Annexin 1: An Endogenous Anti-Inflammatory Protein

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A hallmark of inflammation is the mobilization of blood-borne leukocytes across microvessels to kill and remove the invading pathogen. For its damaging potential, leukocyte movement is finely regulated, and endogenous pathways exist to ensure the time dependency of this process. Annexin 1 and its receptor(s) are one example of these pathways.

An important aspect in the field of cardiovascular inflammation is the process of leukocyte migration and tissue infiltration that causes the tissue damage associated with ischemia-reperfusion injury and other vascular injuries. Leukocyte extravasation is in itself a protective process that the host sets up to control pathogen invasion and other insults. Several proinflammatory mediators operate to ensure the perfect functioning of this process, and these include cytokines and chemokines, as well as several classes of adhesion molecules. Direct observation of an inflamed microcirculation has allowed the characterization of the leukocyte extravasation process, with the definition of the phases of cell rolling, adhesion, and emigration (or diapedesis) (10). The life-saving role of this process is demonstrated by the poor life expectancy of individuals with a genetic deficiency in specific adhesion molecules. Thus an important concept is that inflammation is a protective process and its perfect functioning is crucial for health.

In view of the disruptive power that leukocytes migrated into tissue can exert, because of the production and release of several toxic enzymes and radicals, the time dependency of this process is nearly as crucial to survival as its prompt activation following an inflammatory/pathological insult. Thus several anti-inflammatory mediators and mechanisms operate in the host to promote and control the phase of resolution that switches off leukocyte egress from the blood vessel into the extravascular tissue.

Figure 1 highlights the time dependency of the inflammatory process, with the involvement of pro- and anti-inflammatory mediators. From a theoretical point of view, disease can be explained as an exacerbation of a given proinflammatory pathway, or it can be caused by a malfunctioning, or non-functioning tout-court, of one or more anti-inflammatory pathways. Acceptance of this point will help in making the subsequent statement: the study of anti-inflammatory mediators may lead to innovative drug discovery with the development of compounds that may be effective in curing chronic inflammatory pathologies (e.g., inflammatory bowel disease, rheumatoid or gouty arthritis). Compounds depicted on the mechanism of action of a given anti-inflammatory mediator will be safer, since they will be acting by mimicking the way the body controls inflammation.

Annexin 1: an anti-inflammatory mediator

Thus, together with a few other groups, we propose that resolution is an active phenomenon. There are several examples of anti-inflammatory mediators, some of which have already been associated with the clinical efficacy of known drugs. Figure 2 shows a nonexhaustive list of anti-inflammatory mediators, divided into nonpeptides and peptides/proteins.

The purine adenosine is the first endogenous anti-inflammatory mediator studied in some detail. Adenosine can be released form leukocytes and/or endothelium. It acts at the adenosine A$_{2a}$ receptor to reduce the extent of cell adhesion and emigration. Importantly, mice deficient in A$_{2a}$ receptors have an exacerbated experimental inflammatory response. Anti-rheumatic drugs such as methotrexate and sulfasalazine have been proposed to act by increasing endogenous adenosine levels and/or action (2).

Over the last 10 years, we have been studying the anti-inflammatory actions of the glucocorticoid-inducible protein annexin 1. Blood neutrophils and monocytes have abundant levels of annexin 1. Figure 3 shows that human neutrophils have high cytoplasmic levels of annexin 1, the protein representing between 3 and 4% of total cytosolic proteins. Annexin 1 levels are augmented in response to glucocorticoid induction, as shown in human volunteers. Cytokines such as tumour necrosis factor, interleukin-1, and interleukin-6 can also increase cellular and tissue annexin 1 expression. With respect to the process of leukocyte extravasation, briefly summarized above, it is now clear that when blood-borne neutrophils adhere to the vessel endothelium, annexin 1 exported from the neutrophil cytoplasm to the cell surface (12). The mechanism underlying the externalization of the protein is still unclear. However, its preferential localization in certain cytoplasmic granules suggests that a process of control exocytosis may be responsible for its translocation on the plasma membrane. Figure 4 highlights this mechanism and points to leukocyte adhesion as the optimal stimulus to activate this process. Once on the adherent neutrophil cell surface, annexin 1 operates in an autocrine/paracrine fashion to downregulate the process of leukocyte diapedesis. Within this microenvironment, a catabolic process may also take place thus terminating annexin 1 inhibitory control (12).
Effect of exogenous annexin 1 and annexin 1-derived peptides

In common with all annexins, the larger portion of annexin 1 is formed by four repeats (each one 70–80 amino acids long) that are grouped together to form a globular structure with a convex and concave face. In contrast to the core formed by the four repeats, the NH2-terminal regions are unique to each annexin. Therefore, it is likely that specific actions displayed by a given annexin (there are 13 mammalian annexins) might be mediated by the NH2-terminal sequence. Figure 4 shows a schematic annexin 1 structure (enlarged at right; notice the four globular repeats folded together and the long NH2 terminus).

Following this lead, we demonstrated that a peptide spanning the first 24 amino acids of annexin 1, termed peptide Ac2-26, mimicked human recombinant annexin 1 for its ability to inhibit neutrophil extravasation in models of acute inflammation. On a molar basis, peptide Ac2-26 was less potent than annexin 1, but it was equally effective (i.e., it produced a similar degree of inhibition of neutrophil accumulation into specific tissue sites), indicating that this short region of the protein was fully responsible for this specific effect of the parent protein (11). Application of techniques of intravital microscopy confirmed annexin 1 and peptide Ac2-26 inhibition of blood-borne leukocyte extravasation. In addition, these experiments allowed us to pinpoint the specific step affected by the pharmacological treatment with these agents. Intravenous treatment of animals with annexin 1 or peptide Ac2-26 did not affect the leukocyte rolling phenomenon, nor did it inhibit the extent of leukocyte adhesion to the inflamed postcapillary venule endothelium. In contrast, this treatment affected the fate of the adherent leukocyte. Thus a rapid detachment of adherent leukocytes was observed on injection of human recombinant annexin 1 and its peptides (7, 11). The fact that endogenous annexin 1 could also control the fate of adherent neutrophils was demonstrated with specific neutralizing antibodies; in this context, the antibody also affected the antimigratory effect of the synthetic glucocorticoid dexamethasone, providing a functional link to glucocorticoid-mediated annexin 1 synthesis mentioned before. Together, these experimental observations fitted well with the mobilization of endogenous annexin 1 selectively detected at the stage of leukocyte adhesion and illustrated above (see also Fig. 3). This line of research was delineating a clearer picture in which neutrophil adhesion was the key stimulus required to bring about activation of the annexin 1 pathway. It remained to address the role of annexin 1 proteolysis and the mechanism of action.

It must be said that very little progress has been made toward clarifying the pathophysiological impact of annexin 1 proteolysis. Annexin 1 can act as an endogenous produg to release the NH2-terminal peptide once exported on the plasma membrane, but it is also possible that the protein acts as an intact entity and its proteolysis terminates its effects. More progress has been made in relation to the molecular mechanism regulating the control exerted by the protein, and the NH2-terminal peptide, on the process of neutrophil extravasation.

The mechanism of action of annexin 1

It is important to highlight, at this stage, that the protective role of exogenous annexin 1 and its mediation of glucocorticoid actions have also been demonstrated in models of rheumatoid arthritis and myocardial infarct (5, 15), suggesting a potential therapeutic application for drugs that might be developed from this line of research. However, the answer to a specific question has remained elusive for many years: what is the mechanism of action of annexin 1, and can it be exploited for drug discovery?
Evidence that the parent protein annexin 1 would actually act through a similar mechanism, and more importantly, it did not provide binding data to unequivocally demonstrate interaction between these agents (annexin 1 and annexin 1-derived peptides) with human FPR (for example, performing classical experiments of binding using cells transfected with the receptor). Nonetheless, Walther et al. (14) have the merit to have filled a gap not only in annexin 1 biology (assuming that the findings are relevant to the parent protein, including the endogenous one) but also in the biology of FPR.

Annexin 1 and FPR: a new axis in controlling leukocyte migration?

FPR is the prototype of a group of G protein-coupled receptors (GPCR), of which three members have been described in the human system (6). FPR, the first receptor to be cloned, was identified because it is activated by the bacteria-derived peptide formyl-Met-Leu-Phe (fMLP). The hypothesis that bacteria-derived fMLP would activate neutrophil chemotaxis and migration to the site of infection was then put forward. However, there is no clear evidence that fMLP can actually be released during bacterial infections. Thus it is possible that fMLP activation of FPR, despite occurring at nanomolar concentrations, is an experimental artifact. One spin-off idea from this line of research is that leukocytes “smell” bacterial infections via FPR and can direct themselves toward them. Because the process of leukocyte extravasation is crucial to host survival (effected by walling off, disrupting, and removing the inflammogen), FPR would then be a sort of protective receptor. Finally, the xenobiotic nature of fMLP opens another interesting scientific query that is the nature of the endogenous ligand for human FPR. The study discussed in detail above suggests that annexin 1 may be an endogenous agonist at the receptor, although experimental data with the full-length protein are not reported (14). Another issue to consider in context is the widespread distribution of FPR (1), indicating potential involvement of this receptor in several of the biological actions ascribed to annexin 1, i.e., even outside the closed camp of inflammation and the process of leukocyte migration.

In murine systems the FPR story is more complex, since there are at least six related genes and possibly three distinct proteins (4). Mouse FPR displays significantly lower affinity than fMLP, such that micromolar concentrations of the formylated tripeptide are required to activate it. We have recently used an integrated approach using FPR antagonists (active in the mouse) and animals deficient in the mouse FPR gene to test the antimigratory action of full-length annexin 1 and peptide Ac2-26 using a validated model of peritonitis. However, an apparent discrepancy emerged from these experiments: peptide Ac2-26 inhibition of neutrophil recruitment into the peritoneal cavity in response to local injection of zymosan was absent in FPR knockout mice. In contrast, the antimigratory effect of human recombinant annexin 1 was merely slightly attenuated, but still significantly present, in these mice (13). Similarly, in vitro and ex vivo binding of full-length annexin 1 to cells taken from these mice was reduced (by ~30%) yet not

FIGURE 3. Annexin 1 and the neutrophil. Annexin 1 is a major constituent of neutrophil cytoplasmic proteins, with other members of the annexin superfamily being expressed to a much lower extent. (Approximate annexin contents shown in the figure have been taken from Ref. 3.)

The fact that annexin 1 and its NH2-terminal-derived peptides caused detachment of adherent leukocytes (7) indicated to us the possibility that a controlled activation of the adherent cell had to take place. In other words, the process of detachment could be an active phenomenon requiring cell activation, thereby promoting leukocyte dislocation from the site of adherence: locomotion on the endothelium followed by detachment and rejoining of the blood flow. One way to challenge the “detachment hypothesis” was to test whether annexin 1 and peptide Ac2-26 could activate human or rodent neutrophils in vitro. The first study to have done so was published in the year 2000. Walther et al. (14) confirmed the antimigratory effects of the annexin 1-derived peptide Ac2-26. More importantly, for the first time a link was proposed between peptide Ac2-26 and the receptor for formylated peptides (FPR). Antagonists to this receptor blocked the antimigratory effects of peptide Ac2-26 (as tested in assays of transendothelial passage in vitro). Also, in agreement with the detachment hypothesis illustrated above, peptide Ac2-26 provoked transient changes in intracellular calcium dications and shedding of L-selectin (CD62L) from the plasma membrane. Both of these experimental techniques (fluorimetry for calcium fluxes and flow cytometry for L-selectin expression) are widely applied in laboratories of leukocyte biology as tests of neutrophil activation. Importantly, commercially available FPR antagonists prevented these in vitro actions of the annexin 1-derived peptides (14). This study, however, failed to provide

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Abundance in the cytosol:

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- ANX2  - 0.02 %
- ANX3  - 0.75 %
- ANX4  - 0.05 %
- ANX5  - 0.19 %
- ANX6  - 0.80 %
- ANX7  - not detectable
abolished. Finally, commercially available FPR antagonists abrogated the antimigratory actions of both peptide Ac2-26 and annexin 1. Together these data can be explained by the fact that, in the mouse, FPR can only partially mediate the antimigratory actions of annexin 1 and that other receptor(s) of the family, sensitive to the antagonists used, can be involved in this action. Interestingly, a similar conclusion has also been reached in a model of rat myocardial infarct (5). It remains to be seen if the same applies to human systems.

Concluding remarks

Annexin 1 is an endogenous anti-inflammatory mediator that operates in the host to counteract the process of leukocyte extravasation. More than a decade of research has investigated the pathophysiological nuances of the annexin 1 system with important, yet in some cases inconclusive, demonstrations (e.g., annexin 1 localization in human and rodent leukocyte; its mechanism of externalization; its site of action; role and importance of annexin 1 proteolysis). Pharmacological analyses have identified the most important region of the >300-amino acid protein, showing that peptide Ac2-26 (and in some cases also peptide Ac2-12) retained full anti-inflammatory and protective (in the case of models of ischemia-reperfusion injury) activities.

In the last few years, an innovative link has been made between annexin 1 and annexin 1-derived peptides and the fMLP receptor, or FPR. It is still too early to say that the functioning of the annexin 1/FPR axis is fully understood, although the putative involvement of this axis can explain several experimental data (and satisfy the detachment hypothesis). As explained above, it is possible that other receptor(s) of this family can interact with annexin 1 and annexin 1-derived peptide. Whereas this specific issue will be clarified by further experimentation, it is without doubt that the identification of FPR, a canonical GPCR, as an annexin 1 target is a great incentive for pursuing this line of research.

After a decade of research on annexin 1 and the process of neutrophil extravasation, today we are much closer to exploiting the biological actions of annexin 1 for drug discovery. The definition of the molecular target is clearly a necessary hurdle on the way to a novel annexin 1 mimetic, i.e., a new drug. If and once discovered, this compound would likely be modulatory, rather than fully inhibitory, in its actions and would be...
devoid of major side effects, since it will be mimicking the modus operandi of an endogenous anti-inflammatory mediator.

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References


