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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

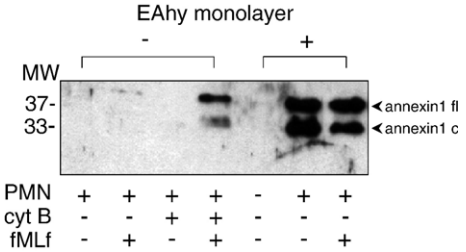

Fig. 1. Release of annexin 1 upon adhesion of PMN to the EAhy monolayer. PMN isolated from buffy coats were treated as indicated and either kept in suspension or incubated on EAhy monolayers for 30 min at 37 °C. Proteins of the cell-free supernatants were separated by SDS-PAGE and immunoblotted using 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.