JLB

Article

Functionally and morphologically distinct populations of extracellular vesicles produced by human neutrophilic granulocytes

Ákos M. Lőrincz, Maria Schütte,* Csaba I. Timár,* Daniel S. Veres,† Ágnes Kittel,‡ Kenneth R. McLeish,§ Michael L. Merchant,§ and Erzsébet Ligeti*,1*

*Department of Physiology and †Department of Biophysics and Radiation Biology, Semmelweis University, Budapest, Hungary; ‡Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary; and §Department of Medicine, University of Louisville, Louisville, Kentucky, USA

RECEIVED OCTOBER 28, 2014; REVISED FEBRUARY 28, 2015; ACCEPTED MARCH 23, 2015. DOI: 10.1189/jlb.3VMA1014-514R

ABSTRACT

EVs in the microvesicle size range released during spontaneous death of human neutrophils were characterized and their properties compared with previously described EVs with antibacterial effect (aEVs, generated on specific activation) or produced spontaneously (sEVs). The 3 vesicle populations overlapped in size and in part of the constituent proteins were stained with annexin V and were impermeable to PI. However, none of them produced superoxide. In contrast, remarkable differences were observed in the morphology, abundance of proteins, and antibacterial function. EVs formed spontaneously in 30 min (sEVs) were more similar to EVs released during spontaneous death in 1–3 d than to EVs formed in 30 min on stimulation of opsonin receptors (aEVs). Spontaneously generated EVs had no antibacterial effect despite their large number and protein content. We hypothesized 2 parallel mechanisms: one that proceeds spontaneously and produces EVs without antibacterial effect and another process that is triggered by opsonin receptors and results in differential sorting of proteins into EVs with antibacterial capacity. Our results call attention to the functional and morphologic heterogeneity within the microvesicle/ectosome fraction of EVs. *J. Leukoc. Biol.* 98: 583–589; 2015.

Introduction

Cells produce different types of extracellular vesicles (EVs) [1, 2], even prokaryotes are secreting EVs [3]. Exosomes are released by fusion of the membrane of the multivesicular bodies, a specialized endosomic compartment, with the plasma membrane. They are of the smallest size, around or <100 nm [4–7]. Microvesicles, microparticles, and ectosomes are generated by shedding from the plasma membrane [8–10]. These vesicles are generally larger than the exosomes, although their size represents a continuous spectrum [11]. Apoptotic vesicles, released by apoptotic cells, are regarded as the largest population of EVs, but neither their size nor their composition has been well characterized [9, 12–14].

Neutrophilic granulocytes are short-lived cells that undergo spontaneous death within a couple of days both in vivo and in vitro under cell culture conditions [15–20]. In previous studies PMN-derived EV formation has been shown by several groups [21–26]. In those studies, the increase of IL-6 secretion from and tissue factor production by endothelial cells [21, 22] and increase of TGF-β and resolvin production in macrophages [23, 24, 26] have been revealed. We, and others, have demonstrated that both the composition and the function of EVs generated from isolated PMN in short-term incubation depend on the type of stimulus applied [25, 26]. Specifically, opsonized particles induced the generation of EVs that were able to impair the growth of bacteria but EVs released on soluble stimuli did not have any antibacterial effect [25]. We also observed spontaneous formation of EVs, from both isolated and circulating PMNs that lacked the antibacterial effect [25].

The aim of the present work was to investigate the EVs generated during spontaneous death of PMN and to define their relation to the EV types described previously.

MATERIALS AND METHODS

Preparation of EVs from PMNs

PMNs were prepared from venous blood of healthy volunteers as described previously [27] according to procedures approved by the institutional review board of the Semmelweis University. The preparation contained >95% neutrophils and <0.5% eosinophils.

Abbreviations: aEV = opsonized particle induced extracellular vesicle, CD11b = cluster definition 11b, integrin α M, DLS = dynamic light scattering, EM = electron microscopy, emPAI = exponentially modified protein abundance index, EV = extracellular vesicle, LTF = lactoferrin, PI = propidium iodide, PMN = polymorphonuclear cell, PS = phosphatidylserine, ROS = reactive oxygen species, sEV = spontaneous formed extracellular vesicle, SOD = superoxide dismutase

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: Department of Physiology, Semmelweis University, 1094, Budapest, Tűzoltó u. 37-47, Hungary. E-mail: ligeti@puskin.sote.hu

Spontaneously generated and antibacterial EVs released from PMNs were isolated, as described previously [25]. Zymozan A particles (Sigma-Aldrich, St. Louis, MO, USA) opsonized in human serum [25] were used for activation of PMNs.

Spontaneous cell death was initiated in DMEM at 37°C by the presence of 5% carbon dioxide [18, 19]. EVs were collected and isolated after 1, 2, and 3 d of incubation. The remaining cells were labeled with PI and FITC-Annexin V and analyzed using flow cytometry.

RNA and DNA detection

RNA was extracted with the TriPure isolation reagent (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. The presence and amount of RNA were measured with NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA).

EV detection using flow cytometry

The method described recently in detail by Lorincz et al. [28] was followed. To avoid swarm detection, the samples were measured at 2 different dilutions.

EM and DLS

The procedures described previously [28] were followed.

Measurement of bacterial survival and $O_2^{\bullet -}$ production

The methods detailed in [27] were applied.

Mass spectrometry analysis

EV samples (45 μg) were lysed using 2% SDS at 65°C for 30 min, reduced, alkylated, and digested using trypsin (Promega, Madison, WI, USA) as described previously [29], with modifications for the filter-assisted sample preparation protocol [30]. Before trypsinization, the samples were pipetted into a Microcon-10 Ultracel YM-10 10,000 NMWL centrifugal filter (EMD Millipore, Billerica, MA, USA) and rinsed (3 times) using 250 μl of 8 M urea/0.1 M Tris-HCl (pH 8.5) to remove SDS. Before digestion, the sample was diluted to 0.8 M urea/0.1 M Tris-HCl (pH 8.5). Peptides isolated through the YM-10 filter were desalted and concentrated using C18 PROTO Ultra-MicroSpin columns (Nest Group, Southborough, MA, USA). Desalted samples were separated offline into 7 strong cation exchange (SCX) fractions using SCX MicroTrap (Michrom-Bruker, Auburn, CA, USA) before analysis using 1D-RP (C18) nanoflow ultra high performance liquid chromatography and nanoelectrospray-mass spectrometry (MS), as described previously [31], using the LTQ-Orbitrap Elite MS platform (Thermo Fisher Scientific).

Data were acquired using Orbitrap Elite (Thermo Scientific) in the electron transfer dissociation decision tree method. All MS1 data were acquired using Fourier transform ion cyclotron resonance MS, and MS2 data using ion trap mobility spectrometry. All MS data were searched using Proteome Discoverer 1.4 (Thermo Scientific) with Sequest (The Scripps Research Institute, San Diego, CA, USA) and Mascot, version 2.4 (Matrix Science, Boston, MA, USA), in a decoy database search strategy against UniProt Knowledgbase *Homo sapiens* reference proteome canonical and isoform sequences current as of July 22, 2014. The searches were performed with a fragment ion mass tolerance of 1.2 Da and a parent ion tolerance of 50 ppm. The iodoacetamide derivative of cysteine was specified as a fixed modification. Oxidation of methionine was specified as a variable modification.

The search data results file were imported into Scaffold, version 4.3.4 (Proteome Software Inc., Portland, OR, USA) to control for <1.0% false discovery rates with PeptideProphet and ProteinProphet (Institute for Systems Biology, Seattle, WA, USA). The peptide and protein identifications were accepted if they could be established at >95.0% probability by the PeptideProphet [32] or ProteinProphet algorithm [33], respectively. A comparison of protein abundance among the EV groups was determined in Scaffold as the emPAI, as described by Ishihama et al. [34].

Immunoblotting

The method described in [25] was followed.

Statistical Analysis

Statistical analysis was performed with Statistica, version 8.0, software (Statsoft Inc., Tulsa, OK, USA) with 2-sample *t* test. All samples were compared with sEV. The results were considered significant at a 2-sided *P* value ≤ 0.05.

RESULTS AND DISCUSSION

EV production during spontaneous death of PMNs

First, we compared the properties of PMNs isolated freshly or kept in cell culture medium for a few days. PMNs were regarded

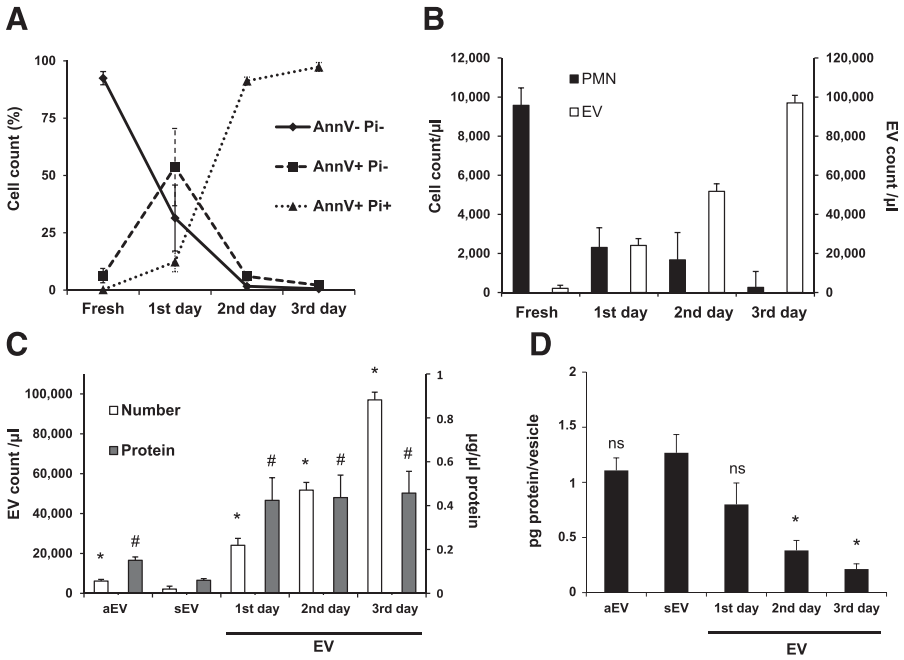

Figure 1. Basic properties of initiating cells and released vesicle populations. (A) Viability changes of PMN population in a 3 d incubation. (B) Cell count (left axis) and EV production (right axis) during 3 d of incubation. (C) Comparison of number (left axis) and protein content (right axis) of freshly produced EVs to spontaneous death derived EVs. (D) Comparison of protein content per vesicle of different types of EVs. (Bars represent standard error of the mean; $n = 3$; in the case of protein measurements, $n = 5$; * and # represent $P < 0.05$ compared with sEV; ns, nonsignificant.) AnnV, annexin V; Pi, propidium iodide.

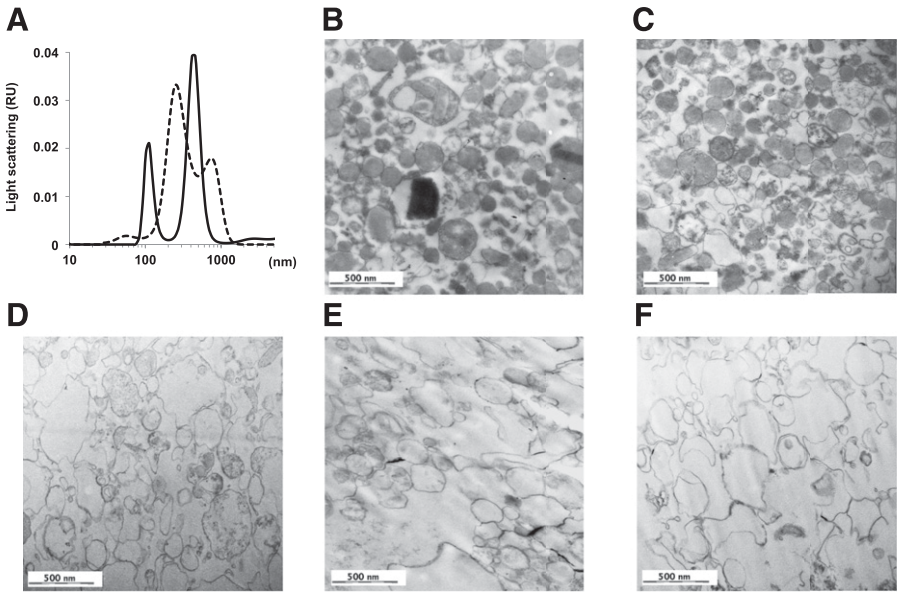

Figure 2. Morphology of different vesicle populations. (A) Size distribution spectra of EVs measured by DLS. sEVs represented by black line; third day EVs represented by broken line. (B–F) Representative electron microscopic images of aEVs (B), sEVs (C), first day EVs (D), second day EVs (E), and third day EVs (F). Original magnification ×30,000. Representative views of 1 of 3 similar experiments.

healthy if they could not be stained with either Annexin V-FITC (BD Biosciences, Franklin Lakes, NJ, USA) or PI (Invitrogen, Eugene, OR, USA). As summarized in **Fig. 1A**, freshly isolated PMNs were 95% healthy and only 5% showed annexin V binding. On the first day, most of the cells (55%) bound to annexin V, indicating enrichment of PS on their surface. Approximately 30% of the cells proved to be healthy and only 10% were PI positive (i.e., sufficiently leaky to enable PI to enter the nucleus). On the second and later days, PMNs positive for both annexin V and PI predominated.

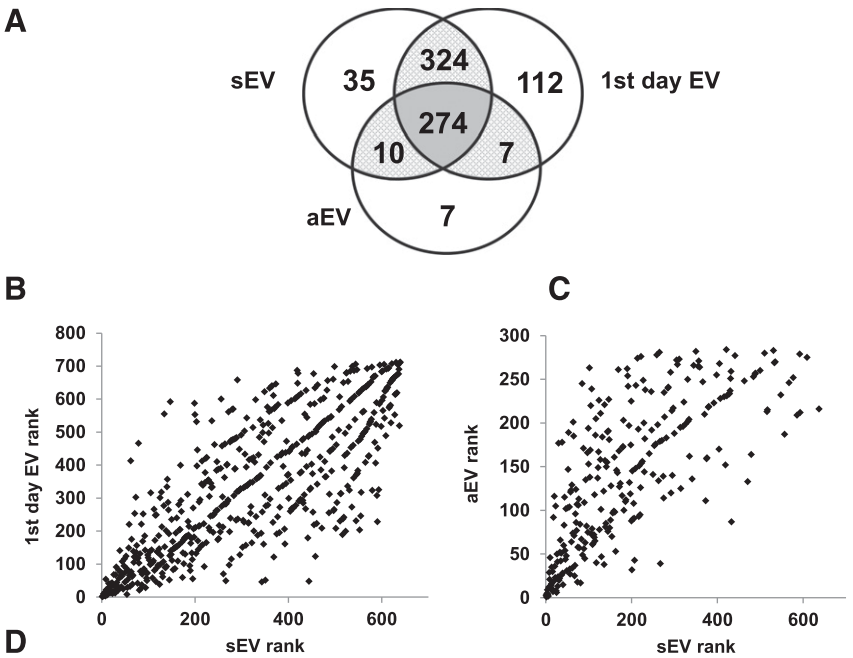

Figure 3. Protein distribution profile of different vesicle populations. (A) Protein distribution identified by MS in aEVs, sEVs, and first day EVs. (B) Spot diagram on proteins identified by MS ranked according their abundance in sEVs (*x* axis) and in first day EV (*y* axis). (C) Spot diagram on proteins identified by MS ranked according their abundance in sEVs (*x* axis) and aEVs (*y* axis). (D) Heat diagram of each R^2 value determined by protein rank correlation (shown in B and C).

R^2	aEV	sEV	1st day EV	2nd day EV	3rd day EV
aEV	1	0.52	0.4	0.44	0.3
sEV	0.52	1	0.62	0.59	0.63
1st day EV	0.4	0.62	1	0.81	0.73
2nd day EV	0.44	0.59	0.81	1	0.78
3rd day EV	0.3	0.63	0.73	0.78	1

Next, we investigated the number and overall composition of EVs produced in short (30 min) or longer term incubations in cell culture medium. The number of EVs carrying the CD11b marker (detected by anti-CD11b-RPE, 1 μg/ml, Dako, Glostrup, Denmark) increased dramatically as the count of healthy PMNs decreased (Fig. 1B). On the third day, EVs outnumbered the initial PMN count 10-fold. The protein content of spontaneously formed vesicles was about 10 times higher after 1 d (first day EVs) than after 30 min of incubation (sEVs) but did not further increase on the subsequent days (Fig. 1C). Accordingly, the protein content per vesicle showed a significant decrease from sEVs to EVs released during 3 d of culture (Fig. 1D). aEVs were significantly more numerous and contained more protein than sEVs did, although both vesicles were generated in 30 min (Fig. 1C). The proportion of EVs that were able to bind annexin V was similar in all the investigated vesicle populations (data not shown).

Morphologic properties of different PMN-derived EV populations

The size of different PMN-derived EV populations was first investigated by DLS (**Fig. 2A**). The 2 populations of EVs formed in short incubation (sEVs and aEVs) did not differ in size, and they showed 2 maximums around 100 and 500 nm. The size scattering distribution curve for EVs issued from spontaneously dying cells was shifted to the right, and the peaks were around 200 and 800 nm.

Next, we investigated the EVs using electron microscopy (Fig. 2B–F). All the fractions contained heterogeneously size vesicles surrounded by intact membrane. However, differences were found in the density of the vesicle content. The frequency of densely packed vesicles was the highest in the aEV fraction (Fig. 2B). This is in accordance with their high protein content per vesicle. EVs generated by spontaneously dying PMNs exhibited increasing number of empty looking large vesicles (Fig. 2D–F), consistent with the decreasing protein content per vesicle (Fig. 1D).

Composition of different PMN-derived EV populations

Our detailed proteomic analysis identified 798 unique proteins with 2 peptides and 95% confidence (Supplemental Table 1). First, we investigated protein overlap among the 3 most characteristic populations: aEVs, sEVs, and first day of spontaneous death (**Fig. 3A**). A total of 274 proteins were present in all 3 populations. In addition, 324 proteins were shared between sEVs and first day EVs, the 2 types of spontaneously formed EVs. Thus, a total of 598 proteins were shared by sEVs and first day EVs. In contrast, aEVs shared only 10 additional proteins with sEVs (for a total of 284 common proteins) and 7 with the first day EV population (for a total of 281 common proteins). The first day EV fraction contained 112 unique proteins, but only 7 proteins were specifically distributed to aEVs.

Next, we listed the proteins in all 5 populations in decreasing order of abundance. Figure 3B and C present a graphic representation of that distribution. The comparison of the abundance rank between the sEVs and first day EVs (2 populations of spontaneously formed EVs) gave a symmetrical distribution, suggesting that the 2-vesicle populations contained the same proteins in similar relative quantities. In contrast, comparing the abundance rank of aEVs with that of sEVs (2 populations of short-term generated EVs) resulted in an asymmetrical distribution, indicating significant differences in the relative amount of constituting proteins.

Similar graphs were created for each possible pair of vesicle populations, and the obtained square of the coefficients of multiple correlation (R^2) are summarized in Fig. 3D. The mathematical analysis clearly showed that the protein pattern is more similar among all the EV populations formed spontaneously than among any spontaneously formed vesicle population and the activation-induced aEV population.

Next, we compared the amount of specific proteins with distinct localization to neutrophils and the role in PMN functions among the 5 EV populations (**Fig. 4** and **Table 1**). We have previously reported that granule proteins were enriched in aEVs compared with sEVs [25]. The present study confirmed the difference in EV expression of granule proteins between aEVs and sEVs (Fig. 4A and B). Surprisingly, the amount of granule proteins was also increased in EVs released from spontaneously dying cells (Fig. 4A). In Western blots, an increase in the amount of lactoferrin could be detected, but the amount of actin did not change significantly, resulting in a clear and gradual increase in

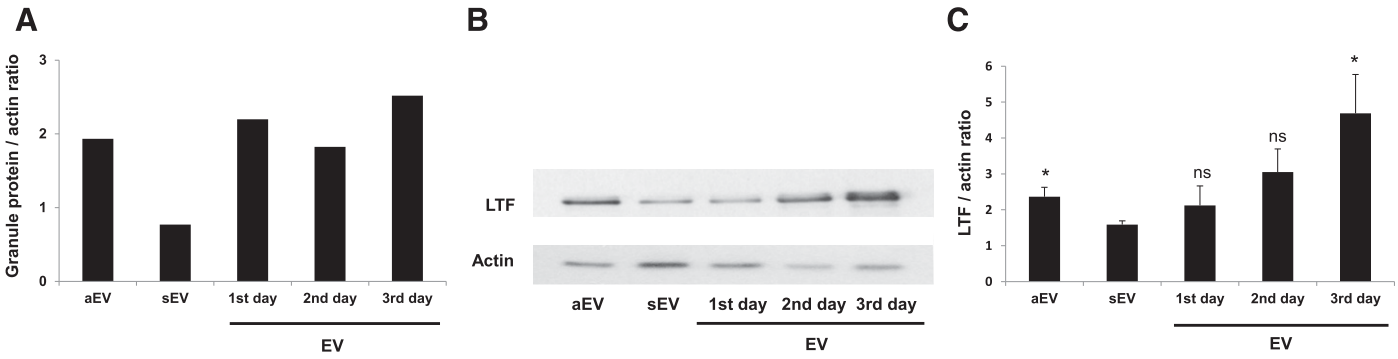

Figure 4. Analysis of specific proteins in different vesicle populations. (A) Amount (emPAI values) of all identified granule proteins related to actin. The results were based on statistical evaluation of relative protein expression of proteomic analysis of 1 set of EVs. (B) Representative Western blot of lactoferrin and actin. (C) Densitometric analysis of lactoferrin related to actin (±SEM; $n = 4$; *$P < 0.05$ compared with sEV; ns, nonsignificant).

TABLE 1. emPAI values of NADPH oxidase subunits in different EV samples

Variable	gp91phox	p22phox	p67phox	p47phox	Rac1/2	p40phox
aEVs	0.77	0.78	0.12	—	4.96	—
sEVs	0.68	0.68	0.14	0.40	5.22	0.22
First day EVs	0.35	0.41	0.23	0.77	3.37	0.13
Second day EVs	0.57	1.89	0.32	0.93	5.06	0.09
Third day EVs	0.44	0.57	0.17	0.83	3.00	0.12

The results were based on statistical evaluation of relative protein expression of proteomic analysis of 1 set of EVs.

the lactoferrin/actin ratio from sEVs toward EVs released from spontaneously dying PMNs (Fig. 4B).

As another group of proteins with specific function, we investigated the presence of the subunits of the NADPH oxidase (Table 1). As previously observed, aEVs did not contain 2 essential cytosolic components ($p47^{phox}$ and $p40^{phox}$) [25]. In contrast, all the spontaneously formed EV populations contained all 6 subunits.

Finally, we assessed the nucleic acid content of the different vesicle types. As summarized in **Table 2**, all vesicle types contained RNA, although a 3-fold difference was found in the amount. In contrast, only vesicles from spontaneously dying cells contained a detectable amount of DNA.

Functional properties of different PMN-derived EV populations

Finally, we investigated 2 major PMN functions: superoxide production and impairment of bacterial growth (**Fig. 5**). In both cases, we tested intact neutrophils as a positive control. The strongest stimulation of superoxide production can be reached using the pharmacological agent phorbol myristate acetate. As demonstrated in Fig. 5A, even this agent was not able to induce any detectable superoxide generation in any of the EV populations. No superoxide was detectable with more sensitive, luminescent techniques, either (data not shown).

In the bacterial survival test, 18% of the initial bacteria survived in the presence of intact PMNs and ~60% in the presence of aEVs generated from the same number of PMNs (Fig. 5B). In contrast, sEVs did not impair bacterial survival, and bacteria even grew in the presence of EVs released from PMNs cultured for 3 d. Both the number and protein content of the applied "third day EV" fraction was significantly greater than that of the aEV fraction (Fig. 1C).

Next, we analyzed the relation of the antibacterial effect to the antimicrobial granule protein content of the different EV types. In Fig. 5C, bacterial survival is shown in the function of the total granule protein content obtained by proteomic analysis. In Fig. 5D, bacterial survival correlated with the lactoferrin content determined by immunoblotting. In both analyses, aEVs represent a clearly distinct population from all spontaneously formed EV types.

Taken together, our data, obtained on microvesicle size EVs released from PMN under different conditions, indicate that some properties, such as annexin V binding, RNA content, heterogeneous size distribution, absence of PI staining, and superoxide production, are similar in the investigated EV populations (Table 2). They also shared ~50% of the identified proteins. In addition to the similarities, important differences were found, in the morphologic properties, protein composition, and, most significantly, in the antibacterial capacity. Comparing the properties and composition of the 5 investigated EV populations indicates that the 4 spontaneously formed EV types are more similar to each other than any of them to the aEVs, the vesicles induced by receptor activation.

On the basis of these findings, we propose that microvesicle size EVs are formed from PMNs by (at least) 2 distinct mechanisms. One is a spontaneously propagating process that produces an increasing number of EVs with decreasing protein content per vesicle as the cells proceed to spontaneous death. The trigger or the mechanism of spontaneous vesicle formation is not known at present. It could be a neutrophil-specific property related to the short lifespan of these cells or to the

TABLE 2. Comparison of properties of different PMN-derived EV types

Variable	Mother cell staining	PS expression	PI staining	PMN specific markers	EM	Size	ROS production	Antibacterial effect	RNA ng/μl (n = 5; SEM)	DNA ng/μl (n = 3; SEM)
aEVs	$AnnV^-$ PI^-	+	−	+	Dens, intact	Peaks: 100, 500 nm	−	+	25.7 ± 4.21	ND
sEVs	$AnnV^-$ PI^-	+	−	+	Dens, intact	Peaks: 100, 500 nm	−	−	22.83 ± 10.46	ND
Third day EVs	$AnnV^+$ PI^+	+	−	+	Empty, intact	Peaks: 200, 800 nm	−	−	66.26 ± 14.79	1.35 ± 0.87

AnnV, annexin; Dens, high electron density; ND, not detected.

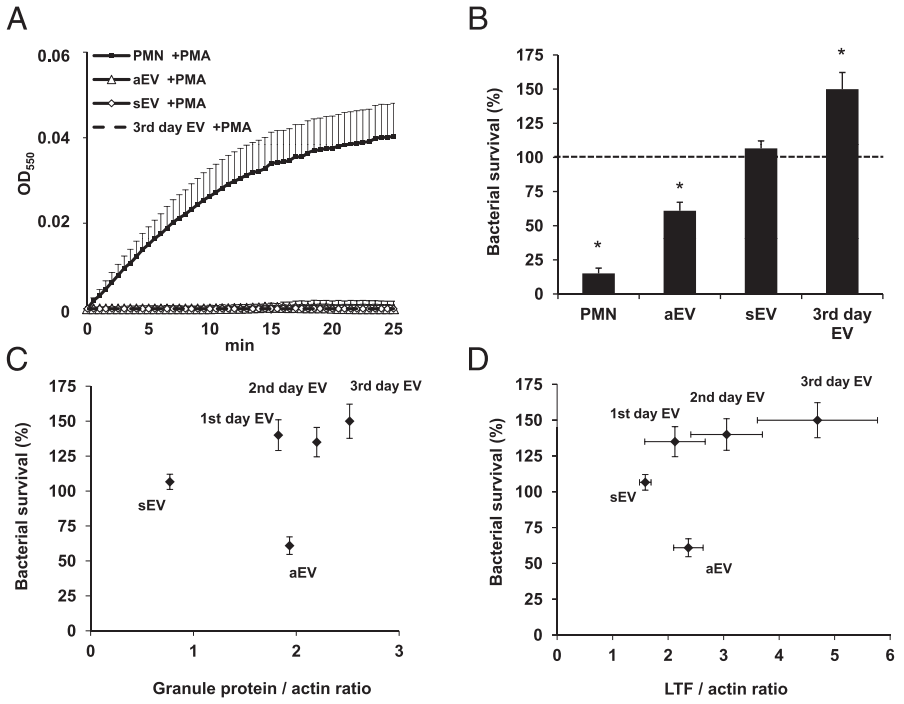

Figure 5. Functional properties of different vesicle populations. (A) Superoxide production of EVs and control PMNs after PMA stimulation. (B) Effect of EVs and PMNs on bacterial growth after a 40 min coincubation; 100% represents the initial bacterial count ($n = 4$ or $n = 8$ in the case of third day EVs ± SEM; $^*P < 0.05$ compared with sEV; ns, nonsignificant). (C) Correlation between bacterial survival and granule protein/actin ratio of the proteomic analysis. (D) Correlation between bacterial survival and lactoferrin/actin ratio of Western blot densitometry.

heterogeneity of the age of the circulating cells or to priming effects. The spontaneously formed vesicles have no antibacterial function. The other mechanism is initiated by opsonin receptors and produces EVs with high protein content and definitive antibacterial capacity. As shown previously [25], the antibacterial capacity of aEVs is exhausted at a significantly lower bacterial load than that of PMNs; however, under certain conditions, aEVs can be similarly effective or even better than PMNs themselves. In our proteomic analysis, we revealed only a few proteins with a low abundance that were specific to aEVs. In contrast, 290 proteins were shared with spontaneously formed vesicles (Fig. 3A). However, significant differences were found in the abundance of these shared proteins (Fig. 3B–D), suggesting a selective sorting mechanism [5, 35]. The importance of differential protein sorting in EV formation is supported by the surprising pattern of NADPH oxidase subunits. Despite the presence of membrane bound subunits and other cytosolic members, 2 essential cytosolic activators were completely missing from aEVs (Table 1). Furthermore, no ROS production in the spontaneously formed vesicles, perhaps because of inhomogeneous vesicle composition or the lack of signaling elements (Fig. 5A). This highlights how carefully ROS production is controlled, even during cell death [36]. Only aEVs were able to impair bacterial growth—despite the high granule protein content in EVs from spontaneously dying PMNs—clearly indicating that the quantity of granule proteins by itself does not determine the biologic activity of EVs (Fig. 5C and D). The large quantity of granule proteins in EVs produced during spontaneous death might be a mechanism for sequestered removal of the dangerous granule enzymes packed in vesicles with "eat me" signals as PS [15, 37].

Beyond the observations specifically relevant for neutrophilic granulocytes, our results have demonstrated that many easily and widely determined properties were similar between EVs formed in short-term incubations or during spontaneous cell death. The size distribution of vesicles released from cultured PMNs shifted toward higher values, supporting the notion that apoptotic vesicles are larger, but the size range was widely overlapping (Fig. 2A). The staining properties and nucleic acid content were similar, and a large number of proteins were shared (Fig. 3A and Table 2). Our data indicate that, similar to the exosome fraction [38], EVs sedimented in the microvesicle/ectosome fraction are also heterogeneous, and the boundary between specifically triggered EVs and apoptotic bodies is not sharp. On the basis of our results, The protein amount per vesicle, electron microscopic images, DNA content, and mainly specific biologic functions might be useful in distinguishing various EV types.

AUTHORSHIP

A.M.L. designed and performed most of the experiments, prepared the figures, and wrote part of the manuscript. M.S. participated in experiments determining the number, protein content, and antibacterial effect of different EV populations. C.I.T. established the methodology of EV preparation and detection of antibacterial effect. D.S.V. performed the DLS measurements. Á.K. performed the electron micrographs. K.R.M. analyzed proteomic data. M.L.M. performed mass spectrometry and analyzed proteomic data. E.L. directed and financed the experiments, organized discussions, wrote and edited the manuscript.

ACKNOWLEDGMENTS

The authors are indebted to Professor Edit Buzás, Mr. X. Osteikoetxea for stimulating discussions, and Ms. Regina Tóth-Kun

and Edit Fedina for devoted and expert technical assistance. The experimental work was financially supported by Hungarian Research Fund (OTKA K108382).

DISCLOSURES

The authors declare no competing financial interests.

REFERENCES

1. Théry, C., Zitvogel, L., Amigorena, S. (2002) Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* **2,** 569–579.
2. Raposo, G., Stoorvogel, W. (2013) Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* **200,** 373–383.
3. Mashburn, L. M., Whiteley, M. (2005) Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* **437,** 422–425.
4. Bobrie, A., Colombo, M., Raposo, G., Théry, C. (2011) Exosome secretion: molecular mechanisms and roles in immune responses. *Traffic* **12,** 1659–1668.
5. Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., Manel, N., Moita, L. F., Théry, C., Raposo, G. (2013) Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J. Cell Sci.* **126,** 5553–5565.
6. Colombo, M., Raposo, G., Théry, C. (2014) Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol.* **30,** 255–289.
7. Johnstone, R. M. (2006) Exosomes biological significance: a concise review. *Blood Cells Mol. Dis.* **36,** 315–321.
8. Buzas, E. I., György, B., Nagy, G., Falus, A., Gay, S. (2014) Emerging role of extracellular vesicles in inflammatory diseases. *Nat Rev Rheumatol* **10,** 356–364.
9. György, B., Szabó, T. G., Pásztói, M., Pál, Z., Misják, P., Aradi, B., László, V., Pállinger, E., Pap, E., Kittel, A., Nagy, G., Falus, A., Buzás, E. I. (2011) Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell. Mol. Life Sci.* **68,** 2667–2688.
10. Zwaal, R. F., Schroit, A. J. (1997) Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* **89,** 1121–1132.
11. Heijnen, H. F., Schiel, A. E., Fijnheer, R., Geuze, H. J., Sixma, J. J. (1999) Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* **94,** 3791–3799.
12. Crescitelli, R., Lasser, C., Szabo, T. G., Kittel, A., Eldh, M., Dianzani, I., Buzas, E. I., Lotvall, J. (2013) Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. J Extracell Vesicles 2, 20677.
13. Turiák, L., Misják, P., Szabó, T. G., Aradi, B., Pálóczi, K., Ozohanics, O., Drahos, L., Kittel, A., Falus, A., Buzás, E. I., Vékey, K. (2011) Proteomic characterization of thymocyte-derived microvesicles and apoptotic bodies in BALB/c mice. *J. Proteomics* **74,** 2025–2033.
14. Sadallah, S., Eken, C., Schifferli, J. A. (2011) Ectosomes as modulators of inflammation and immunity. *Clin. Exp. Immunol.* **163,** 26–32.
15. Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M., Haslett, C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J. Clin. Invest.* **83,** 865–875.
16. Luo, H. R., Loison, F. (2008) Constitutive neutrophil apoptosis: mechanisms and regulation. *Am. J. Hematol.* **83,** 288–295.
17. Savill, J., Haslett, C. (1995) Granulocyte clearance by apoptosis in the resolution of inflammation. *Semin. Cell Biol.* **6,** 385–393.
18. Tortorella, C., Piazzolla, G., Spaccavento, F., Pece, S., Jirillo, E., Antonaci, S. (1998) Spontaneous and Fas-induced apoptotic cell death in aged neutrophils. *J. Clin. Immunol.* **18,** 321–329.
19. Colotta, F., Re, F., Polentarutti, N., Sozzani, S., Mantovani, A. (1992) Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* **80,** 2012–2020.
20. McCracken, J. M., Allen, L. A. (2014) Regulation of human neutrophil apoptosis and lifespan in health and disease. *J Cell Death* **7,** 15–23.
21. Mesri, M., Altieri, D. C. (1999) Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. *J. Biol. Chem.* **274,** 23111–23118.
22. Mesri, M., Altieri, D. C. (1998) Endothelial cell activation by leukocyte microparticles. *J. Immunol.* **161,** 4382–4387.
23. Gasser, O., Schifferli, J. A. (2004) Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* **104,** 2543–2548.
24. Gasser, O., Hess, C., Miot, S., Deon, C., Sanchez, J. C., Schifferli, J. A. (2003) Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp. Cell Res.* **285,** 243–257.
25. Timár, C. I., Lorincz, A. M., Csépányi-Kömi, R., Vályi-Nagy, A., Nagy, G., Buzás, E. I., Iványi, Z., Kittel, A., Powell, D. W., McLeish, K. R., Ligeti, E. (2013) Antibacterial effect of microvesicles released from human neutrophilic granulocytes. *Blood* **121,** 510–518.
26. Dalli, J., Montero-Melendez, T., Norling, L. V., Yin, X., Hinds, C., Haskard, D., Mayr, M., Perretti, M. (2013) Heterogeneity in neutrophil microparticles reveals distinct proteome and functional properties. *Mol. Cell. Proteomics* **12,** 2205–2219.
27. Rada, B. K., Geiszt, M., Káldi, K., Timár, C., Ligeti, E. (2004) Dual role of phagocytic NADPH oxidase in bacterial killing. *Blood* **104,** 2947–2953.
28. Lorincz, A. M., Timar, C. I., Marosvari, K. A., Veres, D. S., Otrokocsi, L., Kittel, A., Ligeti, E. (2014) Effect of storage on physical and functional properties of extracellular vesicles derived from neutrophilic granulocytes. *J. Extracell. Vesicles* **3**, 25465.
29. Uriarte, S. M., Rane, M. J., Merchant, M. L., Jin, S., Lentsch, A. B., Ward, R. A., McLeish, K. R. (2013) Inhibition of neutrophil exocytosis ameliorates acute lung injury in rats. *Shock* **39,** 286–292.
30. Wiśniewski, J. R., Zougman, A., Nagaraj, N., Mann, M. (2009) Universal sample preparation method for proteome analysis. *Nat. Methods* **6,** 359–362.
31. Baba, S. P., Hoetker, J. D., Merchant, M., Klein, J. B., Cai, J., Barski, O. A., Conklin, D. J., Bhatnagar, A. (2013) Role of aldose reductase in the metabolism and detoxification of carnosine-acrolein conjugates. *J. Biol. Chem.* **288,** 28163–28179.
32. Keller, A., Nesvizhskii, A. I., Kolker, E., Aebersold, R. (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **74,** 5383–5392.
33. Uriarte, S. M., Powell, D. W., Luerman, G. C., Merchant, M. L., Cummins, T. D., Jog, N. R., Ward, R. A., McLeish, K. R. (2008) Comparison of proteins expressed on secretory vesicle membranes and plasma membranes of human neutrophils. *J. Immunol.* **180,** 5575–5581.
34. Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., Mann, M. (2005) Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol. Cell. Proteomics* **4,** 1265–1272.
35. Timar, C. I., Lorincz, A. M., Ligeti, E. (2013) Changing world of neutrophils. *Pflugers Arch.* **465**, 1521–1533.
36. Decoursey, T. E., Ligeti, E. (2005) Regulation and termination of NADPH oxidase activity. *Cell. Mol. Life Sci.* **62,** 2173–2193.
37. Erwig, L. P., Henson, P. M. (2008) Clearance of apoptotic cells by phagocytes. *Cell Death Differ.* **15,** 243–250.
38. Bobrie, A., Colombo, M., Krumeich, S., Raposo, G., Thery, C. (2012) Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation. *J. Extracell. Vesicles* **1**, 18397.

KEY WORDS:
apoptotic bodies · microvesicles · bacterial survival · spontaneous death · protein profile