The Laryngoscope

Exosomes Mediate Interepithelial Transfer of Functional P-Glycoprotein in Chronic Rhinosinusitis With Nasal Polyps

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Objective: P-glycoprotein (P-gp) drives type-2 helper T-cell inflammation in chronic rhinosinusitis with nasal polyps (CRSwNP) through unknown posttranslational mechanisms of overexpression. A recent randomized clinical trial demonstrated that inhibition of P-gp was as effective as oral steroids and biologics in treating CRSwNP. Exosomes are 30- to 150-nm vesicles capable of intercellular membrane protein transfer. The aims of this study were 1) to determine whether CRSwNP mucus exosomes are enriched with P-gp, and 2) whether exosomal P-gp can be functionally transferred to autologous epithelial cells as a putative mechanism for the proinflammatory overexpression of P-gp in CRSwNP.

Study Design: Institutional review board-approved study in CRSwNP and control patients (n = 10 per group).

Methods: P-gp content of purified mucus exosomes was characterized by transmission electron microscopy and enzyme-linked immunosorbent assay. Epithelial transfer of exosomal P-gp was determined by time-lapse fluorescent microscopy and calcein acetoxymethylester functional P-gp assay.

Results: CD63+/P-gp+ exosomes were detected in both groups. P-gp was significantly enriched in CRSwNP exosomes relative to control (median 198.5; interquartile range 123.6–270.5 vs. 74.4; 41.3–95.0 pcg P-gp/10^9 exosomes, $P = 0.002$). Exosomes were absorbed by epithelial cells within 10 minutes, resulting in a significant increase in P-gp activity in CRSwNP patients relative to control ($P = 0.006$).

Conclusion: Here we demonstrate the presence and P-gp enrichment of mucus-derived exosomes, or *rhinosomes*, in CRSwNP. These rhinosomes are capable of rapid intercellular transfer of P-gp, leading to increased P-gp function within recipient cells. This represents a novel mechanism for maintaining P-gp overexpression in CRSwNP, and more generally for interepithelial transfer of other proteins between mucosal epithelial cells.

Key Words: Chronic rhinosinusitis with nasal polyps, epithelium, exosome, P-glycoprotein, sinonasal mucus, rhinosome.

Level of Evidence: NA.

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INTRODUCTION

Permeability-glycoprotein(P-gp) is a membrane-bound efflux pump encoded by the multidrug resistance 1 gene located on chromosome 7q21.12.[1] P-gp–mediated transport has been observed in the regulation of cytokine secretion in multiple human cell types,[2,3] implicating a potential immunomodulatory role. Multiple studies examining P-gp function within sinonasal mucosa have shown that this function can regulate the secretion of type 2 helper T-cell (Th2)-associated cytokines in a concentration-dependent manner.[3–5] Epithelial P-gp protein further has been shown to be overexpressed in patients with chronic rhinosinusitis with nasal polyps (CRSwNP),[6–8] suggesting a key role in the immunopathophysiology of Th2-mediated sinonasal inflammation. This role was confirmed by a recent, double-blind randomized placebo-controlled clinical trial (DBRCT) demonstrating that P-gp inhibition was as effective as oral steroids and biologic agents in controlling both subjective and objective measures of CRSwNP.[9] P-gp upregulation in CRSwNP has been shown to be post-translational,[5] possibly involving a secreted isoform[8]; however, the mechanism responsible for maintaining the field effect of P-gp overexpression remains unclear.

Exosomes are 30- to 150-nm vesicles, surrounded by a lipid bilayer, that have a density of 1.13 to 1.19 g/mL. Exosomes have been detected in a wide range of body fluids, including blood, lymph, cerebrospinal fluid, urine, and nasal lavage fluid.[10–12] Biophysically, exosomes are equivalent to cytoplasm enclosed in a lipid bilayer, with the external domains of transmembrane proteins exposed to the extracellular environment.[13] The biogenesis of exosomes is controlled by the endosomal sorting complex required for transport. These events lead to the development of late endosome/multivesicular bodies, which can

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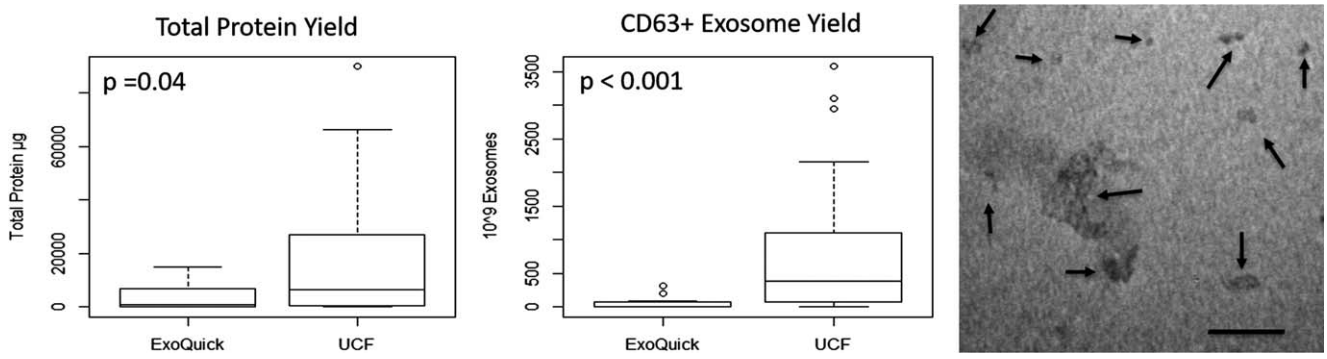

Fig. 1. Comparison of protein and CD63$^+$ exosome concentration by isolation method (UCF; ExoQuick: commercially available precipitation method [System Biosciences, Palo Alto, CA]) demonstrating enhanced yield using UCF. TEM image (right) demonstrating proteinacious debris (black arrows, bar 100 nm) contamination using the ExoQuick (System Biosciences) isolation method.
TEM = transmission electron microscopy; UCF = ultracentrifugation.

then be recycled back into the plasma membrane and released as exosomes. As a result of this process, exosomes become strongly enriched in markers, including the tetraspanins CD63, CD9, CD81, and CD82, which can be used to detect their presence and quantity.[10] Exosomes can transport a wide range of cargo, including growth factors and their receptors, DNA, mRNA, and microRNA. Further studies have demonstrated that exosomes are able to shuttle this cargo, including integral membrane proteins such as the chemokine receptor CCR5,[14] to adjacent cells. Exosome-mediated transfer of functional P-gp also has been previously demonstrated in several cell populations, including the MCF-7 human breast cancer cell line[15] and the CCRF-CEM human acute lymphoblastic leukemia cell line.[16]

Based these previous studies, we hypothesized that 1) exosomes would be detectable in human nasal mucus; 2) exosomes purified from patients with CRSwNP would be enriched with P-gp; and 3) CRSwNP-derived exosomes would be able to transferring functional P-gp to autologous epithelial cells.

MATERIALS AND METHODS

Sinonasal Mucosa and Mucus Sampling

Patient consent for tissue and mucus sampling was approved by the Massachusetts Eye and Ear Infirmary Institutional Review Board. All samples were taken from patients undergoing sinonasal surgery and had not been exposed to antibiotics or steroids for at least 4 weeks prior to harvest. Inclusion criteria included patients diagnosed with CRSwNP by European Position Paper on Rhinosinusitis and Nasal Polyps[17] criteria and healthy patients (i.e., controls, n = 10 per group) without CRS undergoing endoscopic sinus surgery for orbital or skull base pathologies. CRS was ruled out by history, endoscopy, and imaging in control patients. Exclusion criteria included ciliary dysfunction, autoimmune disease, cystic fibrosis, immunodeficiency, or smoking. Allergy was diagnosed by skin prick or allergen-specific IgE in addition to history. Aspirin-exacerbated respiratory disease was diagnosed by aspirin challenge in all positive patients by the referring allergist. Mucus samples were taken from the middle meatus by placing a compressed polyvinyl alcohol sponge (PVA) (Medtronic, Minneapolis, MN) against the ethmoid bulla for 5 minutes, taking care not to abrade the mucosa or contaminate the sponge with blood. Mucosal samples were then taken from the ethmoid bulla.

Exosome Purification From Whole Mucus

The exosome purification procedure was adapted from the ultracentrifugation (UCF) procedure described by Théry et al.[18] This technique was compared with a commercially available precipitation method (ExoQuick, System Biosciences, Palo Alto, CA) and provided greater purity with higher protein and exosome yield in agreement with van Deun et al.[19] (Fig. 1). Mucus samples were extracted from the PVA sponge by centrifugation (1500 g at 4°C for 30 minutes). The mucus was then diluted in 150 μL of 1× phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA) with Protease Inhibitor Cocktail (1:100, Sigma, St. Louis, MO). Cellular debris was pelleted by centrifugation at 45 minutes at 12,000 × g at 4°C. The supernatant was then suspended in 4.5 mL of PBS in polypropylene tubes (Thinwall, 5.0 mL, 13 × 51 mm, Beckman Coulter, Indianapolis, IN) and ultracentrifuged for 2 hours at 110,000 × g at 4°C. The supernatant was collected, and the pellet was resuspended in 4.5 mL 1× PBS. The suspension was filtered through a 0.22-μm filter (Fisher Scientific, Pittsburgh, PA) and collected in a fresh ultracentrifuge tube. The filtered suspension was then centrifuged for 70 minutes at 110,000 × g at 4°C. The supernatant was collected, and the pellet was resuspended in 200 μL PBS with protease inhibitor. Prior to cell culture dosing, the exosome concentration of each pellet was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) for the established exosome markers CD63 and CD9 (ExoELISA, System Biosciences, Palo Alto, CA), as previously described.[20]

Transmission Electron Microscopy of Mucus-Derived Exosomes

The exosome transmission electron microscopy (TEM) procedure was adapted from Théry et al.[18] Isolated exosomes were fixed for 1 hour at room temperature in 2% paraformaldehyde in 0.1 M sodium phosphate buffer (Electron Microscopy Sciences, Hatfield, PA). Next, 5 μL of the exosomes were absorbed onto Formvar/carbon-coated electron microscopy grids (Electron Microscopy Sciences) for 20 minutes. After absorption, the grids were rinsed in PBS three times and then transferred to PBS/50 mM glycine (Sigma Aldrich, St. Louis MO) for four washes. The grids were blocked in 5% bovine serum albumin (BSA) (Fisher Scientific) in 1x phosphate-buffered saline (buffer) for 10

TABLE I.
Patient Demographics.

	Control (n = 10)	CRSwNP (n = 10)	*P* Values
Age-Median Years (interquartile range)	57 (24.8–70.5)	53.5 (40.3–60.8)	0.55
Sex			
Male	2 (20%)	6 (60%)	0.67
Female	8 (80%)	4 (40%)	
Ethnicity			
African American	1 (10%)	0 (0%)	0.46
Asian	1 (10%)	3 (30%)	
Caucasian	7 (70%)	5 (50%)	
Hispanic	1 (10%)	2 (20%)	
Asthma	2 (20%)	5 (50%)	0.67
Allergy	4 (40%)	3 (30%)	0.67
AERD	0 (0%)	1 (10%)	0.67

AERD = aspirin exacerbated respiratory disease; CRSwNP = chronic rhinosinusitis with nasal polyps.

minutes at room temperature. The grids were incubated at 4°C overnight in the primary antibody (1:25, Purified Mouse Anti-Human CD63 Clone H5C6, BD Biosciences) diluted in 1% BSA buffer. Next, the grids were rinsed in 0.1% BSA buffer and then rinsed in 0.5% BSA buffer six times each. The secondary Protein-G antibody (1:20 in 1% BSA buffer, EM grade, 10 nm, Electron Microscopy Services, Hatfield, PA) in 5% BSA buffer was applied for 1 hour at room temperature and rinsed eight times with 1× PBS. The grids were incubated in 1% glutaraldehyde in 0.1 M sodium phosphate buffer (Electron Microscopy Services) for 5 minutes. After rinsing eight times in deionized water, the grids were contrasted in uranyl-oxalate solution, pH 7 (Uranyl Acetate (UA), Electron Microscopy Services) for 5 minutes. The grids were blotted on filter paper and air-dried prior to imaging. The exosomes were observed using a FEI Tecnai G2 Spirit transmission electron microscope (FEI, Hillsboro, Oregon) at an accelerating voltage of 100 kV interfaced with an AMT XR41 digital CCD camera (Advanced Microscopy Techniques, Woburn, MA) for digital TIFF file image acquisition. Rabbit IgG (Vector Laboratories, Burlingame, CA) and CD63 lysate (Novus Biologicals CD63 Overexpression Lysate [Native], Fisher Scientific) were used as negative and positive controls, respectively.

In Vivo Quantification of Mucus-Derived Exosomal P-gp Concentration

Mucus was collected from both control and CRSwNP patients for in vivo characterization of exosomal P-gp concentration (see Table I). The mucus was collected using a PVA sponge followed by exosome purification, as described above. The purified exosome fraction was subjected to P-gp, CD63, and CD9 (Systems Bioscience) ELISAs to determine the relative P-gp concentration within the purified exosomal fraction. All values were normalized to the total protein concentration within the same sample using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL).

Primary Human Sinonasal Epithelial Cell Culture

Human sinonasal epithelial cell cultures (HSNECCs) were grown as previously described.[21] Briefly, mucosal biopsy samples were washed and digested in Pronase for 90 minutes at 37°C. Cell suspensions were separated from particulate matter by centrifugation and resuspended in bronchial epithelial growth media (BEGM) (Lonza, Basel, Switzerland). Cells were plated for 2 hours on standard tissue culture plates to remove contaminating fibroblasts. Cells were then expanded for 3 to 5 days on human collagen type IV-coated (collagen from human placenta, Bornstein and Traub Type IV, Sigma Aldrich, St. Louis, MO) 75 cm^2 dishes (Corning Life Sciences, Corning, NY). Once confluent, the HSNECCs were trypsinized and re-seeded evenly in human collagen type IV-coated black walled 96-well (Corning 96-well Plates) tissue culture plates in BEGM and grown to 80% confluence prior to analysis. All in vitro experiments were associated with less than 20% cytotoxicity, as determined by the ReadyProbes Cell Viability Imaging Kit (Blue/Green, Life Technologies, Carlsbad, CA) (see Supporting Fig. S1).

Determination of In Vitro Internalization of Mucus-Derived Exosomes by HSNECCs

Autologous isolated exosomes were dyed using 10× commercially available Exo-Red Acridine Orange (AO) and Exo-Green carboxyfluorescein succinimidyl diacetate ester fluorescent labels (Systems Bioscience), according to the manufacturer's instructions, to characterize RNA and protein (respectively) internalization into HSNECCs derived from the same patient. After labeling, 25 μL of the purified autologous exosomes (1.25×10^9 exosomes/mL) were added to the HSNECCs in a black walled 96-well plate. The wells were imaged every 10 minutes for 30 minutes using a Leica DM IL LED fluorescent microscope (Leica, Buffalo Grove, IL) and a 20× objective. The same field of view was used for all time points.

Quantification of Exosome-Mediated Transfer of Functional P-gp

A calcein acetoxymethylester (AM) assay[22] was performed on the HSNEC cultures following dosing with autologous purified mucus-derived exosomes to quantify the relative acquisition of functional P-gp activity. HSNECCs were exposed to BEGM-containing exosomes (1.25×10^9 exosomes/mL) or exosomes, along with 0.625 μM of the third-generation P-gp inhibitor Zosuquidar 3HCl (Medkoo, Chapel Hill, NC).[23] Prewarmed calcein AM (Life Technologies) was added to each well for a final concentration of 2.5 μM, as previously described.[22] After 15 minutes, each well was washed 3× with cold PBS, and the wells were imaged in triplicate. Fluorescence was quantified using the corrected total cell fluorescence method, as previously described by McCloy et al.[24] A reduction in calcein fluorescence corresponds to a gain in P-gp function, whereas an increase in fluorescence corresponds to successful inhibition.[22]

Statistics

Statistical analysis was performed using R v3.3.0. The Shapiro-Wilk test was used for assessing normality. For the nonparametric data, the Kruskal-Wallis test was used to examine statistical differences between multiple groups, and the Mann-Whitney rank sum test was used to examine statistical differences between two independent groups. Correlations were tested using the two-tailed Spearman's rank-order correlation. Values falling outside 1.5 times the interquartile range of their respective data set were considered outliers and indiscriminately excluded from analysis. Results were considered significant when a P value of ≤ 0.05 was obtained. All in vitro studies were performed in technical duplicates.

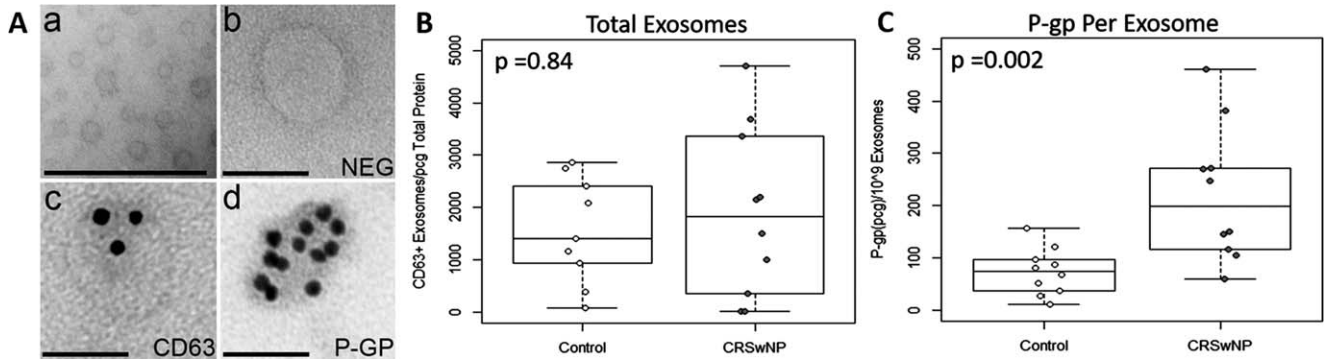

Fig. 2. Characterization of mucus-derived exosomes. (A) TEM images demonstrating (a) whole-mounted exosomes purified from nasal mucus (bar 500 nm); (b) negative control (bar 100 nm for b–d) confirming the typical exosome size and morphology; (c–d) immunogold labeling of exosome marker CD63 and P-gp localizing to the exosome membrane. (B) Scatter/boxplots (median and interquartile range; bars represent 1.5 times the interquartile range) demonstrating similar total exosome concentrations between CRSwNP and control patients. (C) Scatter/boxplots (median and interquartile range; bars represent 1.5 times the interquartile range) demonstrating a significantly higher P-gp per exosome concentration in CRSwNP patients relative to control.
CRSwNP = chronic rhinosinusitis with nasal polyps; P-gp = P-glycoprotein; TEM = transmission electron microscopy; UCF = ultracentrifugation.

RESULTS

Exosomes Are Present in Sinonasal Mucus and Are Enriched With P-gp Among Patients With CRSwNP

The first series of experiments were designed to quantify the presence of exosomes within nasal mucus and to characterize the relative abundance of exosomal P-gp by patient group. CD63 is one of the most commonly utilized exosome markers and correlated strongly with CD9, another exosome-associated tetraspanin (see Supporting Fig. S2). CD63 therefore was used as the primary exosome marker throughout the study.[10] Among both the control and CRSwNP-purified exosome fractions, we found structures corresponding to the expected 30- to 150-nm size and spheroid morphology of exosomes using TEM.[13] Subsequent immunogold labeling then confirmed the presence of both CD63 and P-gp epitopes within the exosome superstructure (Fig. 2A). Having confirmed the presence of P-gp–containing exosomes in nasal mucus by TEM, we next sought to determine whether there was a difference in secreted exosome volume or P-gp composition between the mucus of control and CRSwNP patients (n = 10 per group) (see Table I) by ELISA. We found no significant difference between median total exosome concentration between the CRSwNP (1831.0, interquartile range, IQR, 519.3–3073.4 exosomes/pcg total protein) and control group (1405.3, IQR 934.4–2403.7; $P = 0.84$) (Fig. 2B). However, among the CRSwNP patients, the median concentration of P-gp per exosome was significantly greater (198.5, IQR 123.6–270.5 pcg P-gp/10^9 exosomes) than that of the control patients (74.4, IQR 41.3–95.0, $P = 0.002$) (Fig. 2C).

Autologous Exosomes Are Capable of Transferring Functional P-gp to Cultured Sinonasal Epithelial Cells

After establishing the presence of P-gp within mucus-derived exosomes from both control and CRSwNP patients, we next sought to determine whether these exosomes could be transferred from the mucus to naïve cultured epithelial cells. After exposing the cells to purified autologous exosomes, we demonstrated a rapid and progressive uptake of labeled exosomal protein and RNA as early as 10 minutes (Fig. 3A). This time scale is consistent with the possibility of the interepithelial transfer of exosomes resulting from the physiologic mucociliary clearance transport. We next studied whether uptake of these exosomes was associated with the transfer of functional P-gp. Utilizing an established calcein AM P-gp activity assay,[22] we found that exosome exposure resulted in a significant reduction in calcein fluorescence, indicating a gain of P-gp function. The median reduction was significantly greater in the CRSwNP group (75.6% baseline, IQR 74.1%–81.5%) relative to the control (83.8% baseline, IQR 77.3%– 87.3%; $P = 0.007$), which is consistent with the relative enrichment of exosomal P-gp evident in the ELISA findings. Furthermore, this gain in function was abrogated by the addition of Zosuquidar, a highly potent and specific inhibitor of P-gp[22] (Fig. 2B), providing confirmatory evidence for the exosome-mediated transfer of functional P-gp.

DISCUSSION

Over the past several years, posttranslational P-glycoprotein overexpression has emerged as a significant feature of CRSwNP.[8] A recent DBRCT demonstrated that direct inhibition of P-gp using Verapamil hydrochloride in patients with CRSwNP led to a significant 28-point reduction in the 22-item Sinonasal Outcome Test,[25] as well improvements in radiologic (i.e., Lund-Mackay[26]) and endoscopic (i.e., Lund-Kennedy[27]) scores.[9] Because these improvements were commensurate with those reported in oral steroid and biologic therapies,[28,29] elaboration of the mechanisms of P-gp overexpression could yield deeper insights into the immunopathophysiology of CRSwNP, and thus lead to novel upstream therapeutic strategies targeting P-gp expression and exosome trafficking.

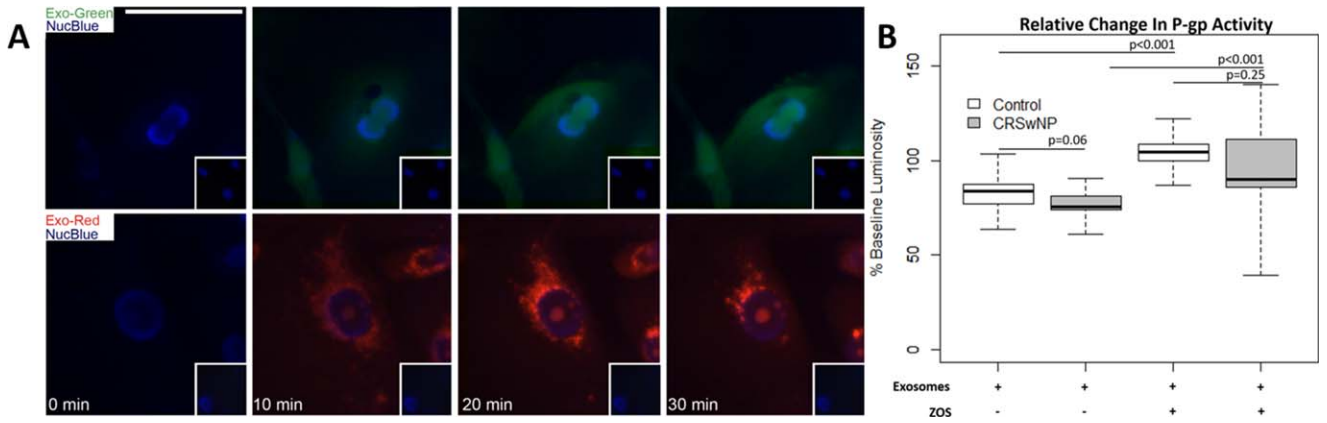

Fig. 3. Epithelial cell internalization of autologous exosomes. (A) Time-lapse fluorescent images of exosomal uptake by autologous epithelial cells (bar 50 μm; blue: nuclear stain, green: CFSE exosome protein stain, orange: AO exosome RNA stain). Within 10 minutes of exposure, the exosomes can be clearly seen within the membrane and cytoplasm of the cell. Over the subsequent 20 minutes, the protein signal spreads throughout the cell, whereas the exosomal RNA concentrates around the nucleus (insets represent time-matched unstained exosome negative controls). (B) Boxplot (median, interquartile range; bars represent 1.5 times the interquartile range) of Calcein AM fluorescence demonstrating a differential gain of P-gp function following exosome exposure in CRSwNP patients relative to control, which is abrogated by P-gp specific inhibition with Zosuquidar (Medkoo, Chapel Hill, NC).
AM = acetoxymethylester; AO = acridine orange; CFSE = carboxyfluorescein succinimidyl diacetate ester; CRSwNP = chronic rhinosinusitis with nasal polyps; P-gp = P-glycoprotein.

Our first set of experiments demonstrated that nasal mucus-derived exosomes are present in the sinonasal mucus of both healthy patients and those with CRSwNP. This confirms the findings of Wu et al.,[30] the only other group to demonstrate the presence of sinonasal mucus exosomes and report an upregulation in ATP-binding cassette (ABC) transporter pathway microRNAs present in allergic rhinitis. Using two complimentary analyses of TEM and ELISA, we were then able to confirm that P-gp protein is relatively enriched within the exosomes of patients with CRSwNP.

We next sought to determine whether these exosomes can transfer functional P-gp to cultured epithelial cells. Previous studies have demonstrated that major histocompatibility class II molecules may be expressed within the exosomal membrane.[20] Thus, to avoid any confounding immunogenic responses, purified exosomes only were exposed to epithelial cultures derived from the same patient. Our uptake studies demonstrated that epithelial cells were capable of avidly internalizing the exosomes into both their cell membrane and cytoplasm using separate exosomal protein and RNA tags, which is consistent with previous findings.[15] Furthermore, the rapidity of uptake suggests that in vivo interepithelial transfer of exosomes could occur as a consequence of physiologic mucociliary transport flow, which would otherwise clear the exosomes within 15 to 20 minutes.[31] We then used an established fluorescent P-gp activity assay[22] to demonstrate that this exosomal transfer was associated with a significant increase in P-gp activity within the recipient cells. P-gp acquisition via intercellular transfer previously has been described by several authors. Levchenko et al.[32] were among the first to demonstrate the transfer of P-gp between cells in a human neuroblastoma BE (2)-C cell line, rendering the recipient cells resistant to colchicine. Lv et al.[15] then expanded on this work, implicating exosomes as the probable mechanism of P-gp transfer in an MCF-7 breast cancer cell line leading to docetaxel resistance. These findings in cancer cell lines have been further confirmed by other studies.[33,34]

The P-gp activity assay demonstrated that the sequelae of exosome-mediated P-gp transfer was more pronounced in the CRSwNP group. The concentration of exposed exosomes was held constant, which links this effect to the higher P-gp/exosome concentration evident in the CRSwNP patients rather than an effect related to total exosome release. There are several strengths and weaknesses within this study that bear consideration. One principle strength is that this study is the first to demonstrate epithelial transfer of both autologous exosomes and their functional protein cargo within primary human samples. Secondly, this study provides novel evidence for the role of this mechanism in the maintenance of inflammation in CRS. A weakness of our study design is that it cannot distinguish whether the enhanced P-gp function resulted from direct P-gp transfer from donor exosomes or an increase in intrinsic P-gp activity. However, the rapid time-frame excludes the possibility of transcriptional upregulation as a mechanism, thereby favoring extragenetic protein transfer. Although this weakness could be addressed using immortalized cell lines, as previously reported,[15,32] the data would have far less clinical relevance than the results presented herein from primary patient samples. A second weakness is that our study design did not directly examine the impact of exosomal P-gp transfer on Th2 proinflammatory cytokine production. Flow cytometric and multiplexed ELISA-based studies to address this question are planned. Finally, although the control group consisted of healthy patients without CRS, 40 did have concomitant allergy, which may have unknown effects on exosomal transport of P-gp.

CONCLUSION

Taken as a whole, this study points to a novel mechanism for the maintenance of P-gp overexpression in CRSwNP. Our data suggests that exosomes within the nasal mucus of patients with CRSwNP are enriched with P-gp, which can be functionally transferred to adjacent epithelial cells through mucociliary flow. To differentiate these exosomes from other serum- and tissue-derived exosomes, we propose that nasal mucus-derived exosomes may be termed *rhinosomes*. These results not only suggest the potential for novel rhinosome-directed therapeutic strategies but also open the door to further research on both the physiologic and pathophysiologic roles of rhinosomal mediated protein transport.

Acknowledgment

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