect of ANXA1 or its derived peptides in models of ischemic stroke.[72,75,118,119] Rats exposed to ischemia and Ac2–26 administration showed reduced infarct sizes and limited cerebral edema after 2 and 24 hours and this effect was most prominent when Ac2–26 was directly given upon reperfusion.[118] Correspondingly, Ac2–26 reduced leukocyte adhesion in the cerebral microvasculature though FPR2. Similar protective properties where observed using full length ANXA1.[119] Equivalently, Ac2–26 inhibited neutrophil-platelet aggregate formation and in consequence the adhesion of neutrophils to the endothelium again *via* binding though FPR2 and thus reduced the size of the infarcted area, decreased neurological score and abrogated cytokines secretion.[72]

Conclusions

Inhibiting inflammation has proven to be beneficial in animal models of atherosclerosis, however translation into clinical practice has failed up to now[120,121] probably caused by divergent leukocytes behavior since inflammation is instigated to clear the affected area, but uncontrolled or aggressive inflammation causes tissue damage. Therefore, stimulating resolution of inflammation instead might be an attractive strategy.

Substantial progress has been made in ANXA1 research, exposing ANXA1 as a potential therapeutic drug facilitating a wide range of pro-resolving responses. In cardiovascular diseases the protein has been shown to be beneficial in protecting against inflammation in atherosclerosis, myocardial infarction and stroke. As discussed, ANXA1-instructed mechanisms are multidisciplinary and affect leukocytes as well as endothelial cells and tissue resident cells like macrophages and mast cells. In summary, ANXA1 and its derived peptide Ac2–26 are important modulators of the leukocyte adhesion cascade and limit leukocyte recruitment. Furthermore, ANXA1 promotes apoptosis of neutrophils and subsequently efferocytosis by macrophages. Additionally, it polarizes macrophages toward an anti-inflammatory phenotype with the additional effect of an enhanced anti-inflammatory cytokine secretion and a suppressed secretion of inflammatory mediators. Importantly, much research is focused on pharmaceutical tools to improve pharmacokinetics and drug targeting. For example, polymeric nanoparticles have been developed to deliver ANXA1 directly at the side of inflammation. These strategies will favor the translation of ANXA1 to clinical practice as an attractive protein to halt disease progression and restore homeostasis.

Abbreviations

ANXA1	annexin A1
ATP	adenosintriphosphat
CRAMP	cathelicidin-related antimicrobial peptide
CCL	CC-chemokine ligand
Col IV	collagen IV
CXCL	chemokine (C-X-C motif) ligand
CR-AnxA1$_{2-50}$	cleavage resistant annexin A1$_{2-50}$
Del-1	developmental endothelial locus-1
FPR	formyl peptide receptor
fMLF	formyL-met-leu-phe
GDF-15	growth differentiation factor-15
GP	glycoprotein
HDL	high density lipoprotein
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
LDL	low density lipoprotein
LXA4	lipoxin A4
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NPs	nanoparticles
P2X7R	P2X purinoceptor 7
PCSK9	proprotein convertase subtilisin/kexin type 9
PECAM-1	platelet endothelial cell adhesion molecule 1
PS	phosphatidylserine
SAA	serum amyloid A
TNFα	tumor necrosis factor alfa
VCAM-1	vascular cell adhesion molecule 1

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