Biochimica et Biophysica Acta 1820 (2012) 1705–1714

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Proteome analysis identified human neutrophil membrane tubulovesicular extensions (cytonemes, membrane tethers) as bactericide trafficking

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ARTICLE INFO

Article history:
Received 10 February 2012
Received in revised form 7 June 2012
Accepted 25 June 2012
Available online 2 July 2012

Keywords:
Neutrophil
Secretion
Bactericides
Cytonemes
Membrane tubulovesicular extensions
Membrane tethers

ABSTRACT

Background: Following adhesion to fibronectin neutrophils can develop membrane tubulovesicular extensions (TVEs) that can be 200 nm wide and several cell diameters long. TVEs attach neutrophils to the other cells, substrata or bacteria over distance. To understand the physiological significance of TVEs we performed proteome analysis of TVE content in neutrophils plated to fibronectin in the presence of compounds known to induce TVE formation (nitric oxide donor diethylamine NONOate, 4-bromophenacyl bromide, cytochalasin D).
Methods: Development of TVEs was confirmed by scanning electron microscopy. TVEs were disrupted following removal of inductors and biochemical, high-performance liquid chromatography and mass spectrometry investigations were employed to characterize the proteins within the incubation media.
Results: TVE disruption released (a) the granular bactericides lactoferrin, lipocalin, myeloperoxidase, cathepsin G and defensins; (b) energy metabolism enzymes; (c) actin cytoskeleton proteins; (d) S100 proteins; and (e) annexin 1.
Conclusions: The data confirm that TVEs represent a means of secretory bactericide trafficking, where the protrusions fuse with the plasma membrane upon neutrophil adhesion or extend from the cell surface when fusion is impaired. It is proposed that proteins abundantly presented in TVE (energy metabolism enzymes, actin cytoskeleton and S100 proteins, annexin 1) play an important role in fusion of TVE with the plasma membrane.
General Significance: Our study confirms TVEs as neutrophil secretory protrusions that make direct contacts with cells and bacteria over distance. The membrane-packed content and outstanding length of TVEs might allow targeted neutrophil secretion of aggressive bactericides over a long distance without dilution or injury to surrounding tissues.

1. Introduction

The ability of neutrophils to penetrate inflamed tissue, scavenge and kill microorganisms is essential to the role they play in host defense. Adhesive interactions of neutrophils with bacteria lead to phagocytosis (ingestion) of microorganisms and their intracellular killing by neutrophil bactericides secreted into phagosomes. Previously we have demonstrated the new type of neutrophil adhesive interactions with cells and bacteria achieved through the formation of very long and thin membrane tubulovesicular extensions (TVEs, membrane tethers, cytonemes) [1]. The protrusions anchor the cells to the substrata and to other cells and catch and hold remote extracellular bacteria over distance, acting as temporarily adhesive organelles. TVEs have a uniform diameter (150–250 nm depending on conditions) that is maintained along their length, a rapid rate of growth (1–5 μm/min), a high degree of flexibility and mobility (particularly in non-attached tips) and the capacity of shedding from the cell surface [1–5]. Study of TVEs has been limited as a consequence of their size (which is on the threshold of optical resolution); however, use of cytoplasmic and lipid fluorescent dyes has revealed that TVEs have a membrane covering and a cytoplasmic core [1,5]. In size and structure, TVEs resemble membrane tethers, which can

Abbreviations: TVE, tubulovesicular extensions; BPB, 4-bromophenacyl bromide; MPO, myeloperoxidase; NGAL, neutrophil gelatinase-associated lipocalin; TKT, transketolase; G6PDH, glucose-6-phosphate dehydrogenase; PGI, phosphoglucose isomerase (neuroleukin); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; *ov*-serpin, serine (or cysteine) proteinase inhibitor, clade B (ovalbumin); S100A8, S100A9, the S100 calcium-binding proteins, known also as calgranulins A and B or myeloid-related proteins MRP8 and MRP14, or cystic fibrosis antigen; S100A8/A9, calprotectin; MMP-9, matrix metalloproteinase 9 or gelatinase B, 92 kDa; HNP 1–3, human neutrophil peptides 1–3 or defensins; EM1, EM2, extracellular medium collected after first 20 min and second 15 min of neutrophil adhesion to fibronectin respectively

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doi:10.1016/j.bbagen.2012.06.016

be pulled from the cell bodies upon neutrophil flowing over P-selectin or endothelium at the physiological rate [6–8] or as a result of micropipette manipulation [9,10]. In vitro, formation and shedding of membrane tethers have been shown to stabilize neutrophil rolling velocities [7].

Neutrophil TVEs develop on the cell surface during first 10–20 min of neutrophil adhesion to fibronectin-coated substrata in the presence of some agents. Inhibition of glucose metabolism, as well as blocking of vacuolar type ATPase (V-ATPase) can induce TVEs formation [2,4]. Glycolytical enzymes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [11–15], and vacuolar ATPase [16–18] are intimately involved in membrane fusion events and can be employed to initiate TVE formation. Glycolytic enzymes and V-ATPase are known to interact directly with actin [19,20] [21]. Disruption of actin cytoskeleton with cytochalasin D also promotes TVE formation [2,5]. The alkylating agent 4-bromophenacyl bromide (BPB), which affects actin cytoskeleton through a leukocyte-specific actin-bundling protein, L-plastin [22], is a reliable inducer (>90% of neutrophils) of cytoneme formation [2]. Nitric oxide (NO), a powerful physiological regulator of neutrophil adhesive interactions [23], is both an inhibitor of V-ATPase [24] and GAPDH [25], as well as an inducer of TVE formation [1,3].

A greater understanding of the structural and functional changes that describe the process of TVE formation may serve to provide us with new insights into their role in neutrophil function We describe herein the combination of scanning electron and fluorescent microscopy analysis with a comprehensive range of biochemical techniques, including electrophoresis, high-performance liquid chromatography (HPLC) and mass spectrometry, to better describe the process of TVE formation.

2. Materials and methods

2.1. Materials

Bicarbonate-free Hank's solution BPB and cytochalasin D were purchased from Sigma (Steinheim, Germany). Ficoll-Paque was obtained from Pharmacia (Uppsala, Sweden), fibronectin from Calbiochem (La Jolla, USA) and diethylamine NONOate from Cayman (Massy, France).

2.2. Isolation of neutrophils

Experimental procedures were reviewed and approved by the Institutional Ethics Committee of the A. N. Belozersky Institute. Healthy volunteers who gave informed consent and who had not taken any medication in the previous 2 weeks preceding sampling underwent vena puncture (using methods approved by the Ministry of Public Health Services of the Russian Federation) to provide blood samples for neutrophil isolation. Neutrophils were isolated from the freshly drawn blood on a bilayer gradient of Ficoll-Paque (1.077 and 1.125 g/mL). Washed neutrophils were resuspended in bicarbonate-free Hank's solution containing 10 mM HEPES (pH 7.35).

2.3. Adhesion of neutrophils to fibronectin and tubulovesicular membrane extension formation

Hank's solution containing 5 μg/mL fibronectin was incubated for 2 h at room temperature in 6-well culture plates and then washed thoroughly with buffer, leaving the wells coated with fibronectin. Neutrophils were plated in these protein-coated wells (3×10^6 cells in 2 mL per well) in corresponding buffer and incubated for 20 min at 37 °C. 4-Bromophenacylbromide (BPB, 15 μM), diethylamine NONOate (1 mM) and cytochalasin D (10 μg/mL) were added to the cells before plating. The control neutrophils spread over substrata during the 20 min incubation period. Incubation in the presence of BPB, diethylamine NONOate or cytochalasin D was associated with the development of TVEs on the cell surface of neutrophils. The extracellular media (EM1) from the control and the diethylamine NONOate- and BPB-treated wells were collected and it was confirmed that the TVE remained attached to the neutrophil surfaces following this process. Identical volumes of buffer (minus the induction factors) were added to each sample and incubated for 15 min at 37 °C. This procedure caused disruption and shedding of TVEs from the cell surface into the medium. Extracellular medium (EM2) was collected and inhibitors of metalloproteinase (EDTA 5 mM), serine (PMSF 200 mM) and cysteine (E64 10 μM) proteinases and myeloperoxidase (sodium azide 0.025%) were added to the samples. Samples were centrifuged at 5000*g* to remove unattached neutrophils and the supernatant from each of the six analogous wells combined.

To test viability of neutrophils at the end of experiment cells were stained with trypan blue dye and the Hoechst fluorescent DNA dye. After collection of EM2, neutrophils were incubated with 0.5 mM trypan blue (0.5 mM) in Hank's solution (15 min at 37 °C) and washed, and the number of dead cells (percent stained) was counted. In the other experiments, after collection of EM2 neutrophils were fixed with paraformaldehyde (4% in PBS, pH 7.4). For DNA staining cells were washed, permeabilized with Triton X-100 (0.1% for 5 min), washed again and incubated with 50 μg/mL Hoechst DNA dye for 30 min.

2.4. Concentration of proteins and sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis

Two procedures were used to concentrate proteins within EM1 or EM2 for further analysis. Proteins were either:

- Concentrated on Microcon centrifugal filters (10,000 MWCO) through centrifugation at 11,000*g* and 4 °C (Beckman Coulter Microfuge 22).
- Mixed with an equal volume of chloroform–methanol mixture (2:1, v/v), vortexed for 1–2 min, agitated in a shaker at 4 °C for 30 min and centrifuged at 11,000*g* for 20 min. The chloroform and methanol–water phases were collected and the solvents evaporated. Concentrated proteins of both phases were subjected to electrophoresis or HPLC analysis. The chloroform phases contained nearly all of the proteins detected, whereas the water–methanol fractions contained only trace amounts of protein.

The protein profile of the chloroform fraction matched the protein profile of samples concentrated using Microcon filters.

A one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed according to the methods described previously using MiniPROTEAN 3 Cell (Bio-Rad) equipment and a 15% polyacrylamide gel under non-reducing conditions [26]. Equal aliquots of preparations were boiled for 3 min in a lysing buffer [Tris–HCl 30 mM (pH 6.8); SDS 1%; urea 3 M; glycerol 10%; bromophenol blue 0.02%] and loaded into different lanes of the same gel. Gels were stained with Coomassie Brilliant Blue G-250 0.22% (Serva).

2.5. Mass spectrometry identification of proteins and sample preparation

The excised Coomassie-stained protein bands generated from the SDS-PAGE underwent trypsin in-gel hydrolysis. Gel pieces (1×1 mm^2) were washed twice with 100 μL acetonitrile 40% in NH_4HCO_3 100 mM (pH 7.5) for 30 min at 37 °C, dehydrated with 100 μL acetonitrile and air-dried. They were incubated with 4 μL modified trypsin 12 μg/mL (Promega) in NH_4HCO_3 50 mM for 6 h at 37 °C. Peptides were extracted through incubation with 6 μL trifluoroacetic acid solution 0.5% in acetonitrile 10% for 30 min and were subjected to mass spectrometric analysis.

For mass spectrometry, sample aliquots (1 μL) were mixed on a steel target with 0.3 μL 2,5-dihydroxybenzoic acid (20 mg/mL in acetonitrile 20% and trifluoroacetic acid 0.5%) and the droplets were left to dry at room temperature. Matrix-assisted laser desorption ionization mass spectography (MALDI-MS) and tandem mass spectography