CUSTOM-MADE NEUTROPHILIC GRANULOCYTE DERIVED EXTRACELLULAR VESICLES

Ph.D. Theses

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Budapest
2021

I. Introduction

Extracellular vesicles (EV) are phospholipid bilayer delimited particles released spontaneously, upon different stimuli and during apoptosis by both pro- and eukaryotic cells.

When eukaryotic EVs are released from multivesicular bodies, they tend to be smaller in size (diameter: 30-100 nm) and are often called exosomes, while a medium-sized (100-1000 nm) population referred to as microparticles, microvesicles or ectosomes can be released by blebbing of the plasma membrane. Large EVs (1-5 μm) are typically generated during apoptosis.

EVs can transfer their content to other cells or stimulate receptors of target cells, as well as affect different extracellular proteins and microorganisms directly, usually by utilizing their specific surface structure. There is a very large amount of information about countless physiological and pathophysiological effects of EVs. They can complement the known humoral and direct contact-based communication pathways between immune cells by carrying pro- or anti-inflammatory potential or by transferring antigens for presentation. EVs are also known to influence coagulation: both

pro- and anticoagulant effects have been characterised in great detail.

Neutrophilic granulocytes belong to the fast-reacting innate immune response, and they are the principal cells against bacteria and fungi. When they encounter danger signals, they leave the vessels and move to the infection site guided by diverse chemotactic stimuli. They encounter the pathogens recognised by opsonin receptors and pattern recognition receptors. If the pathogen can be internalised, phagocytosis occurs, and intracellular killing mechanisms get activated. By producing newly manufactured cytokines, neutrophils also play a role in finely regulating the development and the evolution of inflammatory and immune responses.

Neutrophil-derived EVs were first identified in 1991. Since then, countless direct (e.g., antimicrobial, prothrombotic) and indirect (anti- or pro-inflammatory) effects have been attributed to them. Basic physical and chemical characteristics of neutrophil-derived EVs are similar in many of these studies, while the variety of the described effects is rather puzzling. The inconsistent data regarding the EVs' effects could arise from the different quality and purity of the PMN isolates, protocols used for EV production and preparation, storage conditions of the EV samples and experimental environment of the investigated

target cells. Data are more comprehensible when one considers the different stimulating agents used for EV production, which is the most crucial factor in our opinion. However, there is considerable inconsistency in the results even if we compare the reports using the same activators. We can hardly find any comparative studies, where two or more types of EVs are parallelly investigated. Without these the effect of specific environmental factors and stimuli on the behaviour of neutrophil EVs cannot be determined, as none or very few basic experimental procedures are matching.

II. Objectives

The aim of my Ph.D. work was to use three, in our laboratory's previous publications well defined, after isolation freshly applied neutrophil EV populations to evaluate their effects on different physiological processes under comparable conditions.

First, I intended to clarify whether these neutrophil-derived EVs are taken up by other neutrophils, monocytes and lymphocytes.

Second, I aimed to investigate the effect of spontaneously released EVs (sEVs), EVs released upon stimulation with opsonised zymosan particles (activated EVs, aEVs) and EVs released upon spontaneous cell death in the course of incubation without activating factors for 24 h (apoEVs) on other neutrophilic granulocytes, endothelial cells and blood plasma.

Finally, it was also important to show, that neutrophils remain viable and do not release significant amounts of neutrophil extracellular traps (NET) during our EV production step, so that the detected effects can entirely be attributed to EVs.

III. Results

1. Interaction of neutrophil EVs with other neutrophils, monocytes and lymphocytes

The fate of fluorescently labelled neutrophil EVs was followed upon encounter with neutrophils, monocytes or lymphocytes. EVs produced upon stimulation with opsonised zymosan (aEVs) or spontaneously from fresh (sEVs) or apoptotic cells (apoEVs) were labelled with the membrane-localised stain PKH67 and fluorescence was detected by flow cytometry.

In 45 min measurable increase of mean fluorescent intensity (MFI) occurred with aEV and apoEV populations in all three cell types. On the other hand, sEVs seem to associate with neutrophils only.

With confocal microscopic imaging I could verify that EVs are engulfed in neutrophils, as opposed to staying only attached on the surface of the cells.

2. Effect of neutrophil EVs on the migratory potential of neutrophilic granulocytes

I pretreated cells with previously produced sEVs, apoEVs and aEVs for 45 min and put them in a transwell chamber filled with fMLP as chemoattractant.

After one hour of migration, there was no difference in the absolute numbers of transmigrated cells between the different groups.

3. Effect of neutrophil EVs on the phagocytotic capacity of neutrophilic granulocytes

Pretreated cells were incubated for 20 min with opsonised fluorescent *S. aureus* bacteria. Either one concentration of bacteria was used (neutrophil:bacteria ratio 1:30) and samples were taken every 5 min (kinetic measurements), or five different concentrations of bacteria were used (neutrophil:bacteria ratios 1:10, 1:30, 1:100, 1:300 and 1:1000) and the sample was analysed at the end of the incubation period only (maximal capacity measurements).

Neither the kinetics nor the maximal capacity of neutrophil phagocytosis was affected by pretreatment with the indicated EV populations.

4. Effect of neutrophil EVs on the ROS production of neutrophilic granulocytes

I pretreated neutrophils with the indicated EV populations and their controls for 45 min, followed by a secondary activation with PMA (or leaving the cells unstimulated), and a 90 min luminescent detection of ROS. I analysed the ROS production rate at an early stage (10 min after the secondary activation) and at the maximal capacity of the cells (typically occurring between 30 to 40 min after the secondary activation).

While aEVs consistently enhanced both the early and maximal ROS production, sEVs decreased them. Early-stage ROS production was decreased by apoEVs, but the peak of the curve was unaffected. This represents a third kind of EV-related action, i.e., the rightward shift of the curve.

EV pretreatment alone without secondary activation did not trigger ROS production.

5. Effect of neutrophil EVs on the cytokine production of neutrophilic granulocytes

I incubated neutrophils with the indicated EV populations and their controls for 3 h and determined the IL-8 concentration in the supernatant after centrifugation of the cells.

aEVs enhanced the IL-8 secretion in every single experiment, and a similarly consistent lowering effect was seen with sEVs. On the contrary, apoEVs showed no significant effect on the IL-8 secretion.

Importantly, I failed to detect any measurable amount of IL-8 in the aEV and sEV isolates themselves.

6. Effect of neutrophil EVs on endothelial cells

We incubated confluent HUVEC layers with the indicated EV populations and their controls for 24 h. IL-8 concentration of the supernatant was determined at 24 h with sandwich ELISA, while E-Selectin expression at 6 h and vascular cell adhesion protein 1 (VCAM-1) expression at 24 h were measured after fixation with cellular ELISA.

HUVECs exposed to aEVs showed signs of pro-inflammatory activation regarding IL-8 release and adhesion molecule

expression. In contrast, we did not obtain any consistent effects with sEVs and apoEVs.

7. Effect of neutrophil EVs on coagulation

I incubated EVs with recalcified citrated pooled human plasma and measured the change of absorbance (turbidity) to elucidate the effect of our EV populations on plasma clotting.

First, I analysed the impact of EVs without the addition of thromboplastin (TP). Under these circumstances, coagulation did not necessarily occur, so I examined the number of coagulated wells. Frequency of coagulation was almost the same in the aEV and lysed aEV treated samples, while sEVs and apoEVs significantly promoted the occurrence of coagulation.

Second, we were curious whether EVs could influence the plasma clotting when it is activated by TP and coagulation occurs in every well. Therefore, I measured the average clotting time in recalcified citrated pooled human plasma after the addition of TP and EV samples. Only apoEVs had a significant accelerative effect in this experimental setting. The presence of sEVs also elicited a minor decrease of the clotting time, albeit this effect was not statistically significant.

8. Assessment of the contribution of NET formation and other cell death forms to the described effects

There are multiple neutrophil cell death forms, which can exhibit both pro-inflammatory and anti-inflammatory properties. It is also possible, that NET formation affects the investigated cell and plasma functions. We wanted to elucidate the possible extent of their contribution to the effects described above.

Apart from early apoptosis, all of these cell death forms are accompanied by increased permeability of cell membranes and often also by the release of DNA into the extracellular space. Therefore, I stained my cell isolates with the membrane-impermeable nucleic acid dye PI and measured the intensity of fluorescence for 210 min in not stimulated, opsonised zymosan and PMA stimulated samples. As PMA was shown to induce NETosis, it served as positive control in this measurement. Detectable PI staining is only possible in case of DNA release into the extracellular space or when the permeability of the cell membrane is increased.

At 20 min, which is the end of the incubation period of neutrophils with or without the used stimuli before the isolation

of our EV populations, virtually no PI positivity can be detected. Relevant amount of PI positive cell death forms (late apoptosis, necrosis, necroptosis, pyroptosis or NETosis) occur earliest during the second hour of incubation.

I analysed representative confocal microscopic images of PI-stained neutrophils after 20 min and 3 h when not stimulated, stimulated with opsonised zymosan or with PMA. Only PMA treated cells after 3 h of incubation show signs of NETosis, i.e., fibrillar PI positive structures, which was not detectable when DNase was also present.

IV. Conclusions

I investigated the functional differences of previously described neutrophilic granulocyte derived EV populations. Based on the results described above, I make the following conclusions:

1. Neutrophil-derived aEVs and apoEVs are taken up by neutrophils, monocytes and lymphocytes. However, sEVs are only taken up by neutrophils themselves. Based on confocal microscopic images, EVs are internalised in neutrophils.

2. Neutrophil-derived EVs do not affect the chemotactic migration of neutrophils to fMLP.

3. Neutrophil-derived EVs do not influence the kinetics nor the maximal capacity of neutrophil phagocytosis.

4. The maximal and early-phase ROS production capacity of neutrophils upon PMA stimulation is enhanced by aEVs and decreased by sEVs. On the other hand, apoEVs only inhibit the early phase of ROS production, leading to a delayed release with the same maximal capacity.

5. IL-8 release of neutrophils is enhanced by aEVs, reduced by sEVs and not affected by apoEVs.

6. Endothelial activation based on IL-8 secretion, E-Selectin and VCAM-1 expression is triggered by aEVs but not by sEVs or apoEVs.

7. Spontaneous coagulation of blood plasma is triggered by apoEVs and sEVs, but not by aEVs. Blood plasma clotting induced by TP is accelerated by apoEVs but not by sEVs or aEVs.

8. Pro-inflammatory, PI positive cell death types (e.g., pyroptosis, necroptosis or necrosis) are not detectable in our neutrophil population within the time frame of PMN preparation and EV production steps, i.e., they are not responsible for the described pro-inflammatory effects.

Based on these data, we propose that neutrophil-derived EVs are custom-made and can have divergent, selective, and sometimes even antagonistic effects depending on the environmental conditions prevailing at the time of the EV production.

V. Bibliography of the candidate's publications

Publications related to the present thesis:

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