Antibacterial effect of human neutrophilic granulocyte derived microvesicles

Ph.D Dissertation

Csaba István TIMÁR

Semmelweis University
Ph.D School of Molecular Medicine

Supervisor: Erzsébet Ligeti, Professor, member of the Hungarian Academy of Science

Official opponents: János Matkó, Professor, doctor of the Hungarian Academy of Science
Erna Pap, Associate professor, Ph.D.

Chair of the compherensive examination cometee:
András Falus, Professor, member of the Hungarian Academy of Science

Members of the compherensive examination cometee:
Gabriella Sármay, Professor, member of the Hungarian Academy of Science
Ákos Zsembery, Associate professor, Ph.D.

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Introduction

Human neutrophilic granulocytes (PMN) are the most abundant leukocytes in our blood. These cells play fundamental role in the immune system. They are the first to migrate to the site of infections, where their main function is to eliminate microorganisms (such as bacteria and fungi). The process involves engulfment and segregation of the microorganisms intracellularly, in phagolysosomes. The NADPH-oxidase of the PMN plays a leading role in the antimicrobial properties of the phagolysome: it is responsible for superoxide production towards the phagolysome, and also involved in pH regulation of the lumen. The absence or dysfunction of this enzyme complex results in dysfunctional bacteria elimination against some bacteria strains (such as *Staphylococcus aureus*), but far not against all (such as *Escherichia coli*). Since the extracellular pH and potassium ion concentration also have influence to the functionality of the NADPH-oxidase, the extracellular environment also have effect on the intracellular bacteria elimination of the PMN.

Beside the phagolysomal microbe elimination, the PMN also have extracellular antimicrobial properties. The neutrophil extracellular trap (NET) have been described in the last decade. The cornerstone of this process is formation of an extracellular fibrillar net from DNA, incorporated with histones and some antimicrobial peptides and proteins. The effect of the net is based on the immobilization (with the DNA fibrils) and direct attack (by the antimicrobial peptides and proteins) of the bacteria.

In recent years the existence and effects of cell-derived extracellular vesicles (e.g. exosomes and microvesicles) have been revealed in several physiological functions. These cell derived vesicles are small, double layer phospholipid membrane bounded particles, containing a handful type selected proteins, and also a lot kind of RNA, such as mi-, m-, t-, or non-coding RNA sequences. The classification of these vesicles based on their size, and also on their generating processes. The exosomes are the smaller ones (10 to 100 nm), produced by exocytosis of multivesicular bodies. Microvesicles (MV) are larger (100 nm to 1 μm), they are formed by blebbing of the plasma membrane. The largest (1μm to 4μm) are the apoptotic bodies, produced during apoptosis. The importance of these vesicles have been demonstrated in intercellular communication, transfer of different type of RNA or proteins, modulation of haemostasis, or in metastatization of tumour cells.

Neutrophilic granulocytes were also shown to create such particles, but little was known about their functions, and there was absolutely no information about the effect of these particles to the bacteria, the original target of the PMN. There was also only poor data about the importance of different agents, which provokes MV production, and little was known about differences and similarities between distinct MV populations.

AIMS:

- We wanted to examine the importance of different pharmacological and bacterial agents to both quantitative and qualitative properties of the MV production of human PMN.

- We wanted to investigate the effect of the PMN derived MV to different bacteria strains, both *in vitro* and *ex vivo* experimental circumstances.

- We examined the importance of the extracellular milieu to the phagolysosomal and to the MV mediated antibacterial properties of the PMN

MATERIALS AND METHODS

Preparation of MVs from PMNs and human serum

Venous blood was drawn from healthy adult volunteers according to procedures approved by the Institutional Review Board of the Semmelweis University. Neutrophils were obtained by dextran sedimentation followed by Ficoll-Paque gradient centrifugation. The preparation contained more than 95% PMN and less than 0.5% eosinophils, controlled with Giemsa stain. PMNs were incubated with or without activating agent for 20 minutes at 37°C on a linear shaker (80 rpm/min), unless indicated otherwise. After incubation, PMNs were sedimented (500g, 5 minutes, 4°C) and the supernatant was filtered through a 5µm pore sterile filter. The filtered fraction was sedimented again (15 700g, 10 minutes, 4°C). The sediment was resuspended in HBSS at the original incubation volume unless indicated otherwise. Serum of healthy donors and bacteremic patients was filtered and sedimented as described. This study was conducted in accordance with the Declaration of Helsinki. Amount of separated MV were estimated on the basic of their protein content (determined by the Bradford protein assay with BSA standard) and also with Flow Cytometer. An initial microvesicle-size gate was set with the help of calibrating GFP-expressing bacteria (*S. aureus*, diameter 0.8 µm) and 3.8 µm SPHERO rainbow alignment particles (Spherotech). The absolute count of MVs was measured for 15 s. We defined PMN-derived MVs as particles less than 1.5 µm in diameter and expressing surface marker CD11b.

Opsonization of bacteria

Bacteria were opsonized with pooled normal human serum for 15 minutes at 37°C. After opsonization, bacteria were centrifuged (5 minutes, 4°C, 5000g), and washed by HBSS. The concentration of bacteria was set to $OD_{600nm} = 1.0$.

Measurement of bacterial growth

Bacteria were incubated with MV or with PMN for 30 minutes, at 37°C, with gentle shake. Samples were taken in every 10 minutes, and moved to ice cold, saponin containing HBSS medium to stop further reactions. After the last sampling, samples were frozen to unfold the PMN or MV. In the end, to optimize bacteria growt, samples were moved to Luria-Bertani media. Bacterial growth was followed in a plate reader (Labsystems iEMS Reader MF, Thermos Scientific) on 96 well plate, on the basis of changes in optical density at 650 nm. Original bacteria counts were calculated with calibration bacteria samples.

Measurement of superoxide production

Production of superoxide anion was determined with superoxide dismutase-inhibitable cytochrome c reduction assay.

Fixation and staining of MVs and PMNs

MVs or PMNs were centrifugated with 15700 g, and were layered on cover slips. When indicated, samples were permeabilized with 0.5%

Triton X-100 for 3 minutes at room temperature after fixation. After washing with HBSS, MVs were visualized by R-PE conjugated anti-CD11b monoclonal antibody, or with FITC-conjugated anti-CD18 monoclonal antibody. Myeloperoxidase (MPO) was detected with anti-MPO monoclonal antibody, and FITC-conjugated anti–mouse goat secondary antibody. F-actin was labeled with Alexa 488–Phalloidin, phosphatidylserine was stained with FITC-conjugated annexin-V (used as indicated by the manufacturer; Becton Dickinson Bioscience). As nucleic acid marker propidium iodide was used. On each sample, autofluorescence and isotype antibodies were measured as controls.

Transmission, confocal, and video microscopy

For confocal and video microscopy experiments, Zeiss LSM510 and 710 confocal laser scanning microscopes were used. In video microscopic experiments stage and objective were heated to 37°C and slips were coated with BSA (10 wt/vol %) for 1 hour at room temperature. Images were analyzed with LSM Image Browser software (Zeiss) and with ImageJ software.

Electron microscopy of MVs

Pelleted MVs were fixed at room temperature for 60 minutes with 4% paraformaldehyde in PBS. The preparations were postfixed in 1% OsO_4 (Taab) for 30 minutes. After rinsing with distilled water, the pellets were dehydrated by ethanol, followed by block staining with 1% uranyl-acetate in 50% ethanol for 30 minutes, and embedded in Taab 812 (Taab). After overnight polymerization at 60°C and

sectioning for EM, the ultrathin sections were analyzed with a Hitachi 7100 electron microscope equipped by Veleta, a 2k x 2k MegaPixel side-mounted TEM CCD camera (Olympus). Electron micrographs were edited by Photoshop CS3 (Adobe).

Dynamic light scattering

Dynamic light scattering (DLS) measurements were performed at room temperature, using an ALV goniometer with a MellesGriot diode-pumped solid-state laser (CVI MellesGriot) at 457.5 nm wavelength. The radius of the particles was calculated using sphere approximation.

Immunoblotting

MVs were lysed in 4 _ Laemmli sample buffer, boiled, run on 10% (wt/vol) polyacrylamide gels and transferred to nitrocellulose membranes. After blocking for 1 hour in PBS containing 5% albumin and 0.1% (wt/vol) Tween 20, blots were incubated with anti–lactoferrin polyclonal antibody in 1:1000 dilution or anti–_-actin mAb (both from Sigma-Aldrich) in 1:10 000 or anti-MPO antibody (Cell Signaling, Danvers) in 1:500 dilution in PBS containing 5% ovalbumin. Bound antibody was detected with enhanced chemiluminescence using horseradish peroxidase–conjugated anti–rabbit-Ig (from donkey) or anti–mouse-Ig (from sheep) secondary antibodies (GE Healthcare) used in 1:5000 dilution.

RESULTS:

Human PMN, prepared from peripheral blood samples are able to produce subcellular vesiculi both spontaneously and upon different stimuli in our experimental surroundings. The size of the separated vesiculi revealed that our preparation method – well correlating to other examinations – separate the microvesicle (MV) fraction of the PMN derived subcellular vesicles (average diameter was about 400 nm). It was a great surprise that the MV produced upon different stimuli did not differ in their size. We also confirmed that our MV bounded with phospholipid bilayer, and the direction of this bilayer is similar to the donor cell's membrane. The two chains of the complement receptor 3 (CR3), the CD11b and the CD18 have been found in large concentration in MV membrane, so we used this two protein for routine marker of PMN derived MV. Strong contrast to their diameter, the amount and the protein composition of the separated MV strongly depended on the inductor stimuli – the largest differences between MV were in the amount of antibacterial peptides and proteins. I have to bring the opsonized *Staphylococcus aureus* out in bold relief, since it led to the production of the largest amount MV (bacterial-MV, or bMV hereunder), and only these b-MV had well detectable antibacterial capacity against *S. aureus* an *Eserichia coli*.

Detailed examination of the antibacterial effect of bMV revealed several strong differences to the NET, and also to the phagolysosomal bacteria elimination processes of the PMN. First, the

bMV did not required the opsonization of the aimed bacteria. The bMV – in contrast to the phagocytosis – attach only to the surface of the bacteria, but did not engulf it. The antibacterial effect of the bMV did not depended from pH , potassium concentration or from the superoxide production of the extracellular environment, strong contrast to phagolysomal bacteria killing. The effect of bMV also was insensitive to DN-ase treatment, in contrast to the effect of the NET. The bMV was bacteriostatic to the bacteria, in contrast to the bactericide effect of the phagolysosome. The development of bMV-based effect developed in minutes, while the NET requires hours. On the other hand, bMV is inhabitable with blockade of the CR3, while the NET is insensitive to this blockade. Another important difference is the induction of the processes: MV generated to the strongest inductors of NET (such as lipopolysaccharide or phorbol ester) did not led to the production of antibacterial MV.

Taken together, the effect ant the process of the bMV differed in many points from other antibacterial mechanism of the PMN. But what is the basic of this effect? Since there is a well detectable difference in the composition of bMV to other MV, and also only intact bMV has antibacterial effect, we examined this two flavor to get closer to the identical mechanism.

The identification of the direct connection of NV and bacteria revealed a strong negative correlation between the numbers of the attached bacteria to the detected antibacterial effect. Namely, the more bacteria was in clump, the less bacteria divided. Other MV, such as spontaneously formed MV (sMV), did not made as much attachment

as the bMV did. We also observed aggregation of bMV with bacteria, when the bacteria itself were treated with bactericide agents, suggesting that this effect did not require alive bacteria. On the other hand, ditruption of bMV with e.g. detergents inhibited the antibacterial effect of the preparation, and also did not lead to the aggregation of the bacteria. On the other hand, the amount of the aggregated bMV to bacteria also determined the antibacterial effect: the more MV to one bacteria resulted in the fewer dividing bacteria. The importance of this difference is well demonstrated, when we compared the bMV to phorbol ester induced MV (pMV): they attach nearly the same amount of bacteria, but pMV lack formation of large aggregates, in controst to bMV, which also correlated to the detected antibacterial effect. These results suggest that not only the attachment, but the amount of carried antibacterial effectors (such as antimicrobial peptides or proteins) is responsible for the antibacterial effect. This finding underlines the importance of the carried antimicrobial proteins of the bMV.

To understand the detailed process of bMV-bacteria aggregation, we wanted to investigate the effect of non-antimicrobial proteins in details on the basic of their pharmacological inhibitors. The inhibition of the CR3, the actin cytoskeleton or the phophatidyl inositol 3 kinase, or withdrawing glucose all led to depressed attachment, and also to decreased antibacterial effect. Taken together these results suggest that the attachment itself is an active process. Since the affinity of CD18 depends on the conformation, and the change of conformation requires active cytoskeletal movements,

which requires energy, we also aim to investigate these intravesicular processes, but till now we do not have answer to these questions.

We also wanted to investigate whether have bMV importance in the human body. To answer this question, we separated PMN derived MV from the blood plasma of healthy volunteers and also from septic patients, treated with new and verified *Staphylococcal* infection, examined their amount and also their attitude to bacteria. MV from healthy donors did not attached to bacteria and did not created large aggregates, while the same amount MV from septic patients formatted a large number of large aggregates. On the other hand, PMN derived MV accumulated aprox. 10 times higher concentration in the blood of septic patient, in contrast to healthy volunteers.

Taken together, our experiments revealed an earlier unknown bacteriostatic mechanism of the PMN, which is independent from the opsonization of the bacteria and also from the extracellular pH, and bear on the direct attachment of bacteria to the MV.

COCNLUSIONS

During my Ph.D. works I examined a special population of the neutrophilic granulocyte derived subcellular vesicles, the microvesicles. I determined their size, composition, also investigated on of their potential biological role, their effect to bacteria survival. I also investigated the importance of the extracellular environment to the antibacterial effect the phagolysosomal, and also to the MV dependent antibacterial mechanisms of the PMN.

As a conclusion, I can say that PMN produce MV spontaneously and upon different stimuli. The composition an the antibacterial effect of these MV strongly depends on the agent which induces the production of the MV. The opsonized bacteria is one of the most important inductor of the MV production of the PMN, since it led to the largest amount of produced MV, and, on the other hand, these MV have a well detectable antibacterial effect.

The antibacterial effect of these MV did not depends from the opsonization of the attacked bacteria, bur depends on the direct connection between the MV and the bacteria, which process depends on the CR3 and on some intravesicular processes (e.g. PI3K or actin.cytoskeletal movements). The antibacterial effect also depends from amount of the MV what attach to one bacteria, suggesting the role of the carried antimicrobial peptides and proteins.

The effect of bMV also was relatively independent from the extracellular enviroment, since it was insensitive to the extravesicular acidosis, cloride or potassium ion concentration changes, while the

phagolysosomal bacteria elimination strongly depends on the extracellular pH.

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