Suppression of Lymphocyte Functions by Plasma Exosomes Correlates with Disease Activity in Patients with Head and Neck Cancer

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Abstract

Purpose: Head and neck cancers (HNCs) often induce profound immunosuppression, which contributes to disease progression and interferes with immune-based therapies. Body fluids of patients with HNC are enriched in exosomes potentially engaged in negative regulation of antitumor immune responses. The presence and content of exosomes derived from plasma of patients with HNC are evaluated for the ability to induce immune dysfunction and influence disease activity.

Experimental Design: Exosomes were isolated by size-exclusion chromatography from plasma of 38 patients with HNC and 14 healthy donors. Morphology, size, numbers, and protein and molecular contents of the recovered exosomes were determined. Coculture assays were performed to measure exosome-mediated effects on functions of normal human lymphocyte subsets and natural killer (NK) cells. The results were correlated with disease stage and activity.

Results: The presence, quantity, and molecular content of isolated, plasma-derived exosomes discriminated patients with HNC with active disease (AD) from those with no evident disease (NED) after oncologic therapies. Exosomes of patients with AD were significantly more effective than exosomes of patients with NED in inducing apoptosis of CD8+ T cells, suppression of CD4+ T-cell proliferation, and upregulation of regulatory T-cell (Treg) suppressor functions (all at P < 0.05). Exosomes of patients with AD also downregulated NKG2D expression levels in NK cells.

Conclusions: Exosomes in plasma of patients with HNC carry immunosuppressive molecules and interfere with functions of immune cells. Exosome-induced immune suppression correlates with disease activity in HNC, suggesting that plasma exosomes could be useful as biomarkers of HNC progression.

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allows for an efficient, high-throughput isolation of morphologically intact, functionally active exosomes from plasma of patients with cancer (20). This method has been used to obtain exosome fractions from plasma of patients with HNC and to evaluate their effects on normal human immune cell subsets. Furthermore, our data indicate that exosomes present in the peripheral circulation of patients with HNC play a key role in immune regulation during cancer progression and response to therapy. Our data suggest that monitoring the protein content, molecular profiles, and suppressive functions of exosomes isolated from patients’ plasma offers an opportunity for defining the extent and levels of immune suppression prior to and during therapy. In aggregate, we demonstrate that exosome-mediated immune suppression can be reliably measured \textit{ex vivo} and may represent a clinically useful biomarker for the integrity of the immune system in patients at diagnosis and during oncologic therapies.

### Materials and Methods

Plasma specimens and isolation of peripheral blood mononuclear cells

Peripheral venous blood specimens were collected from patients with HNC (n = 38) or healthy volunteers (n = 14) after informed consent was obtained from all individuals. The study was approved by the Institutional Review Board of the University of Pittsburgh (IRB #960279, IRB#0403105, and IRB #0506140) and was conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). The patients with HNC were seen at the UPMC Otolaryngology Clinic between years 2014 and 2016. Samples were obtained from 19 patients with active disease (AD) prior to any therapy, 15 patients with NED following oncological therapies (selected at random in respect to time since last therapy) and four patients with recurrent disease (REC). The blood samples were obtained from healthy volunteers (selected at random in respect to time since last therapy) and four patients with recurrent disease (REC). The blood samples were obtained from healthy volunteers (selected at random in respect to time since last therapy) and four patients with recurrent disease (REC). The blood samples were obtained from healthy volunteers (selected at random in respect to time since last therapy).

### Exosome isolation from plasma by mini size-exclusion chromatography (mini-SEC)

Thawed plasma samples were differentially centrifuged 2,000 \( \times g \) for 10 minutes at room temperature (RT) and then at 14,000 \( \times g \) for 30 minutes at 4°C. Next, plasma was ultrafiltrated using a 0.22-μm filter (EMD Millipore). Self-made mini-SEC columns were prepared as previously described (20). The void volume fractions (1 mL) were collected, with exosomes eluting in fractions #3–5. The fraction #4 contained the bulk of eluted exosomes as previously reported (20). The eluted exosomes were characterized for the protein content, size, particle numbers, morphology, molecular profiles, and suppressive functions as described in the information to follow.

### Protein determination

Protein content and concentration of the isolated exosomal fractions were determined using the Pierce BCA protein assay kit (Pierce Biotechnology) following the manufacturer's instructions. The protein concentrations are calculated as μg protein/1 mL plasma loaded onto the mini-SEC column.

### Exosome size and concentration assessment by tunable resistive pulse sensing (TRPS)

Size ranges and concentrations of isolated exosome fractions were measured using TRPS as recommended by the system manufacturer Izon. Nanopores NP150 were coated with different buffers from the reagent kit supplied by Izon. Immediately before and after each experiment, calibration beads provided in the kit (200 EV, at the 1:1 ratio) were tested under the same conditions used for the samples. A small volume (10 μL) of the exosome fraction #4 was diluted 1:10 in 0.03% Tween-20 in PBS and loaded on the nanopore. The measurement conditions for the sample were as follows: NP37266, stretch 45.6 mm, voltage 0.68 V, current 144–150 nA, and two pressure steps 5–12 mbar. Each particle was measured by a short drop of the current (blockade). At least 500 particles and two pressure levels were recorded for both, samples and calibration beads. The Izon software version 3.2 was used for data recording and for calculating nanoparticle size ranges and concentrations.

### Transmission electron microscopy (TEM)

TEM was performed as previously described (23) at the Center for Biologic Imaging at the University of Pittsburgh. Freshly isolated exosomes were layered on copper grids with 0.125% Formvar in chloroform and stained with 1% uranyl acetate in ddH₂O. Immediately, exosomes on the grids were visualized by the transmission electron microscope JEOL, JEM-1011.

### Western blots

For analyses by Western blots, exosome fraction #4 was concentrated on Vivaspin 500 (VS0152, 300,000 MWCO, Sartorius, Göttingen, Germany). Exosomes were tested for the presence of exosomal markers, including TSG101 and of other protein markers of interest as previously described (20). Briefly, exosomes were lysed in Lane Marker Reducing Sample Buffer (Pierce, Thermo Scientific) separated on 7–15% SDS/PAGE gels (Bio-Rad)
always applying 10 µg protein/lane and transferred onto an Immobilon-P PVDF membrane (EMD Millipore) for Western blotting. Membranes were incubated overnight at 4°C with antibodies purchased from various vendors as indicated and used at the following dilutions: from Abcam: TSG101 (1:500, ab30871), Fas (1:1000, ab133619), Fasl (1:500, ab68338), CTLA-4 (1:1000, ab134090), TRAIL (1:500, ab2056), PD-L1 (1:400, ab58810), CD80 (1:1000, ab134120), OX40L/CD252 (1:1000, ab108083), CD70 (1:1000, ab134623); from Santa Cruz Biotechnology: CD39 (1:400, sc-33558), CD73 (1:400, sc-256603), COX-2 (1:500, sc-1745), Bel-2 (1:500, sc-509); from R&D: PD-1 (1:1500, MAB1086); from Cell Signaling: TGF-β1 (50), Bcl-2 (1:500, sc-1745), Bcl-xL (1:500, sc-3711) or from Sigma Aldrich: PRAME (1:1000, SAB1401390). HRP-conjugated secondary antibody (1:3000–15000, Pierce, Thermo Fisher) was added for 1 hour at room temperature (RT), and blots were developed with ECL detection reagents (GE Healthcare Biosciences). The blots were semiquantitated by densitometry using software Image J. The integrated pixel value was determined for each protein band by multiplying image intensity and the band area after subtracting the mean background value.

**Functional studies**

**Apoptosis assays in CD8+ cells.** Freshly isolated CD8+ T cells (10^5/mL) were activated with CD3/CD28 T-cell activator (105/100 µL, Stemcell,) and IL2 (150 IU/mL, PeproTech, Bionity, Rocky Hill) in freshly prepared RPMI for 48 hours. In some experiments, CD8+ Jurkat cells (10^5/mL) were plated in a 96-well plate (10^5 cells/well) in MV-depleted RPMI for 24 hours. Next, exosomes (2–3 µg in 50 µL PBS) isolated from plasma of patients with HNC with AD, REC, or NED and from plasma of NDs were added and cocultures were incubated for 24 hours. Cocultures containing no exosomes (=50 µL PBS) served as controls. Apoptosis of CD8+ T cells was measured by flow cytometry using an Annexin V assay (Beckman Coulter) and Accuri flow cytometer (BD Bioscience). Additionally, Caspase-3/7 activity was measured in some experiments using Caspase-3/7 Glo and Cell TiterGlo Viability assays following the manufacturer’s instructions (Promega) and assessed in GloMax 96- Microplate Luminometer (Promega).

**CFSE-based CD4+ proliferation assay**

CD4+ T cells isolated from PBMCs of normal donors were labeled with 1.5 ±µmol/L CFSE (Cell Trace, Thermo Scientific) in 0.1% BSA in PBS (v/v) for 10 minutes at 37°C. The staining was quenched with an equal volume of exosome-depleted FBS (21). CFSE-labeled T cells (10^5 cells/well) were activated with CD3/28 beads (cell to bead ratio 1:1, T-cell activation/expansion kit, Miltenyi) for 24 hours and coincubated with exosomes (2–3 µg of fraction #4 in 50 µL PBS) for 3 days at 37°C. Proliferation of T cells was measured on day 4 by flow cytometry, and the data were analyzed by Modfit (Verity Software House). The percentage suppression of proliferation in cocultures with exosomes was calculated as described by us (24) and compared with control T cells incubated alone or with exosomes isolated from plasma of NC.

**CD39 induction in CD4+ T cells by patients’ exosomes**

Freshly isolated, normal human resting CD4+ T cells (10^7/100 µL) were coincubated with exosomes (2–3 µg in 50 µL PBS) isolated from patients with AD or NED and from NDs’ plasma in the presence of 20 µmol/L of exogenous ATP. Samples incubated with no exosomes (PBS only), and no ATP were used as controls. CD4-PE (Beckman Coulter) and CD39-FITC (eBioscience) expression and appropriate isotype controls were used to phenotype the T cells by flow cytometry after 20 hours of coincubation.

**Adenosine production by CD4+CD39+ Treg coincubated with exosomes**

25,000 freshly isolated, normal human resting CD4+CD39+ in 50 µL PBS were coincubated with exosomes isolated from plasma of patients with AD, NED, and NDs under conditions described above in the presence of 20 µmol/L ATP for 1 hour (25). Concentrations of 5'-AMP and adenosine (ADO) and their degradation products (Inosine, Hypoxanthine and Xanthine) were measured by mass spectrometry as previously described (25). As controls, Treg incubated without ATP and ATP in PBS only were used.

**Exosome-induced downregulation of NKG2D expression on NK cells**

Resting PBMCs obtained from healthy donors were coincubated with or without exosomes as described above for 24 hours. Exosome-induced downregulation of NKG2D expression levels (MF) and percentages of NKG2D+ cells were determined by gating on CD3+CD56+ NK cells as previously described (20). NKG2D-PE antibody and matching isotype control were purchased from Beckman Coulter.

**Cytotoxicity assay**

Flow cytometry-based cytotoxicity assays (26) with K562 serving as target cells and activated NK cells treated or not with ± exosomes as effector cells were performed as described by us with minor modifications. NK cells (10^5 cells/mL) isolated from PBMCs of NDs were activated with IL2 (1000 IU/mL, Peprotech) for 48 hours. On day 2, exosomes (2–3 µg of fraction #4 in 50 µL PBS) isolated from plasma of patients with HNC or ND were added to 150 × 10^3 activated NK cells placed in wells of a 96-well plate. Aliquots of PBS (50 µL; No exosomes) were added to control wells. Following 24-hour coincubation, 30 × 10^5 CFSE-labeled K562 target cells were added to the wells to obtain the NK:K562 ratio of 5:1. In preparation for the assay, K562 cells were labeled with CFSE for 10 minutes, washed twice with complete RPMI medium and reconstituted to the concentration of 10^5 cells/mL. Plated K562 targets and effector NK cells ± exosomes were coincubated for 4 hours at 37°C in the atmosphere of 5% CO2 in air. Next, cells were stained with 7-AAD (eBioscience). Cytotoxicity was determined by gating on CFSE-positive K562 cells and estimating the percentage of these cells that were also stained with 7-AAD. In parallel, NKG2D downregulation was determined in activated NK cells only ± exosomes.

**Data analysis**

Data were summarized by descriptive statistics (IBM SPSS, version 23) such as means and standard errors (SE). Statistical analysis was performed using GraphPad Prism (version 6). As parametric tests unpaired t test or one-way ANOVA and for nonparametric data Mann–Whitney U or Kruskal–Wallis tests were used. T-cell proliferation in CFSE-based assays was assessed with Modfit (Verity software house, version 4). Flow analyses were performed with VenturiOne version 5.0 or Kaluza v1.5 (Beckman Coulter). A P value of <0.05 was considered to be statistically significant.
Results
Clinicopathological characteristics of the study patients

The clinicopathological characteristics of the patients who contributed specimens for the study are listed in Supplementary Table S1. The patient age and gender are representative for patients with HNC, with an average age of 60 years (range, 24–79 years) and predominantly male (76% vs. 24%). Primary tumor sites were oral cavity (61%) and oropharynx (34%). Only two larynx patients (5%) were included. At blood draw, disease status was AD in 19 patients (50%), REC in four patients (11%), and NED in 15 patients (39%).

The numbers of patients with the early-stage (UICC stage I/II) versus late-stage (UICC stage III/IV) disease were at the ratio of 2:3. Most patients (39%) had small tumors (T1 or T2) or a negative nodal status (N0, 55%). No patient had distant metastases at the time of diagnosis. The majority of 23 patients had intermediately differentiated tumors (G2). HPV status, routinely determined by p16 immunohistochemistry, was positive in 10 patients, negative in eight patients, and undetermined in 20 patients with nonoropharyngeal cancer. The majority (50%) consumed alcohol and/or tobacco at the time of diagnosis. As treatment, 19 patients (50%) underwent surgery alone, 18 patients (47%) had surgery with an adjuvant therapy (radiotherapy and/or chemoradiotherapy) and one patient was treated with primary chemoradiotherapy.

Exosomal protein content in plasma of AD and patients with NED

Exosome recovery from plasma (μg protein/mL of plasma in the fraction #4) was significantly greater in patients with AD than in patients with NED or NDs (Fig. 1A). Importantly, in patients with NED after therapy, protein levels in total exosomal fractions were the same as those in ND (Fig. 1A). Exosomes isolated from plasma of patients with AD with early disease (stage I/II) had significantly lower exosome protein levels than those isolated from patients with AD with advanced (stage III/IV) disease (Fig. 1B). Interestingly, somewhat higher exosomal protein levels (NSD) were observed in patients who underwent surgery with adjuvant therapy (chemotherapy alone or chemoradiotherapy) compared with patients who had surgery only (Fig. 1C).

Higher particle concentrations in exosomal fractions isolated from plasma of AD versus patients with NED

Tunable Resistive Pulse Sensing (TRPS or qNano) measurements were performed with exosomes recovered in fractions #4 from plasma of three patients with AD and three patients with NED as well as three NDs. Size ranges and mean sizes were similar in all three groups (size range ~40–150 nm, mean diameter ~71–84 nm). The average particle count was highest in patients with AD (363 × 10^9 particles/mL), intermediate in patients with NED (207 × 10^9 particles/mL), and lowest in ND specimens (7 × 10^9 particles/mL). These differences in particle concentration levels are significant for all cohorts and are in agreement with protein levels for the exosomal fractions in AD, NED, and NDs, as shown in Fig. 2. Thus, both the protein content and exosome numbers were highest in plasma of patients with AD.

Characterization of exosomes recovered from patients’ plasma

Although the recovery of exosomes was greater in patients with HNC than in NDs, it was necessary to prioritize the assays selected for exosome characterization. Thus, not all functional assays could be performed in all patients. In most cases, the results obtained were representative of eight to 10 subjects in each patient cohort (AD vs. NED). In NDs, low levels of plasma-derived exosomes limited exosome analysis to few parameters.

Morphological features of exosomes isolated from plasma of patients with AD or NED

The representative TEM images of exosomes isolated by mini-SEC from plasma of patients with HNC with AD or NED and from plasma of NDs are shown in Fig. 3. Freshly isolated exosomes eluting in #4 fractions appeared as intact vesicles ranging in size from 30 to 100 nm and were morphologically identical.

Western blot profiles of exosomes from plasma of AD and patients with NED

Western blots were performed following exosome concentration by Vivaspin 500 (MWCO 300,000), which was necessary to be able to consistently load 10 μg exosomal protein/lane on SDS gels. The composite Western blot shown in Fig. 4 presents exosome protein profiles for four patients with AD, four patients with NED, and three patients with ND. The data in Fig. 4 match with protein levels for the exosomal fractions in AD, NED, and NDs. AD compared with NDs (P < 0.001), whereas exosome protein levels in NED and ND are similar.

Figure 1.
Protein levels in exosomes isolated from plasma of HNC patients or NDs. Following mini-SEC isolation of exosomes in fraction #4, total exosomal protein was measured in BCA assays. A, Exosome protein levels are significantly higher in patients with AD compared with those with NED and ND. AD compared with NED and ND (P < 0.001), whereas exosome protein levels in NED and ND are similar. B, In patients with AD, exosomes from plasma of advanced HNCs (UICC stage III/IV) have significantly higher protein levels than those from early-stage diseases (UICC stage I/II) (P < 0.001). C, Following surgery alone, the exosomes of patients with NED tend to have lower protein levels than exosomes from plasma of patients treated with surgery plus adjuvant oncotherapy. The data in A–C are mean values ± SEM.
NED, and one ND. The objective was to look for differences in the cargo of exosomes obtained from plasma of patients with HNC with AD versus NED. Among the patients, the first two vertical blot profiles are for exosomes of patients with UICC stage I/II disease, the next two are for patients with UICC stage III/IV disease. Because exosomes are known to carry immunosuppressive as well as immuno-activating proteins (27), we selected and respectively blotted for several of these proteins, as shown in the blot horizontal sections A and B. Section C shows expression of TSG101 used as an exosome marker and of PRAME used here as a marker of exosomes potentially derived from the tumor (28). Figure 4A shows that the protein profiles are different for

Figure 2.
Size distributions and particle concentrations in exosome #4 fractions isolated from plasma of patients with HNC and ND. In A, Representative data obtained with fraction #4 exosomes from AD, NED, or ND plasma show the same size distribution, whereas numbers of the recovered particles are highest in the AD #4 fraction. In B, The combined data for three different exosome donors in each cohort are shown. The mean particle concentration is highest in patients with AD, intermediate in those with NED, and lowest in NDs (*: AD vs. ND \( P < 0.01 \); *: NED vs. ND \( P < 0.05 \)). The data are mean values \( \pm \) SEM.

Figure 3.
TEM images of exosomes isolated from plasma of patients with HNC and an ND. Exosomes appear as vesicles ranging in size from 30 to 100 nm. Exosome morphology is the same for patients with AD and NED and the ND. The shown images are representative for 1/5 exosome fractions examined for each cohort.
lower in exosomes of patients with AD with stage III/IV than stage I/II disease and tended to increase, albeit variably, in exosomes of patients with NED. Exosomal levels of PRAME did not change from patient to patient, and its presence in exosomes of ND indicates it cannot be considered as a potential tumor marker in HNC or a marker of exosome origin from tumor cells. Overall, the cargos of exosomes from plasma of patients with AD with advanced disease were enriched in proteins such as COX-2, TGFβ-LAP, PD-1, CTLA-4, and TRAIL. Their levels were variably decreased in exosomes obtained from plasma of patients with NED (Table 1).

Fasl expression on exosomes and their apoptotic activity

Because Fas and Fasl were often detected on Western blots of exosomes from patients with HNC, biological activity of these molecules was tested by coincubating exosomes with CD8⁺ Jurkat cells previously shown by us to carry CD95 (29). As shown in Fig. 5A, upon coincubation with exosomes from plasma of patients with HNC, CD8⁺Jurkat cells bind Annexin V and undergo apoptosis. Furthermore, Fig. 5B shows that levels of Fasl expression on exosomes isolated from plasma of patients with AD appears to be somewhat higher than those on exosomes of with NED (NSD). Also, Annexin V binding is significantly greater (P < 0.05) in CD8⁺ T cells coincubated with exosomes of patients with AD than that in CD8⁺ T cells coincubated with exosomes of patients with NED (Fig. 5C). However, as illustrated in Supplementary Fig. S2, there was no correlation between Fasl expression on exosomes and Annexin V binding to CD8⁺ Jurkat cells. Thus, Fasl⁺ exosomes from patients’ plasma induced apoptosis of CD8⁺ Jurkat cells and primary activated CD8⁺ T cells, as measured in assays based on Annexin V binding or caspase-3/7 activity (Fig. 5C and D). Further, AD and REC exosomes induced significantly more apoptosis in CD8⁺ T cells than exosomes from plasma of patients with NED or ND (Fig. 5C and D). Caspase-3/7 activity in CD8⁺ T cells was significantly elevated upon coincubation with exosomes from patients with AD compared with the control with no exosomes (P = 0.04). Importantly, caspase-3/7 activity decreased upon incubation with exosomes from plasma of the different patient cohorts and NDs: AD &REC=NED>ND (Fig. 5D), albeit given the small numbers of subjects in each group this trend was not statistically significant.

Suppression of CD4⁺ T-cell proliferation by patients’ exosomes versus disease activity

As previously shown by us, HNC plasma-derived exosomes suppress the proliferation of activated CD4⁺ T cells (20). We

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Semi-quantitative densitometry of Western blot bands shown in Fig. 4A was performed as described in Materials and Methods. The data are mean integrated pixel values (image intensity × band area). Exosomes were isolated from plasma of four patients with AD and four with NED.
suspected that the ability of exosomes to mediate immune suppression was related to disease activity in plasma donors. Therefore, exosomes isolated from plasma of patients with AD or NED were coincubated with normal pre-activated CD4\(^+\) T cells, and proliferation of T cells was measured in CFSE-based assays. Representative data shown in Fig. 6A indicate that exosomes of patients with AD induce significantly more apoptosis than NED exosomes (P < 0.05). In Fig. 6B, flow cytometry data for Annexin V binding in CD8\(^+\) Jurkat cells coincubated with plasma-derived exosomes for 24 hours. There was a significant correlation between T-cell apoptosis and the patients’ disease activity (*, P < 0.05; **, P < 0.01; ***, P < 0.001). In Fig. 6C, combined data (means ± SEM) for caspase-3/7 activation in primary CD8\(^+\) T cells coincubated for 24 hours with exosomes as described above (*, P < 0.05).

Figure 5.
Exosomes-mediated apoptosis in CD8\(^+\) Jurkat cells or primary activated CD8\(^+\) T cells. In A, representative flow cytometry data for Annexin V binding in CD8\(^+\) Jurkat cells coincubated with exosomes isolated from patients with HNC or ND. In B, exosomes of patients with AD carry higher FasL than exosomes of NED (NSD) and AD exosomes induce significantly more apoptosis than NED exosomes (P < 0.05). In C, flow cytometry data for apoptosis of primary activated CD8\(^+\) T cells coincubated with plasma-derived exosomes for 24 hours. There was a significant correlation between T-cell apoptosis with the patients’ disease activity (*, P < 0.05; **, P < 0.01; ***, P < 0.001). In D, combined data (means ± SEM) for caspase-3/7 activation in primary CD8\(^+\) T cells coincubated for 24 hours with exosomes as described above (*, P < 0.05).

Induction by patients’ exosomes of CD39 expression and adenosine production in CD4\(^+\)CD39\(^+\) regulatory T cells
Previous evidence showed that exosomes derived from HNC cell line supernatants upregulated expression of CD39 ectonucleotidase on the surface of CD4\(^+\)CD39\(^+\) Treg (30) and increased adenosine production by these Treg (25). We coincubated exosomes derived from HNC patients’ plasma with isolated resting CD4\(^+\) T cells and found that only exosomes from plasma of...
patients with AD significantly upregulated CD39 expression levels on T cells, whereas exosomes isolated from plasma of patients with NED or NDs did not (Fig. 7A and B). As exosomes derived from plasma of patients with HNC invariably carry CD39 and CD73 (Fig. 4; ref. 25), we wished to determine whether the ectonucleotidases tethered to these exosomes retained enzymatic activity. CD4+CD39+ Treg coincubated with exosomes derived from plasma of patients with AD and NED significantly upregulated conversion of exogenous ATP to adenosine (Fig. 7C). Exosomes of patients with AD had higher CD73 activity and induced significantly more adenosine production than did exosomes from patients with NED. Exosomes from plasma of NDs induced significantly less adenosine production than exosomes from HNC patients’ plasma. These data are consistent with previously observed higher levels of CD39 and CD73 carried by patients’ exosomes relative to ND’s exosomes (25, 30). No ATP conversion to adenosine occurred in the absence of exosomes. Interestingly, levels of 5’AMP production by CD4+CD39+ Treg alone were high and did not change upon coincubation with exosomes, suggesting that the rate of exosome-mediated conversion of ATP to 5’AMP exceeded that of 5’AMP conversion to adenosine. High levels of adenosine produced by CD4+ T cells in the presence of patients’ exosomes could be responsible for inhibition of CD4 T cell proliferation as reported above.

Exosomes of patients with HNC suppress cytotoxic activity of NK cells

Exosomes from plasma of patients with HNC were previously observed to induce suppression of cytotoxicity in normal human NK cells (20). To show that this exosome-mediated suppression was also related to disease activity in HNC patients, we monitored downregulation of NKG2D expression levels on the surface of normal NK cells coincubated with AD or NED exosomes. The data shown in Fig. 8A and B show the strongest and significant NKG2D downregulation in NK cells coincubated with exosomes of patients with AD and no downregulation in NK cells coincubated with exosomes of patients with NED or NDs. Also, cytotoxicity of CD3+CD56+ NK cells against K562 targets measured by flow cytometry was significantly inhibited by AD exosomes relative to no suppression seen with NED or ND exosomes (Fig. 8C).

Discussion

Suppression of antitumor immune responses by tumor-derived soluble factors, including inhibitory cytokines, has long been recognized as a mechanism contributing to tumor progression (31). Although the existence of cross-talk between tumor and recipient immune cells is widely accepted, the mechanisms responsible for sustaining this communication network have remained obscure. With the recent emergence of exosomes as vehicles engaged in conveying information between tissue and hematopoietic cells (32), attention has focused on the role exosomes present in plasma of all patients with cancer might play in promoting tumor progression or modulating response to therapy. Accumulating evidence suggests that exosome fractions in plasma of patients with cancer are enlarged relative to those in plasma of healthy donors (33) and that the protein content, numbers of exosomes present as well as the molecular cargo of exosomes could serve as potential biomarkers of disease progression and even disease outcome following therapy (34). Indeed, our study of exosomes isolated from plasma of patients with HNC shows that plasma levels of exosomal proteins, exosome numbers, their molecular cargo, and their ability to alter functions of normal immune cells reflect the presence and activity of the disease as well as response to oncologic therapies. It is important to note that our studies reported over a decade ago showed that CD8+ T cells freshly isolated from the circulation of patients with HNC with AD were highly sensitive to spontaneous apoptosis and that the fraction of apoptosis-prone CD8+ effector T cells was highest in patients with AD (35–37). At the time, we interpreted these results as evidence that immune effector cells in the circulation of HNC patients with AD were induced in vivo into early apoptosis by...
are readily identifiable in Western blots. This, of course, limits the number of exosome components that can be examined and can only provide a semiquantitative analysis by densitometry.

The presence, quantity as well as molecular and functional characteristics of plasma-derived exosomes appear to correctly differentiate patients with HNC with AD from those with NED after oncologic therapy. Furthermore, among patients with AD studied prior to any therapy measurements of the protein levels in isolated exosome fractions were sufficient to discriminate between patients with stage I/II and III/IV disease. Patients with recurrent disease tended to have exosome protein levels similar to those with AD. We expected that the molecular content of plasma-derived exosomes would be a better discriminator between AD and patients with NED than exosome protein levels. To this end, we began our studies using mass spectrometry analysis of plasma-derived exosomes. Unfortunately, none of the inhibitory proteins responsible for suppression of immune cells by these exosomes were detected among hundreds of the identified proteins (data not shown). These proteins, present on exosomes at femtomolar levels, require antibody amplification for signal detection, and they are readily identifiable in Western blots. This, of course, limits the number of exosome components that can be examined and can only provide a semiquantitative analysis by densitometry measurements of individual protein bands. Nevertheless, even with these limitations, Western blot analyses showed enrichment of several immunoregulatory proteins in exosomes from plasma of patients with AD that discriminated them from patients with NED.

By far the most consistent and convincing results were obtained when we measured effects of the plasma-derived exosomes on various functions of human immune cells. In these assays, patients’ exosomes were titrated into fully functional immune cells isolated from the blood of normal donors. Invariably, exosomes in patients with AD induced significantly stronger apoptosis of CD8+ T cells, greater inhibition of T-cell proliferation or of NKG2D expression on NK cells and better upregulation of suppressor functions in CD4+CD39+ Treg than exosomes of patients with NED. In aggregate, these data provide evidence that exosomes in plasma of patients with cancer could serve as reliable indicators of exosome-mediated immune dysfunction in cancer that correlates with disease activity. We expected that exosome-induced apoptosis, as measured by Annexin V binding in Jurkat cells, would correlate with FasL expression levels in exosomes. However, no correlation was observed, perhaps because FasL expression in exosomes was measured by Western blots, which detect membrane-bound and intra-vesicular proteins. In our previously reported experiments, suppressive effects of exosome-associated FasL, TGF-β1, or CD73 on immune cells were partially but never completely blocked by anti-FasL Ab, anti-TGFβ1 Ab, or pharmacologic inhibitors of CD73, respectively (25, 29). These results suggested that not one but several different inhibitory pathways are activated in immune cells interacting with exosomes that carry multiple inhibitory ligands. Indeed, the Western blot profiles of exosomes of exosomes from HNC plasma indicate these vesicles carry at least nine different immunosuppressive molecules and deliver them to immune recipient cells as “bundles” capable of inducing apoptosis or inhibiting other functions. For example, the presence of FasL and TRAIL in exosome cargo, as shown in Fig. 4A, suggests that efficient exosome-mediated apoptosis of T cells we measured ex vivo was likely induced by simultaneous ligation of both of these death ligands to receptors expressed on T cells. In aggregate, our

Figure 7.
Exosomes from plasma of patients with HNC upregulate CD59 expression levels and adenosine production by Treg. In A, Representative flow cytometry data showing upregulation of CD59 expression levels in resting CD4+ T cells isolated from PBMC of NDs. In B, Data for upregulation of CD59 by exosomes obtained from plasma of AD, NED, or ND after 24-hour coincubation. Only exosomes of patients with AD significantly augmented CD59 expression levels (P < 0.05). In C, Increased adenosine production by resting CD4+CD39+ Treg following coincubation with exosomes from plasma of patients with AD and NED or ND in the presence of 20 μmol/L exogenous ATP for 1 hour. Extracellular adenosine levels are the highest in Treg coincubated with exosomes of patients with AD (P < 0.05), intermediate in Treg coincubated with exosomes of patients with NED (P < 0.05) and lowest in Treg coincubated with exosomes of NDs. The data are from three independent experiments of which two were performed with duplicate cocultures.
Data emphasize the potential of plasma-derived exosomes to serve as noninvasive indicators of disease activity before or after oncological therapies as well as indicators of immune dysfunction that these exosomes are capable of inducing in patients with cancer.

The potential of exosomes to serve as noninvasive biomarkers of cancer progression on the one hand, and as metrics of immune dysfunction that these exosomes are capable of inducing in patients with cancer.

The optimal use of exosomes as biomarkers of tumor progression and/or immune suppression in cancer depends on the ability to isolate exosomes from patients’ plasma and to then separate TEX from non-TEX in order to obtain a tumor-specific exosome signature. This strategy presents a considerable technical barrier requiring reagents specific for tumor antigens such as glypican-1 in the Melo and colleagues report (41). Our study indicates that an alternative strategy to developing plasma exosomes as future cancer biomarkers could be based on functional evaluations of total exosome fractions ex vivo, using assays with normal immune cell subsets to discern and measure immunological abnormalities induced by exosomes. Immune competence of a patient whose tumor produces highly immunosuppressive exosomes is likely to be compromised. One such assay that could be readily standardized for potential clinical development is the flow-based apoptosis assay, where CD8+ Jurkat cells are coincubated with plasma-derived exosomes, providing a relative measure of Fas/FasL-mediated activity these exosomes can deliver. Exosome isolation from plasma using miniSEC is a high-throughput procedure (20). The development and subsequent validation of this platform for linking exosome-induced immune suppression with clinical endpoints is likely to provide useful prognostic information. The results we have presented are a "proof of principle" that could be extended, quantitated and validated in the future to harness plasma exosomes as biomarkers of cancer-related immune dysfunction.

Figure 8.
Exosome-induced downregulation of NKG2D and suppression of NK cell-mediated cytotoxicity. In A, Representative flow cytometry data for downregulation of NKG2D expression levels in normal human NK cells by exosomes of patients with HNC. Exosomes were coincubated with resting PBMCs and gates were set on CD3+CD56+ NK cells. Exosomes of the AD patient induced the most prominent loss of NKG2D expression levels relative to NED or ND exosomes. In B, Combined MFI data for NKG2D downregulation by exosomes of HNC patients and NDs are shown. In C, Cytotoxicity of NK cells against K562 targets was measured using CFSE-labeled K562 target cells by flow cytometry. Only exosomes from patients with AD suppressed NK cell-mediated cytotoxicity (*, P < 0.05).
deficiencies during the cancer progression or as biomarkers of therapy-related immune recovery after immune therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: S. Ludwig, E.K. Jackson, T.L. Whiteside
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References
# Clinical Cancer Research

## Suppression of Lymphocyte Functions by Plasma Exosomes Correlates with Disease Activity in Patients with Head and Neck Cancer

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