Exosome secretion by eosinophils: A possible role in asthma pathogenesis

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Background: Eosinophils secrete several granules that are involved in the propagation of inflammatory responses in patients with pathologies such as asthma.
Objective: We hypothesized that some of these granules are exosomes, which, when transferred to the recipient cells, could modulate asthma progression.
Methods: Eosinophils were purified from peripheral blood and cultured with or without IFN-γ or eotaxin. Multivesicular bodies (MVBs) in eosinophils were studied by using fluorescence microscopy, transmission electron microscopy (TEM), and flow cytometry. Exosome secretion was measured and exosome characterization was performed with TEM, Western blotting, and NanoSight analysis.
Results: Generation of MVBs in eosinophils was confirmed by using fluorescence microscopy and flow cytometry and corroborated by means of TEM. Having established that eosinophils contain MVBs, our aim was to demonstrate that eosinophils secrete exosomes. To do this, we purified exosomes from culture medium of eosinophils and characterized them. Using Western blot analysis, we demonstrated that eosinophils secreted exosomes and that the discharge of exosomes to extracellular media increases after IFN-γ stimulation. We measured exosome size and quantified exosome production from healthy and asthmatic subjects using nanotracking analysis. We found that exosome production was augmented in asthmatic patients.
Conclusion: Our findings are the first to demonstrate that eosinophils contain functional MVBs and secrete exosomes and that their secretion is increased in asthmatic patients. Thus exosomes might play an important role in the progression of asthma and eventually be considered a biomarker. (J Allergy Clin Immunol 2015;135:1603-13.)

Key words: *Asthma, eosinophils, exosomes, multivesicular bodies, biomarker, IFN-γ, endosomes, lysobisphosphatidic acid, CD63, secretion*

Abbreviations used
ECP: Eosinophil cationic protein
EPO: Eeosinophil peroxidase
GFP: Green fluorescent protein
ILV: Intraluminal vesicle
LBPA: Lysobisphosphatidic acid
MBP: Major basic protein
MFI: Mean fluorescence intensity
MVB: Multivesicular body
PKC: Protein kinase C
TEM: Transmission electron microscopy
WB: Western blot

Eosinophils are recruited in large numbers at the site of allergic inflammation, parasitic infections, and other immune responses.[1] These immune leukocytes are not only destructive effectors cells but also active players in immune modulation, tissue repair processes, and normal organ development in both health and disease.[2-4]

Eosinophils have the capacity to synthesize, store within intracellular granules, and immediately secrete a diverse repertoire of cytokines, chemokines, and other important mediators. Secretory granules or vesicles of eosinophils are storage organelles in which molecules from the trans-Golgi network are concentrated and packaged. When appropriately stimulated, secretory granules are selectively mobilized to the plasma membrane. Once there, they undergo complex fusion events to secrete their content to the extracellular space.[5,6]

From the trans-Golgi network, the endosomal system controls the uptake and processing of various macromolecules. Most cells have vesicular organelles, which are interconnected and consist basically of primary endosomes, late endosomes, and lysosomes.[7] $CD63^+$ late endosomes generate intraluminal vesicles (ILVs) rich in lysobisphosphatidic acid (LBPA) into their lumen in a process called maturation. Through this process, late endosomes are transformed into mature $CD63^+/LBPA^+$ multivesicular bodies (MVBs).[8] MVBs not only fuse to lysosomes to degrade their intraluminal cargo but also fuse to the plasma membrane to release ILVs into the extracellular space as exosomes.[7,9] Exosomes are small vesicles that contain bioactive lipids, nucleic acid, and proteins, which are delivered to different locations in the body. One of the most important functions of exosomes appears to be intercellular communication.[10] Exosomes are secreted, constitutively and on stimulation, by different types of cells, and thus their composition differs depending on their cellular origin. They also have specific molecules related to their biogenesis, which allow their characterization (exocarta.org/exosome_markers). Exosomes have been defined by their size, density, and expression of specific biomarkers (eg, tetraspanins).[11] Exosomes can be found in abundance in body fluids,

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such as blood, saliva, urine, breast milk, and bronchoalveolar lavage fluid.[12-17] Thus the exosomal pathway constitutes a mechanism for local and systemic intercellular transfer of information.[10] Because of their abundance and unique composition, they might represent ideal biomarkers for diagnosis and prognosis of a wide variety of diseases.[18]

Asthma is a common chronic inflammatory disorder characterized by variable airflow obstruction in association with airway hyperresponsiveness. The release of potent inflammatory mediators and the remodeling of the airway wall promote airway dysfunction.[19] The late/chronic phase is mainly attributed to the effects of infiltrating eosinophils and their derivatives.[20]

Exosomes released from both immune and structural cells in the lung[21,22] have recently been shown to play an important role in local regulation of asthma pathology.[23] Moreover, exosomes from asthmatic patients differ in quantity, composition, and function compared with those from healthy subjects,[24] which demonstrates the implication of exosomes in regulation of asthma pathology. Therefore it is important to discern which cell types are involved in exosome production.[25]

Recent studies have found vesicles produced by eosinophils in the extracellular space functioning autonomously and independently outside of eosinophils.[26,27] It is possible that these vesicles from eosinophils participate in the exacerbation of asthma. Our study was based on the theory that some of those vesicles could be eosinophil exosomes with a significant implication in asthma progression.

METHODS

Sample collection and eosinophil isolation

Samples of peripheral blood (50 mL) from asthmatic patients (n = 20) and healthy subjects (n = 10) were obtained voluntarily and after obtaining written informed consent. Patients with asthma had a consistent history of the disease and objective evidence of asthma (as defined by the American Thoracic Society)[28] for at least 6 months. These patients either showed a greater than 12% improvement in FEV_1 10 minutes after administration of 500 μg of inhaled terbutaline or had methacholine airway hyperresponsiveness (PC_{20} methacholine <16 mg/mL). Asthmatic patients had mild persistent disease[29] and were clinically stable. None had a history of respiratory tract infections for at least the 6-week period preceding the study. We included both atopic and nonatopic patients in the asthmatic group because no differences in the parameters assessed for both sets of patients had been observed previously. For patients who were receiving inhaled corticosteroids, the drugs were withdrawn for at least 2 weeks before blood extraction. No patient was receiving oral corticosteroids (for at least 6 months before the study), leukotriene receptor antagonists, aspirin, or any other COX inhibitor.

Eosinophils were purified from the peripheral blood of healthy control subject and patient donors by using a 2-step procedure, as previously described.[30] Briefly, the polymorphonuclear cell fraction was obtained by using Ficoll gradient centrifugation. The second step involved removal of residual cells from the polymorphonuclear cell fraction. To purify eosinophil, $CD2^+$, $CD3^+$, $CD14^+$, $CD16^+$, $CD19^+$, $CD20^+$, $CD36^+$, $CD56^+$, $CD123^+$, and glycophorin A–positive cells were discarded by using a magnetic bead separation technique, as described in the manufacturer's instructions (EasySep; StemCell Technologies, Vancouver, British Columbia, Canada). Eosinophil viability and purity were routinely greater than 98%. Purified eosinophils were suspended in RMPI-1640 medium (Sigma-Aldrich, St Louis, Mo) supplemented with 0.1 mmol/L nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L HEPES, 2 mmol/L L-glutamine, and 10% (vol/vol) FBS (Lonza, Basel, Switzerland), which had been stripped of bovine exosomes by means of ultracentrifugation. The culture medium was also supplemented with a cocktail of IL-5 (5 ng/mL) and GM-CSF (10 ng/mL). The culture cells were maintained at 37°C in a 5% CO_2 atmosphere for 24 hours.

Transfection assays and expression vectors

Eosinophils were transiently transfected with 20 μg of the pEFGFP-C1bosCD63[31] plasmid in a total volume of 650 μL. The culture was maintained at 37°C in a 5% CO_2 atmosphere for 24 hours. Routine transfection efficiency was around 75%.

Time-lapse assays

At 24 hours after transfection, eosinophils were attached to glass-bottom 35-mm culture dishes (MatTek, Ashland, Mass) coated with fibronectin. Once the cells were placed in the microscope chamber, eosinophils were stimulated with 20 ng/mL IFN-γ (R&D Systems, Minneapolis, Minn) in culture medium and maintained in an automatic OKO Lab System (Okolab SRL, Naples, Italy) that controls CO_2 levels, temperature, and humidity.[32] Subsequently, epifluorescent images were taken with a Nikon Eclipse TE2000S microscope equipped with a DS-Qi1Mc digital camera and a Plan Apo VC 60 NA 1.4 objective. Time-lapse analysis was performed in living cells by using NIS-AR software (Nikon Instruments, Melville, NY). Time-lapse images were homogeneously taken every minute by using a band pass–specific filter for green fluorescent protein (GFP) to avoid fluorescence quenching with a 1-second exposure time. Sequence image acquisition was coordinated with an automatic fluorescence shutter to avoid fluorescence bleaching. Epifluorescent image improvement was achieved by using Huygens deconvolution software (SVI, Hilversum, The Netherlands).

Fluorescence and confocal laser-scanning microscopy

After 24 hours of culture, the samples were fixed and intracellularly labeled, as described previously,[32] with anti-tetraspanin CD63 (Immunostep S.L., Salamanca, Spain) primary antibody, a reporter of endosomal vesicles, and anti-LBPA primary antibody (Echelon Biosciences, Salt Lake City, Utah), which is a specific marker of MVBs,[33] and an appropriate secondary fluorescein isothiocyanate (BD Biosciences, Franklin Lakes, NJ) and Alexa Fluor 647–conjugated antibody (Invitrogen, Madison, Wis). Epifluorescent images were taken with the same microscope and digital camera described for time-lapse analysis. For quantification, digital images were analyzed with NIS-AR software (Nikon Instruments). The experimental significance of the results obtained at the single-cell level was achieved by analyzing a minimum number of 50 cells per treatment from different microscope fields. Results were expressed as the mean of sum intensity LBPA fluorescence/area of the cells. Confocal microscopy was performed with a Leica TCS SP5 scan head mounted on a Leica microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) and a 63 NA 1.4 Plan Apo objective. Images of the cells were acquired from a 0.7-μm optical section, and no labeling was observed when using the secondary antibody alone. Merged images were performed, taking the z axis maximum intensity projection (which takes the intensity values of individual pixels in all sections and collapses them into a single illuminated image). ImageJ software was used for analysis (National Institutes of Health, Bethesda, Md; http://rsb.info.nih.gov/ij),[34] and colocalization indexes were analyzed with the JACoP plugin for ImageJ software.

Induction of exosome secretion and inhibition assay

For the induction of exosome secretion, 2×10^6 eosinophils per well were treated with or without 20 ng/mL IFN-γ (R&D Systems) for 10 minutes and with or without 100 ng/mL eotaxin (R&D Systems) for 1 hour. Inhibition assays were performed by adding 2 μmol/L protein kinase C (PKC) inhibitor (Gö6985) for 20 minutes before IFN-γ treatment.

Exosome purification

Exosomes produced by an equal number of cells per each experimental condition were isolated from the cell-culture supernatants, as previously