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Proteolytic cleavage of annexin 1 by human leukocyte elastase

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Abstract

Annexin 1 has been shown to participate through its unique N-terminal domain in the recruitment and activation of leukocytes at sites of inflammation. Peptides derived from this domain are true mimetics of the annexin 1 action in all inflammation models tested and most likely serve as the active entities generated at sites of inflammation. To elucidate mechanisms underlying peptide generation we used isolated blood leukocytes and endothelial cell monolayers. We show that following endothelial adhesion, annexin 1 was externalized from leukocytes and rapidly cleaved. Addition of purified annexin 1 to degranulating leukocytes resulted in the truncation of annexin 1, which seemed to depend on the proteolytic activity of human leukocyte elastase (HLE). The capacity of elastase to proteolytically cleave annexin 1 was confirmed using both purified annexin 1 and HLE. The identification of annexin 1 as a substrate for HLE supports the model in which annexin 1 participates in regulating leukocyte emigration into inflamed tissue through N-terminal peptides generated at inflammatory sites.

Keywords: Calcium; Endothelial cell; Inflammation; Elastase

1. Introduction

A key step in inflammation is the migration of neutrophils from the circulating blood into the inflamed tissue. To ensure precise regulation, a complex network of soluble and surface-bound mediators controls leukocyte rolling on and adhesion to the endothelium as well as the subsequent transendothelial passage into the extravascular tissues [1]. Annexin 1, a member of the annexin family of Ca^{2+}/lipid-binding proteins [2], has received considerable attention over the past two decades as an anti-inflammatory mediator, since it was shown to exert anti-inflammatory activities in several models of inflammation, e.g. by inhibiting neutrophil extravasation [3]. The physiologic importance of its extracellular activity has been strengthened by the recent identification of annexin 1 as a specific ligand for the formyl peptide receptor family of chemoattractant receptors [4–6]. It was shown that the anti-migratory property of annexin 1 on the transendothelial migration of granulocytes is at least in part due to receptor desensitization. Receptor activation and desensitization is mediated through the N-terminal part of the annexin 1 molecule [4,5], most likely explaining previous observations that proteolytic removal of the N-terminal part causes inactivation of annexin 1 [7], whereas synthetic peptides corresponding to this part of annexin 1 retain the full pharmacologic activity [8].

Annexin 1 is highly susceptible to proteolytic cleavage within the N-terminal domain where cleavage sites for proteases have been identified. Proteolytic cleavage of precursors is a common step in the activation of bioactive molecules. Many molecules relevant to inflammation such as cytokines and their receptors contain cleavage sites for neutrophil serine proteases and are their natural substrates [9,10]. Modulation of the bioactivity through released neutrophil proteases might be a potent mechanism to control the extent of inflammatory processes. Human leukocyte elastase (HLE) is a major serine protease stored in the primary granules of polymorphonuclear neutrophils (PMN) that are released upon PMN activation [11]. HLE has been shown to accumulate at sites of inflammation and to participate in inflammatory disorders such as rheumatoid arthritis and cystic fibrosis [12]. We therefore analyzed the sensitivity of annexin 1 to proteolytic cleavage by HLE.

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2. Material and methods

2.1. Reagents

Human leukocyte elastase (E.C. 3.4.21.37) was obtained from Calbiochem, the specific human leukocyte elastase inhibitor N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (MeOSuc-AAPV-CMK), cytochalasin B and the chemotactic peptide N-formyl-Met-Leu-Phe (fMLF) were purchased from Sigma.

2.2. Cell culture

The human endothelial hybrid cell line EAhy.926 (obtained from the German Cell Culture Collection, DSMZ, Braunschweig, FRG) was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS, 2 mM glutamine and penicillin/streptomycin. Cells were cultured at 37 °C in a humified atmosphere with 7% CO_2.

2.3. Isolation of human peripheral blood PMN

PMN were isolated from buffy coats using Ficoll-Paque gradient centrifugation [13]. Erythrocytes were removed from sedimented granulocytes through hypotonic lysis.

2.4. Expression and purification of recombinant human annexin 1

To obtain recombinant human annexin 1, the cDNA encoding human annexin 1 was cloned into pET23a(+) vector that has been modified to allow for bacterial expression of untagged recombinant proteins [14]. Recombinant annexin 1 was purified from the *E. coli* lysates as described in [15].

2.5. Adhesion of PMN to EAhy monolayers and detection of released annexin 1

Mobilization assays using granulocytes adhering to EAhy monolayers were performed essentially as described [16]. In brief, 1.5×10^6 PMN/well in adhesion buffer (Hanks' balanced salt solution supplemented with 1.3 mM $CaCl_2$ and 1.3 mM $MgCl_2$) were added to EAhy grown on six-well plates or kept in suspension. After incubation for 30 min at 37 °C, supernatants were aspirated. To remove cell surface-bound annexin 1, EAhy cells were washed with PBS containing 5 mM EDTA. Contaminating cells were removed by centrifugation at 5000×*g* for 5 min and proteins in the recovered cell-free supernatants were TCA-precipitated. Equal amounts of each sample were subjected to SDS-PAGE and subsequent Western blotting. Annexin 1 was detected using the anti-annexin 1 polyclonal antibody r656 [17] which recognizes both intact and cleaved annexin 1.

2.6. Annexin 1 cleavage by activated PMN

For cleavage assays using PMN and recombinant human annexin 1, 1×10^6 granulocytes in RPMI with addition of 20 mM HEPES, were treated as indicated. PMN were primed with 5 μg/ml cytochalasin B for 1 h at 37 °C. The specific inhibitor MeOSuc-AAPV-CMK was added at a final concentration of 10 μM. Subsequently, 2 μg of recombinant annexin 1 were added and PMN were stimulated with 100 nM fMLF. After 45 min at 37 °C, cells were removed by centrifugation. Equal amounts of the supernatants were TCA precipitated and subjected to SDS-PAGE. Proteins were visualized by Coomassie staining.

2.7. In vitro cleavage of recombinant human annexin 1

Purified recombinant human annexin 1 (1 μg) was incubated with recombinant human leukocyte elastase at a final concentration of 0.3 units/ml in reaction buffer (50 mM Tris, pH 7.6; 0.5 M NaCl, 20 mM $CaCl_2$) with or without the specific inhibitor MeOSuc-AAPV-CMK at a final concentration of 100 μM for 1 h at 37 °C. Samples were subjected to SDS-PAGE and the gels were Coomassie-stained.

3. Results

3.1. Annexin 1 is externalized and truncated upon adhesion of PMN to endothelial cells

Isolated peripheral PMN and the human endothelial hybrid cell line Eahy.926 were used as an in vitro model of PMN adhesion to endothelial cell layers. Immunoblotting analysis revealed that annexin 1 was essentially undetectable in the supernatants of either resting PMN in suspension (Fig. 1, lane 1) or EAhy monolayers (Fig. 1, lane 5). Treatment of non-adherent PMN in suspension with fMLF alone was not sufficient to induce annexin 1 externalization (Fig. 1, lane 2), whereas cytochalasin priming and further stimulation with fMLF triggered annexin 1 release to a substantial amount (lane 4). Upon PMN adhesion to the endothelial monolayer, significant annexin 1 release was observed even in the absence of fMLF stimulation (Fig. 1, lane 6). Whereas lysates prepared from resting PMN only show an immunoreactive signal corresponding to the size of the full-length 37 kDa protein (not shown), a large fraction of the annexin 1 found extracellularly had a smaller size of approximately 33 kDa, indicating rapid and effective proteolytic cleavage.

3.2. In vitro proteolysis of purified human annexin 1 by Human Leukocyte Elastase

Human leukocyte elastase (E.C. 3.4.21.37) is one of the major serine proteases stored in the primary granules of PMN. Elastase is effectively released into the extracellular milieu following adhesion of activated PMN. To further characterize the proteolytic activity responsible for the observed cleavage of annexin 1, we chose to investigate the ability of annexin 1 to serve as a direct substrate for HLE. In a first set of experiments, we incubated bacterially expressed recombinant human annexin 1 with purified HLE. Annexin 1 obtained from the *E. coli* expression system was predominantly in the full-length form with a molecular weight of ~37 kDa and contained only minor amounts of smaller fragments (Fig. 2). Exposure of recombinant human annexin 1 to HLE resulted in the total loss of the 37 kDa

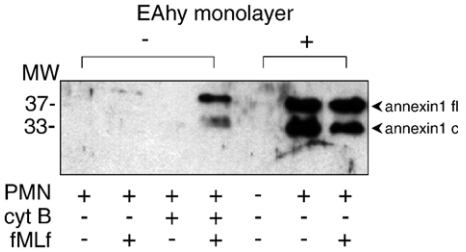

Fig. 1. Release of annexin 1 upon adhesion of PMN to the EAhy monolayer. PMN isolated from buffy coats were treated as indicated and either kept in suspension or incubated on EAhy monolayers for 30 min at 37 °C. Proteins of the cell-free supernatants were separated by SDS-PAGE and immunoblotted using the anti-annexin 1 polyclonal antibody r656 which recognizes both the intact, full-length (fl) and cleaved (c) protein species. Note that in the absence of a EAhy monolayer, annexin 1 is only externalized upon priming with 5 μg/ml cytochalasin B and subsequent fMLF stimulation of PMN. Results are representative of at least three independent experiments.

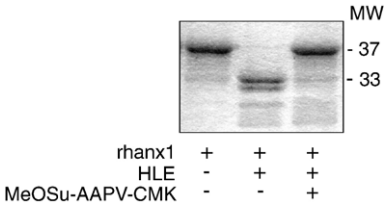

Fig. 2. Proteolytic cleavage of annexin 1 by HLE. Purified recombinant human annexin 1 (rhanx1, 1 μg) was exposed to 0.5 μM HLE for 1 h at 37 °C or left untreated. Proteolysis of annexin 1 was analyzed by SDS-PAGE followed by Coomassie Blue staining of the gel. The gel shown is representative of at least three independent experiments.

species, whereas bands of lower molecular weight, predominantly of 33 kDa, appeared. Addition of N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (MeOSu-AAPV-CMK), a cell-permeable, non-cytotoxic inhibitor of HLE, completely inhibited the ability of HLE to cleave annexin 1 (Fig. 2, lane 3). The observed direct proteolytic effect of HLE on recombinant human annexin 1 identifies annexin 1 as a substrate for HLE.

3.3. Purified annexin 1 is cleaved by PMN stimulated to degranulate

To examine further the specificity of annexin 1 as a HLE substrate and to confirm that HLE proteolytic activity from activated PMN is responsible for the cleavage of annexin 1, we next performed experiments in which purified recombinant human annexin 1 was exposed to PMN. As shown in Fig. 3, co-incubation of annexin 1 with PMN was not sufficient to cause annexin 1 degradation, even when PMN were stimulated with fMLF. Because neutrophils in suspension show only limited degranulation, disruption of the actin cytoskeleton by cytochalasin B is needed to promote elastase release from neutrophil primary granules upon stimulation with activating mediators such as fMLF [18–20]. We found that cleavage of extracellularly added annexin 1 was indeed dependent on priming of PMN with cytochalasin B and subsequent fMLF activation, indicative of a controlled activation-driven process. As addition of the HLE inhibitor MeOSu-AAPV-CMK significantly inhibited the observed degradation of annexin 1, elastase seemed to be the major PMN-derived protease responsible for proteolytic cleavage of annexin 1 (see also Fig. 2).

4. Discussion

In the present study, we characterized the externalization of annexin 1 from PMN. Significant release of annexin 1 was found only when PMN were induced to degranulate. Interestingly, although the nonphysiological agent cytochalasin B that is necessary for the release of primary granules from PMN in suspension also induced externalization of annexin 1, adhesion of PMN to endothelial cells was much more efficient. A vast amount of the externalized protein was of smaller molecular size, indicating rapid proteolytic cleavage by PMN-derived proteolytic activity. We demonstrated the ability of human leukocyte elastase to digest annexin 1. Furthermore, an elastase-specific enzymatic activity released from activated PMN rapidly cleaved annexin 1, suggesting that extracellular cleavage of the anti-inflammatory mediator annexin 1 can occur at sites of inflammation.

The 37 kDa protein annexin 1 belongs to the annexin family of Ca^{2+} binding proteins. Whereas the core modules of annexins are evolutionary highly conserved, the preceding N-terminal domains vary considerably in length and sequence between different annexins and seem to exert important regulatory activities. Annexin 1 is highly abundant in neutrophils. Although it is a cytosolic protein lacking a secretory signal sequence and therefore cannot enter the classical ER/Golgi secretion pathway, it is nevertheless found in extracellular fluids such as human serum, with elevated annexin 1 levels occurring particularly in inflammatory settings [21–24]. Exogenously administered it acts as a potent anti-inflammatory agent that limits the transendothelial migration of leukocytes [25] and thereby the degree of inflammation, a pharmacologic property that has been reported in many different animal models of inflammation [26]. This vast body of literature on its anti-inflammatory activities as well as the observation that annexin 1 deficient mice show an exaggerated inflammatory response in both acute and chronic models of experimental inflammation [27,28] point to the physiological relevance of extracellular annexin 1. The finding that annexin 1 represents a novel endogenous ligand for all known members of the formyl peptide receptor superfamily [4–6], heptahelical, G-protein coupled receptors on the leukocyte surface that mediate cell activation and chemotaxis upon binding of bacterially derived peptides of the fMLF prototype [29], established a mechanistic basis for the annexin 1-mediated anti-inflammatory effects.

Studies on the anti-inflammatory activity of annexin 1 both in in vitro and in vivo models revealed that this anti-inflammatory activity lies within the unique N-terminal part. Peptides corresponding to this sequence act as mimetics that retain the full pharmacologic property of the whole protein [8], and they also serve as ligands for the formyl peptide receptors. Annexin 1 is a relatively unstable molecule and cleavage of the pharmacologically active N-terminal part is commonly observed [30]. Interestingly, a vast amount of the protein externalized upon contact of PMN with the endothelial cells is of smaller molecular size,

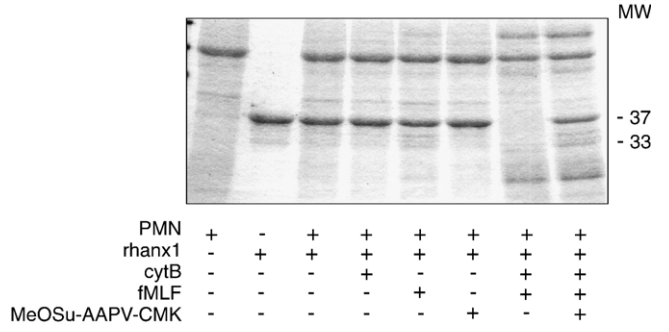

Fig. 3. Activated PMN rapidly degrade exogenously added annexin 1. Purified recombinant human annexin 1 (rhanx1, 2 μg) was added at 37 °C to unstimulated PMN or PMN treated with the agents as indicated. Degranulation was stimulated with 100 nM fMLF for 45 min. Cell-free supernatants were obtained and proteins therein were subjected to SDS-PAGE and Coomassie staining. A representative result is shown.

indicating rapid proteolytic cleavage. The recent analysis of the annexin 1 crystal structure discovered that this N-terminal domain is mostly buried in the protein core and therefore most likely not accessible for FPR interaction [31]. Therefore, N-terminal peptides released by limited proteolysis are most likely the physiologically relevant components in vivo. The pathophysiologic relevance of annexin 1 cleavage is strengthened by the observation of elevated levels of N-terminally cleaved annexin 1 in bronchoalveolar lavage fluids from patients with cystic fibrosis or other lung diseases compared to healthy volunteers [32–34].

Leukocyte proteases play a major role in acute inflammatory disorders with or without bacterial involvement. Neutral serine proteases such as human leukocyte elastase are stored in high amounts in the primary granules of neutrophils. Whereas they were commonly thought to be part of a relatively non-specific degradation machinery for phagocytosed cell fragments or microorganisms within the neutrophil lysosomes, it became evident in recent years that they also function as important regulators in the inflammatory process within the extracellular milieu. Their abilities to proteolytically modulate cytokine bioactivities by cytokine degradation (e.g. IL-6, TNF-α, IL-2), cleavage of inactive precursor molecules leading to the release of active cytokines (e.g. IL-1β, TNF-α, IL-8), cytokine receptor shedding, and cleavage of cytokine binding proteins suggest a role in the complex control of inflammation [9].

Degranulation and release of the cytotoxic degradative enzymes must be tightly controlled to prevent associated damage of the surrounding host tissue. Adhesion of neutrophils to substratum seems to induce neutrophils to rapidly mobilize primary granules upon stimulation with chemokines [20]. Neutrophils in suspension show only limited degranulation. Therefore, priming with cytochalasin B is needed to promote elastase release from neutrophil granules upon stimulation with activating mediators such as fMLF [35]. In this study, we could demonstrate a direct, elastase-dependent proteolytic conversion of annexin 1 into a truncated species by activated PMN. Although we focused here on HLE, we cannot rule out the possibility that other PMN-proteases could also account for the observed cleavage of annexin 1. However, our data showed an almost complete inhibition of annexin 1 cleavage by the specific HLE inhibitor.

Thus, it seems likely that the rapid cleavage of annexin 1 externalized from activated neutrophils that migrate into the extravascular tissues is to a great extent dependent on elastase activity. The controlled cleavage might be part of an appropriate mechanism to control and limit the amount of inflammation through proteolytic generation of anti-inflammatory mediators.

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