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Human Plasma Membrane-Derived Vesicles Halt Proliferation and Induce Differentiation of THP-1 Acute Monocytic Leukemia Cells

Ephraim A. Ansa-Addo,* Sigrun Lange,† Dan Stratton,* Samuel Antwi-Baffour,∗ Igor Cestari,*†‡ I Marcel I. Ramirez,*†§ Maria V. McCrossan,§ and Jameel M. Inal*

Plasma membrane-derived vesicles (PMVs) are small intact vesicles released from the cell surface that play a role in intercellular communication. We have examined the role of PMVs in the terminal differentiation of monocytes. The myeloid-differentiating agents all-trans retinoic acid/PMA and histamine, the inflammatory mediator that inhibits promonocyte proliferation, induced an intracellular Ca^{2+}-mediated PMV (as opposed to exosome) release from THP-1 promonocytes. These PMVs cause THP-1 cells to enter G_{0}-G_{1} cell cycle arrest and induce terminal monocyte-to-macrophage differentiation. Use of the TGF-β receptor antagonist SB-431542 and anti–TGF-β1 Ab showed that this was due to TGF-β1 carried on PMVs. Although TGF-β1 levels have been shown to increase in cell culture supernatants during macrophage differentiation and dendritic cell maturation, the presence of TGF-β1 in PMVs is yet to be reported. In this study, to our knowledge we show for the first time that TGF-β1 is carried on the surface of PMVs, and we confirm the presence within PMVs of certain leaderless proteins, with reported roles in myeloid cell differentiation. Our in vitro findings support a model in which TGF-β1-bearing PMVs, released from promonocytic leukemia cells (THP-1) or primary peripheral blood monocytes on exposure to sublytic complement or after treatment with a differentiation therapy agent, such as all-trans retinoic acid, significantly reduce proliferation of THP-1 cells. Such PMVs also induce the terminal differentiation of primary peripheral blood monocytes as well as THP-1 monocytes. The Journal of Immunology, 2010, 185: 000–000.
carry Wnt-3, which is involved in hematopoietic differentiation, and such PMVs were shown to reprogram hematopoietic progenitor cells
(13). This evidence, that PMVs, albeit from embryonic stem cells, are involved in differentiation in the hematopoietic environment in the bone marrow suggests possible PMV involvement in leukemia myeloid development and possibly monocytic hematopoiesis, all of which need to be investigated further.

In addition to PMA and all-trans retinoic acid (ATRA), inflammatory mediators, such as histamine (this study), and bacterial products fMLP and LPS can also induce microvesiculation, providing that cells have the cognate receptors. Because inducers of PMV release have been described in connection with possible therapies for acute monocytic leukemia, we wanted to see whether PMVs themselves could induce cell cycle arrest/terminal differentiation (the hallmark of differentiation therapy) in the monocytic leukemia cell line THP-1. This was important in view of the recent findings that microvesicles, albeit from neutrophils (termed eco

somes), were shown to interfere with the maturation of immature dendritic cells (14). Furthermore, early success of ATRA in acute promyelocytic leukemia (APL) has not been followed up with new drugs, and resistance can develop with all existing therapies (15, 16). This study aims to show principally whether PMVs can initiate differentiation/stop proliferation of THP-1 cells. From examining the literature, such PMV-mediated differentiation would likely be a multifactorial process involving several proteins and microRNAs. Among the many factors that PMVs harbor and the numerous PMV-associated cytokines revealed by proteomics studies is the multifunctional TGFB-1, which is found principally associated with platelet PMVs (17) and that inhibits the proliferation of various cell types in vitro (18). To our knowledge, we describe for the first time the presence of surface-bound TGFB-1 on PMVs. Given the important role that TGFB-1 plays in the regulation of cellular proliferation, as well as its autocrine inhibition of proliferation in APL cell line HL-60, we asked whether any growth regulation and differentiation of promonocytic leukemia cells could in part be influenced by PMVs bearing TGFB-1, which are released from cells by the action of known differentiation therapeutics. If so, this would be the first report of TGFB-1 carried on the surface of a PMV that was deliverable as a functional signaling molecule.

Materials and Methods

Cell culture and peripheral blood monocyte isolation

All cells used in this study were maintained in growth medium containing RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 pg/ ml streptomycin. Cells were occasionally maintained for a week in growth medium supplemented with 1% kanamycin at 37°C in a humidified atmosphere of 5% CO2 (all reagents were purchased from Sigma-Aldrich, St. Louis, MO). Exponentially growing cells were counted and viability was determined using the Guava EasyCyte flow cytometer (ViaCount assay; Guava Technologies, Hayward, CA). Throughout, after 3 d in culture, cells were split 1:4 and only cultures with at least 95% viability were used. After 3–5 d, cells were recovered from original frozen stocks. For experiments, THP-1 cells were grown in a similar growth medium supplemented with 5% FBS. Throughout, cell morphology was monitored and images were collected using a 1x81 motorized inverted fluorescence microscope (Olympus, Melville, NY). Peripheral blood monocytes (PB mono) were isolated from whole blood using a two-step technique (19) with Ficoll-Hypaque of density 1.070 g/ml and then a Percoll gradient of density 1.066 g/ml. This technique gave a high yield of monocytes with a minimum contamination with platelets and RBCs.

Purification and characterization of PMVs from conditioned medium

PMVs were isolated by a modification of the method of Eken et al. (14). Briefly, conditioned medium from cells (1 × 10⁶) stimulated with 10% normal human serum (NHS), 0.1 µM PMA, 10 µM histamine, or 1 µM ATRA at 37°C for 30 min, unless otherwise stated, was first centrifuged at 160 × g for 5 min to remove cells. The supernatant was then centrifuged twice at 4000 × g for 30 min to remove cell debris. The resultant supernatant was sonicated in a sonicating water bath (Townsion and Mercer, Croydon, U.K.) for 5 × 1 min prior to centrifugation to disperse aggre
gated exosomes. Supernatant was centrifuged at 25,000 × g for 90 min to pellet PMVs, which were then washed once by resuspending in PBS and centrifuging at 25,000 × g for 90 min. The PMVs were then resuspended in PBS and quantified on a Guava EasyCyte flow cytometer using ExpressPlus software, or stained with annexin V (AnV)-FITC to determine surface phosphatidylinserine (PS). The protein concentration of isolated PMVs was determined using the BCA protein assay kit (Pierce, Rockford, IL), according to the manufacturer’s instructions, and PMVs were then used immediately. To confirm the size range of PMVs isolated, and to standardize the flow cytometer (Guava EasyCyte) settings for counting THP-1–derived PMVs. Megamix fluorescent sizing beads (Biocytex, Marseille, France) calibrated to 0.5 and 0.9 µm were used, according to the manufacturer’s instructions.

Growth inhibition assays

Human THP-1 cells in the exponential phase were washed twice with RPMI 1640 and resuspended in growth medium containing 5% FBS. Cells were seeded into 12-well plates at 1 × 10⁶ cells/well in triplicate. Different concentrations of PMVs, or a range of differentiation/proliferation-inhibiting agents (PMA, ATRA, histamine), were added to each well (except controls) and plates were incubated at 37°C for 3 d. On the days indicated, nonadherent cells were removed and counted by ViaCount assay on a Guava EasyCyte flow cytometer. In some experiments, THP-1 cells were incubated with 30 µg PMVs alone or in the presence of the TGF-B receptor antagonist SB-431542 (10 µM) or with 25 µg/ml affinity-purified, neutralizing rabbit anti–TGF-B1 (GenWay Biotech, San Diego, CA).

Measurement of intracellular calcium

Measurements of intracellular calcium were made before and after addition of histamine, ATRA, and PMA with or without 25 µM calcium chelator BAPTA-AM (Sigma-Aldrich). After 30 min of pretreatment with the agents in phenol red-free RPMI 1640 (Invitrogen, Carlsbad, CA), cells (1 × 10⁶/ml) were resuspended in physiological salt solution containing 130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPS, and 1% BSA. The procedure was carried out as described previously (20), but essentially the cells were loaded with 2 µM fura 2-AM (Sigma-Aldrich) with continual stirring, and fluorescence was monitored on a spectrofluorimeter at 505 nm upon excitation at 340 and 380 nm every second. Intracellular Ca²⁺ concentration ([Ca²⁺]i) was calculated using the equation [Ca²⁺]i = Kd(R – Rmin)/(Rmax – R), where R is the ratio of the emission intensities measured at 505 nm on excitation at 340 and 380 nm. Rmin was obtained at [Ca²⁺]i of 0 (by addition of 10 mM EGTA), and Rmax was obtained by lysing the cells with 0.1% Triton X-100, thus allowing Fura-2 measurement in the maximum external calcium concentration of 1 mM. The temperature of the experiment was 37 ± 1°C, and Kd for Fura-2 is 224 nM.

Cell cycle analysis

THP-1 cells (1 × 10⁶) in cold PBS were fixed in cold 70% ethanol. After washing in PBS, cells were stained with propidium iodide (50 µg/ml) for 1 h at 4°C and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions. To determine the percentage of the population in G0-G1, S, and G2-M phases of the cell cycle was determined using CellQuest software (BD Biosciences) and represented in GraphPad Prism 5.0 (Graphpad Software, San Diego CA).

Differentiation assays

Adherence of cells was determined following treatment with 30 µg PMVs. On the day of the experiment, 1 × 10⁶ cells/well were seeded into 12-well culture plates in triplicate. On each of the indicated days, the nonadherent cells were transferred into new plates and incubated at 37°C in a 5% CO₂ incubator. The wells were then washed twice with serum-free RPMI 1640 and the attached cells released with trypsin/EDTA (Sigma-Aldrich). The trypsinized cells, now in suspension, were collected by centrifugation (200 × g, 5 min), stained with ViaCount reagent, and counted by flow cytometry.

To investigate differentiation, THP-1 cells or PB mono were seeded into 12-well plates in triplicate. Cells were left untreated (control) or treated either with 30 µg PMVs or with PMA (0.1 µM) and incubated at 37°C in a humidified atmosphere of 5% CO₂ for 3 d. After this time, cells still in suspension were removed and the plates washed three times with RPMI.
1640. Cells attached to the bottom of the plates were incubated at 37°C for 1 h with the Abs anti-CD14-FITC, anti-CD11b-PE, and anti-DC-SIGN-FITC, with IgG-FITC and IgG-PE isotypes being used as controls. After three washes with PBS, cells were placed in PBS, 1% BSA and relative fluorescence was determined using the FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, U.K.).

NBT (Sigma-Aldrich) was used to determine intracellular O$_2^-$ as a measure of differentiation. For this assay, cells seeded at ~5 × 10$^5$ cells/ml in 24-well plates were treated for 1 h at 37°C with 1 mg/ml NBT and 4 μg/ml PMA. After washing in PBS the cell pellet was dissolved in 100 μl DMSO and the absorbance read at 570 nm on a spectrophotometer.

**SDS-PAGE and Western blotting**

Protein extracts were prepared and either analyzed by SDS-PAGE (for identification by mass spectrometry) or by Western blotting as described previously (21).

**Immunofluorescence staining for cytokines/growth factors**

Cell surface expression of TGF-β1, MIF, FGF-1, and Gal-3 molecules was analyzed by flow cytometry (EasyCyte; Guava Technologies). THP-1 cells (2 × 10$^6$) were washed twice (200 × g, 5 min each) with PBS and resuspended in cold PBS containing 10% PBS and 1% NaN$_3$. Cells were incubated in the dark with primary Abs (2 μg/million cells; R&D Systems, Abingdon, U.K.) at 4°C for 1 h with shaking. After three washes (400 × g, 5 min), cells were stained with the isotype-matched controls (anti-mouse or anti-rabbit IgG-FITC; R&D Systems) diluted 1/320 in PBS with 3% BSA and incubated with shaking, in the dark, at 4°C for 1 h. Cells were again washed three times with cold PBS, resuspended in 200 μl PBS containing 3% BSA, 1% NaN$_3$, and analyzed immediately using flow cytometry and the ExpressPlus assay program.

For staining of intracellular cytokines, THP-1 cells (2 × 10$^6$/reaction) in triplicate were resuspended in permeabilization buffer (0.5% Tween 20 in PBS) at room temperature for 20 min. Cells were permeabilized by washing three times with permeabilization buffer and incubated with 2 μg primary Abs at 4°C for 1 h in PBS with 10% PBS/1% NaN$_3$. Cells were washed three times and incubated with IgG-FITC–labeled secondary Abs (4 μg per 200 μl PBS) at 4°C for 1 h and again washed three times with PBS. Samples were finally resuspended in 200 μl PBS containing 3% BSA, 1% NaN$_3$, and analyzed immediately by flow cytometry.

**Flow cytometric analysis of apoptosis**

To see whether PMV-releasing THP-1 cells were undergoing apoptosis, cells were stained with annexin V (AnV) and 7-aminoactinomycin D (7-AAD) (Guava Nexin Reagent). AnV and 7-AAD-positive cells were then quantified using a Guava EasyCyte flow cytometer over the course of 1 h. To see if adding PMVs to cells rendered them apoptotic, these measurements were made 24, 48, and 72 h after addition of PMVs.

**Fluorescent microscopy analysis**

For fluorescent microscopy, all experimental samples were placed into plates containing coverslips by centrifugation (200 × g, 5 min, using an A-2-DWP rotor in a 5804R centrifuge; Eppendorf, Cambridge, U.K.) and fixed with 4% paraformaldehyde at 37°C for 10 min. Plates were gently washed twice with PBS, and coverslips were mounted on microscope slides with DAPI-Vectashield medium (Vector Laboratories, Burlingame, CA) for fixed cells and mounting medium (Agar Scientific, Essex, U.K.) for PMVs. Images were collected using a fluorescence microscope (IX81 motorized, inverted fluorescence microscope; Olympus).

**Fluorescence microscopy of PMV-cell interaction using octadecyl rhodamine-labeled PMVs**

THP-1–derived PMVs were labeled with octadecyl rhodamine chloride (R18) by treating PMVs with 1.37 mM ethanolic solution of R18 (containing a final concentration of 1% v/v) for 1 h at room temperature in the dark. R18-labeled PMVs were separated from unincorporated R18 by ultracentrifugation at 100,000 × g for 1 h followed by dialysis in 14-kDa dialysis tubes at 4°C in HEPES/NaCl buffer. The ratio of R18 to PMVs was estimated measuring the fluorescence before and after removal of the unincorporated probe. THP-1 cells were incubated with R18-labeled PMVs for 10 min at 37°C, followed by three washes in PBS. The cells were fixed in 4% paraformaldehyde in PBS for 15 min. The cells were then washed five times and mounted on slides with Vectashield mounting medium (Vector Laboratories). For fluorescence microscopy, an Olympus IX81 inverted microscope, equipped with a monochromatic camera U-CMAD3, was used. Images were subsequently colored using the Cell^M imaging software (Olympus).

**Quantitation of cytokines/growth factors by ELISA**

PMVs were isolated from THP-1 cells stimulated with 10% NHS at 37°C for 30 min (22). The concentrations of TGF-β1, MIF, FGF-1, and Gal-3 were measured by using 30 μg protein in ELISA kits (R&D Systems) according to the manufacturer’s instructions. In some experiments, PMVs (1 × 10$^7$ reaction), in triplicate, from THP-1, Jurkat, and MCF-7 cells were lysed before measuring cytokine levels.

**Transmission electron microscopy and negative staining**

THP-1 cells (5 × 10$^6$/ml) either stimulated (5% NHS) or not (control) were fixed in 0.1 M fixative solution (3% glutaraldehyde in 0.1 M sodium cacodylate buffer [pH 7.2]) and incubated at room temperature for 1 h. Samples were postfixed by incubation at 0°C for 1 h in 1% osmium tetroxide solution (1:1 mixture of 2% osmium tetroxide [Sigma-Aldrich] and 0.2 M sodium cacodylate buffer) and block stained in 1% aqueous uranyl acetate. Samples were resuspended in 1% hot aqueous and dehydrated in an ascending ethanol series (from 70 to 100%, absolute ethanol [v/v]; Sigma-Aldrich) and washed twice with propylene oxide (Agar Scientific) and left rocking overnight at room temperature. Infiltrated samples were embedded in capsules using applicators and polymerized at 60°C for 24 h. Ultrathin sections were cut on a Leica Ultracut R ultramicrotome (Leica Microsystems, Deerfield, IL) and stained in Reynolds lead citrate. The sections were examined on a JEOL JEM-1200 EX II electron microscope (JEOL, Peabody, MA).

For negative staining, microscopics isolated without prior sonication were stained with 2% aqueous uranyl acetate or 2% phosphotungstic acid (pH 6.8) plus aqueous bicitratin. Samples were placed on 400-mesh copper grids with a Pioloform support film (grids and Pioloform powder from Agar Scientific) and pretreated with 1% aqueous Alcian blue 8GX for 10 min. Digital images were recorded using the AMT digital camera previously described for the examination of stained ultrathin sections.

**Statistical analysis**

Statistical analysis (unpaired t test) was performed using GraphPad Prism software, version 5.0 (GraphPad Software, San Diego, CA). Differences giving a value of p < 0.05 were considered statistically significant.

**Results**

**Characterization of PMVs isolated from THP-1 monocytes**

PMVs were isolated by differential centrifugation of THP-1 cell-depleted conditioned medium after stimulation of THP-1 cells, and they were quantified using the Guava EasyCyte flow cytometer. The stimulus was sublytic complement (5% NHS), as heat inactivation (Fig. 1A) and C9 depletion of NHS (Fig. 1B) abrogated PMV release. The NHS as a source of complement itself had a negligible quantity of PMVs (Fig. 1C). Quantified PMVs were identifiable by their typical, heterogeneous size and granularity profile, R1, in Fig. 1D, as assessed by the logarithmic amplification of forward light scatter (FSC) and side light scatter (SSC) signals. PS expression on the membrane surface indicates the loss of lipid membrane asymmetry and, as well as being a distinguishing characteristic of cells undergoing very early apoptosis (23), it is found on the external leaflet of PMVs. Using flow cytometry, isolated PMVs were found to be PS-positive by staining with AnV (Fig. 1E), to be enriched for actin (Fig. 1F), and to fall within the expected size range, 0.5–0.9 μm diameter (Fig. 1G). After stimulation, the release of PMVs increased over a 20-min period, without any further increase up to 1 h (Fig. 1H). During this time and coincident with the increased numbers of released PMVs, the increase in the percentage of early apoptotic cells almost doubled (from 4.5 to 10% AnV-positive) and of late apoptotic cells also increased significantly (from 1 to 3% AnV-positive cells) compared with unstimulated controls.
When the stimulus was removed and cells were assessed 30–60 min later (Fig. 1I), the percentage apoptosis levels returned to initial levels (7% early and 1% late). This suggests the PS expression on the outer leaflet of the plasma membrane, detected by AnV binding, to be an early feature of PMV release (7) that does not induce cell death. In fact, upon returning the cells to

**FIGURE 1.** Analysis of isolated PMVs. A, Dose response of PMVs released from THP-1 cells with increasing NHS but not heat-inactivated (HI) NHS. B, PMV release is inhibited by incubating cells with 10 mM EGTA and by depletion of complement C9 from NHS. The amount of PMVs in the stimulating 5 and 10% NHS itself is negligible. C, A representative dot plot distribution of PMVs on a flow cytometer in D showing FSC (size) and SSC (granularity). PMVs are heterogeneous in size, and the region, R1, represents the FSC/SSC scatter gate of the vesicles. E, Isolated PMVs were analyzed for PS expression by staining with AnV; the resulting histogram shows ∼54% of labeled PMVs to be AnV-FITC–positive. In F, the feature that PMVs are enriched for actin, identified by mass spectrometry, is confirmed in SDS-PAGE of equal loadings of THP-1 lysate compared with released PMVs. Using fluorescent sizing beads, G shows most released PMVs to be in the size range 0.5–0.9 μm diameter. H. After stimulation to vesiculate, PMV release increased over the first 20 min only, during which time there was an insignificant rise of early apoptotic cells (from 4 to 10%) and of late apoptotic cells (from 1 to 3%). I, One hour later cells were placed in complete growth medium lacking vesiculation stimulus and returned back to almost basal levels of apoptosis (7% early, 1% late). The release of PMVs from THP-1 cells, by electron or immunofluorescent microscopy, was observed for cells either left uninduced (resting) (J, J′, K) or stimulated with 5% NHS (L, L′, M) and in higher magnification (N, N′). Annexin V Alexafluor 488 and DAPI-VECTASHIELD, original magnification ×10,000 (J, J′, L, L′), ×60 (K, M), ×20,000 (N, N′). Magnified image N′, inset, show details of PMV release close to the cells (asterisks indicate released PMVs; white arrowheads point to exosomes). O, Image, prepared by negative staining, of purified PMVs isolated after sonication of supernatant and centrifugation at 25,000 × g for 90 min. Original magnification ×100,000. P, Negative staining image of purified exosomes isolated from PMV-depleted supernatant by ultracentrifugation at 160,000 × g for 16 h. Original magnification ×100,000. *p < 0.05; **p < 0.01; ***p < 0.001.
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non-microvesiculation conditions, the raised PS levels are found to be only transient, as described for IL-1–containing PMVs (7), with the level of late apoptotic cells being only 1%.

THP-1 cells left untreated (Fig. 1J, 1K) or stimulated with 5% NHS at 37˚C for 30 min (Fig. 1L, 1L′, 1M) were fixed immediately and processed either for transmission electron microscopy or immunofluorescence microscopy. Compared to control cells (Fig. 1J, 1J′), the cells activated with sublytic complement showed increased levels of pseudopodia (Fig. 1L, 1L′, 1L″) and the release of AnV-FITC–labeled PMVs (Fig. 1M compared with control in Fig. 1K). Arrowheads indicate PMVs detached from the plasma membrane (Fig. 1L′, inset) and noted with asterisks under higher magnification (Fig. 1N, 1N′). Ultracentrifugation of samples depleted of debris at 160,000 × g for 90 min without prior water sonication resulted in aggregated exosomes being pelleted together with the PMVs (not shown). This is consistent with earlier reports, which suggested that exosomes are able to form clusters by binding with other exosomal vesicles (24). We show electron microscopy images of more pure PMVs (Fig. 1O) isolated, after sonication of supernatant (five times at 1 min) in a sonicating water bath, by centrifugation at 25,000 × g for 90 min. To obtain pure exosomes, supernatant depleted of PMVs was ultracentrifuged at 160,000 × g for 16 h, resulting in vesicles with the typical cup-shaped morphology of exosomes (Fig. 1P).

Monocyte-differentiating agent PMA, proliferation inhibitor histamine, and differentiating therapeutic ATRA induce PMV release and reduce proliferation

To confirm that ATRA, used in differentiation therapy for APL, limits proliferation of THP-1 monocytes, cell counts were determined for 3 d following addition of ATRA. Histamine and PMA were also used (Fig. 2A). By day 3, the significantly decreased proliferation levels with histamine, ATRA, and PMA correlated inversely with increasing levels of PMV release (Fig. 2B, 2C), and all three caused a marked increase in [Ca2+]i (400 nM in 20 s) in THP-1 cells (Fig. 2D).

PMVs reduce the proliferation of THP-1 cells, alter cell morphology, increase adherence, and induce terminal differentiation

To investigate the effect of PMVs per se, we looked at the effect of separately isolated THP-1 PMVs on the growth of THP-1 cells in culture. Current estimates of microvesicle concentration in the peripheral blood of healthy individuals range from 5 to 50 μg/ml. According to our calculations, this equates to 2 × 105 to 2 × 106 PMVs/ml for PMVs ranging in size from 0.5 to 0.9 μm in diameter. Furthermore, we have found the quantity of PMVs/ml in plasma (from 25 fasting subjects) to range from 2.8 × 105 to 5.8 × 105 PMVs/ml (R. Grant, D. Stratton, S. Lange, S. Antwi-Baffour, E. Ansa-Addo, S. Kholia, J. Naveda, and J. Inal, manuscript in preparation).

FIGURE 2. Differentiating agents induce a calcium-mediated release of PMVs from THP-1 monocytes. A, Histamine, ATRA, and PMA result in increasingly significant reductions in cell growth after 3 d. ATRA, PMA, and histamine induce PMV release. THP-1 cells (1 × 105 cells/ml) in triplicate wells of a 24-well plate were incubated with 0.1 μM PMA, 10 μM histamine, and 1 μM ATRA. Cell numbers were determined using a ViaCount assay on the Guava EasyCyte flow cytometer on days 1, 2, and 3 after addition of these agents. In B, for the day 3 results presented, there is an apparent inverse correlation between cell number and PMVs/ml released after treating with histamine, ATRA, or PMA. This is confirmed in C, which shows a significant negative correlation between cell number on the third day and the released PMVs/ml upon treatment with PMA/ATRA/histamine. To show that these differentiating/proliferation-inhibiting agents are inducing PMV release mediated after a rise in [Ca2+]i, spectrophotometric measurements of [Ca2+]i using fura 2-AM were made after addition of ATRA, PMA, or histamine (dotted line represents control, untreated cells) (D). *p < 0.05; **p < 0.01; ***p < 0.001.
FIGURE 3. PMVs stop the proliferation of THP-1 monocytes and induce their terminal differentiation as well as that of primary monocytes. A, THP-1 cells were seeded at $1 \times 10^5$ cells/well in triplicate in RPMI 1640 supplemented with 5% FBS. Wells were incubated without (○) or with (●) 10 µg of PMVs. At 24-h intervals, cells were harvested and the number was determined by flow cytometry. On days 1, 2, and 3, the percentage apoptosis (AnnV-positive) was determined to be ∼15% whether PMVs had been added (●) or not (○). Addition of PMVs appeared to have halted proliferation by 48h. B, The number of viable cells 3 d after exposure to PMVs did not reduce substantially with amounts of PMVs $\leq 10^4$. Each point represents the mean of triplicate cultures. C, Cell cycle analysis shows a large increase in the percentage of cells in cell cycle arrest G0–G1, from 32% after 12 h to 72% at 48 h, compared with control without PMVs (52% after 48 h). D, THP-1 monocytes and PB mono ($1 \times 10^6$ each) are stimulated to release PMVs after incubation with 5% NHS at 37˚C for 20 min. This release is inhibited upon calcium chelation with 10 mM EGTA. E, Human THP-1 and PB mono ($1 \times 10^5$/well) were seeded in triplicate into 24-well plates with or without PMVs. Cell morphology (unstained) was assessed daily using an inverted light microscope. Arrows
species, using the NBT reduction assay, to be significantly increased in mature phagocytes, we found the ability to generate reactive oxygen.

In a preliminary study, we applied isolated THP-1 PMVs to a culture plate after 3 d (Fig. 3A), black bars). Levels of TGF-β1 were significantly higher when PMVs were lysed (Fig. 3B), suggesting that TGF-β1 is predominantly expressed on the cell/PMV surface, whereas MIF, FGF-1, and Gal-3, for the most part, have an intracellular/vesicular location.

TGF-β1 carried by PMVs inhibits proliferation of THP-1 monocytes

To determine whether THP-1 PMVs can interact with THP-1 cells, we were able to show the appearance of the fluorescent lipid R18 on THP-1 cells after incubation for 10 min at 37°C with R18-labeled PMVs (Fig. 6A). This was indicative of lipid mixing and so potentially of membrane fusion and could be blocked by preincubation of recipient THP-1 cells with AnV.

We also investigated whether the ability to inhibit the rate of cell growth was only characteristic of PMVs released from THP-1 cells. When 30 µg of PMVs (equivalent to ∼1 × 10^6 PMVs) from other cell lines, such as MCF-7 (breast cancer), Jurkat (T cell), as PMVs to carry a range of cytokines, acute phase proteins, and chemokines, including IL-12 p70, IL-13, T-TAC, IL-16, IL-17E, MIF, MIP-1α, and Serpin E1. Given the wide range of cytokines carried by the PMVs, we decided to focus on TGF-β1, which if present on the surface of released PMVs, we surmised, could potentially bind in an autocrine/paracrine-like manner to its cognate receptor on myeloid cells and initiate differentiation. Since TGF-β1 has a signal peptide, we also examined whether cytokines, such as MIF, FGF-1, and Gal-3, which lack a signal sequence (28–30), are transported in PMVs. Although it has previously been shown that such leaderless cytokines are transported via a nonconventional secretory process (6, 7), note that most of these studies are based on exosomes, which, unlike PMVs, which are released through a budding process from the cell’s plasma membrane, are released upon fusion of multivesicular bodies with the plasma membrane (31).

By flow cytometry we found THP-1 cells to strongly express TGF-β1 on the surface (Fig. 4A). This has been described for the propeptide part of the large latent TGF-β complex, often tethered to fibronectin in the extracellular matrix (32). MIF, FGF-1, and Gal-3 are expressed on the surface at lower levels (Fig. 4A). Upon stimulation of these labeled cells with 10% NHS, the PMVs released showed surface expression of TGF-β1, but not of MIF, FGF-1, and Gal-3 (Fig. 4B). When the cells were permeabilized with 0.5% Tween 20, there was an increased labeling of MIF, FGF-1, and Gal-3 (Fig. 4C compared with Fig. 4D), which implies their greater intracellular expression.

The appearance of TGF-β1 on PMVs after stimulation of release from THP-1 cells coincided with a removal of the TGF-β1 signal as detected by Western blot on THP-1 cells (Fig. 4D). We also showed extravascular expression of TGF-β1 by immunofluorescence microscopy (Fig. 5A). Although no significant difference was observed in TGF-β1 levels by ELISA, regardless of whether the PMVs were lysed (Fig. 5B), levels of MIF, FGF-1, and Gal-3 were significantly higher when PMVs were lysed before measurement (Fig. 5B), suggesting that TGF-β1 is predominantly expressed on the cell/PMV surface, whereas MIF, FGF-1, and Gal-3, for the most part, have an intracellular/vesicular location.

PMVs released from THP-1 monocytes carry extravascular TGF-β1 and intravesicular proteins MIF, FGF-1, and Gal-3, which are involved in monocyte-to-macrophage differentiation

In a preliminary study, we applied isolated THP-1 PMVs to a human Ab cytokine microarray (R&D Systems) and found the in cultures treated with PMVs indicate attached cells with pseudopodia-like processes after 3 d of incubation. F. After 3 d, increased numbers of cells (THP-1 promonocytes and primary cells) were found attached to the plates in PMV-treated wells (filled bars), but very few cells adhered to the culture plates in the absence of PMVs (open bars). Mean fluorescence intensity, indicative of surface expression levels, showed THP-1 monocytes (G–I) and PB mono (J–L) treated with PMVs to be significantly positive for CD14 and CD11b, but not DC-SIGN (CD209), as compared with untreated control. PMA-treated cells used as control for monocyte-derived macrophages were also shown to be highly positive for CD14 and CD11b. The NBT reduction assay shows an increase in superoxide activity in THP-1 monocytes and primary monocytes (M and N, respectively) treated with PMVs, indicative of their differentiation. Data represent the mean ± SD of two experiments performed in triplicate. *p < 0.05; **p < 0.01; ***p < 0.001.
as those from THP-1 (monocytic leukemia), was added to THP-1 cells, only those from Jurkat and THP-1 cells, which carried higher levels of TGF-β1 (Fig. 6C; 1900 pg by the 1 × 10^6 [30 μg] THP-1 cells and 2600 pg by the 1 × 10^6 [30 μg] Jurkat PMVs), significantly reduced proliferation (Fig. 6D), a known prerequisite for differentiation (33). In contrast, MCF-7 PMVs, which carry less TGF-β1 (250 pg by the 1 × 10^6 [30 μg] PMVs added), only reduced proliferation marginally compared with untreated control. With increasing amounts of MCF-7 PMVs (90 μg [carrying 750 pg of TGF-β1] or 210 μg [carrying 1750 pg of TGF-β1]), a growth reduction equivalent to the level achieved with 30 μg of THP-1 PMVs (carrying 1900 pg of TGF-β1) was achieved. This apparent correlation between levels of TGF-β1 and growth reduction suggests the involvement of TGF-β1 present on the PMVs.

This was further implied by showing that inhibition of growth rate after PMV addition could be restored to near control rates with SB-431542 (Fig. 6E). This is a specific small molecule inhibitor of TGF-βRI kinase and the associated intracellular mediators, such as Smad, with both being central to TGF-β1–mediated differentiation of myeloid cells (34). As TGF-βRI could also be activated.

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**FIGURE 4.** Flow cytometry of PMVs for TGF-β1, MIF, FGF-1, and Gal-3. A, Flow cytometric analysis of THP-1 cells (2 × 10^6) and of released PMVs (B) after surface labeling with Abs against TGF-β1 and proteins, which lack a signal peptide (MIF, FGF-1, and Gal-3). Intracellular staining after permeabilization with 0.5% Tween 20 in THP-1 cells for the above-mentioned proteins (C). Western blotting of THP-1 cells after stimulating PMV release (+) shows a dramatic reduction of TGF-β1 compared with unstimulated cells (−) (D). In all experiments, PMVs were released by providing THP-1 cells with sublytic complement (5% NHS) for 30 min at 37˚C.

**FIGURE 5.** Extravesicular expression of TGF-β1 and intravesicular expression of MIF, FGF-1, and Gal-3 by immunofluorescence microscopy. A, Surface labeling of TGF-β1 on THP-1-derived PMVs but low levels of MIF, FGF-1, and Gal-3. Original magnification ×60; inset, ×20. Inset, Highest expression of TGF-β1 on cell surface. Bright field (BF) and representative IgG are shown. Isotype control images are shown in B. Original magnification ×60, inset, ×20. Scale bar, 10 μm. C, ELISA measurements of selected proteins in lysed and intact PMVs released from THP-1 cells. PMVs (30 μg) from stimulated cells (10% NHS at 37˚C for 30 min) were either lysed or left unlysed and cytokine/growth factor levels were measured. There was no significant difference between TGF-β1 levels in lysed or intact PMVs. Increased levels of MIF, FGF-1, and Gal-3, however, were measured by ELISA after lysis of PMVs. *p < 0.05; **p < 0.01; ***p < 0.001.
by TGF-β1, 2, or 3, we further used anti–TGF-β1 as a blocking Ab, with similar results (Fig. 6E).

Discussion

The release of PMVs by various cells has been implicated in a wide range of biological activities in both in vitro and in vivo studies (5), with the most characterized function being that of intercellular communication (3, 35). We have shown that PMVs are released from cells stimulated through deposition with either sublytic complement or upon treatment with agents used in differentiation therapy. In the present study, to our knowledge we demonstrate for the first time that PMVs (isolated from THP-1 cells) carry TGF-β1 on their membrane surface. These TGF-β1–bearing PMVs can modulate the growth rate of THP-1 monocytes in vitro, without inducing apoptosis, the cells exiting the cell cycle at G0–G1. Additionally, we observed dramatic changes in the morphology of PMV-treated THP-1 monocytes and PB mono as they became increasingly irregular in shape, with membrane extensions resembling pseudopodia. By day 3 they became completely attached to the culture plate and expressed significantly higher levels of typical macrophage markers, such as CD14 and CD11b, but not the dendritic cell specific marker DC-SIGN (CD209). We also showed that PMVs isolated from Jurkat and THP-1 cells carrying high levels of TGF-β1 (≥200 pg TGF-β1/10^5 PMVs) caused significantly greater reductions in the growth rate of THP-1 cells as compared with PMVs from MCF-7 breast cancer cells, expressing lower levels (~30 pg of TGF-β1/10^5 PMVs).

Because differentiation experiments with freshly isolated, human peripheral blood monocytes, as opposed to a leukemic promonocytic cell line, gave similar results, the possible involvement of PMVs in terminal monocyte-to-macrophage differentiation warrants further study.

Others have previously shown that rTGF-β1 can inhibit proliferation and promote differentiation of human promonocytic leukemic cells into macrophages (31). Our results are in agreement, but we go on to show that the source of macrophage-inducing TGF-β1 to be that bound to the PMV surface. We also showed that attachment of PMVs to THP-1 cells is PS-mediated, similar to contact (and fusion) of monocyte-derived PMVs with platelets (36), but we did not show that such attachment/fusion was a prerequisite for the observed differentiation. Although contact with or phagocytosis of apoptotic cells and PMVs by macrophages has
been shown to release TGF-β1 at levels of 80 pg/ml (37) and 350 pg/ml (38), respectively, importantly, such release and autocrine-like action of TGF-β1 on the THP-1 cells, resulting in the observed reduction in proliferation (and consequent differentiation), is unlikely to have occurred in our study, as low TGF-β1–carrying MCF-7–derived PMVs were unable to elicit significant reductions in proliferation.

PMVs carry the majority of nonconventionally secreted proteins released into culture supernatants, and potentially carry them within the PMVs themselves. We have confirmed that the leaderless, intracellular proteins MIF, FGF-1, and Gal-3 are present within PMVs, and thus they are secreted nonconventionally. As reported for TGF-β1, and as described earlier, these leaderless proteins have also been shown to play important roles during the differentiation of myeloid cells. Although PMVs are known to deliver intravesicular proteins and surface receptors to recipient cells (39), this has not hitherto been demonstrated for a surface-bound cytokine, as we describe in this study, for TGF-β1.

The concept that surface TGF-β1 can stimulate TGF-β–mediated signaling on cell-to-cell contact, or by implication in this study by PMV–cell contact, is not new and has been described in T cells. Regulatory T cells (CD4+CD25+) express active TGF-β1 (40), which interacts with TGF-β receptor on effector cells, thereby mediating immunosuppression (41). Similarly, surface-bound, albeit latent TGF-β on the surface of colorectal cancer cells has also been implicated in immunosuppression following cell-to-cell contact (42). Although activation may occur through various mechanisms upon cell contact, latent TGF-β may be activated by αvβ3 integrins, then mediating Smad signaling through TGF-βRI and TGF-βRII; MAPK and PI3K/Akt pathways may also be modulated.

Several inhibitors of TGF-β1 signaling have been reported as potential therapeutics in cancer immunotherapy. Among these is the TGF-β receptor type I antagonist SB-431542, a selective inhibitor of endogenous activin, of TGF-β1 signaling (43), and of resulting phosphorylation of Smads. The tumor-suppressive functions of TGF-β1 have also been reversed using SB-431542 in studies using the colon cancer-derived FET cells (34), and the antagonist has been shown to inhibit the ligand-dependent growth of HT-29 colon cancer cells (34). We demonstrated TGF-β1–mediated involvement in this study by showing that SB-431542 almost completely abrogated the growth inhibition due to PMV treatment of cells. That TGF-β1, on the surface of PMVs, could mediate such a change was confirmed by also reversing growth inhibition in the presence of anti–TGF-β1.

To ascertain the relative importance of PMV release after stimulation with ATRA, PMA, or histamine, in terms of limiting proliferation, it would be interesting to treat promonocytic cell lines with these agents following pretreatment with calpeptin, which specifically blocks calpain-mediated PMV release. TGF-β1 is unlikely to be the only factor that PMVs from THP-1 cells convey to induce differentiation. By using lentivirus-mediated short hairpin RNA expression, we have now generated a THP-1 cell line stable for human complement inhibitory receptor. This cell line may have potential for the treatment of human peripheral blood monocyte isolates. Mem. Inst. Oswaldo Cruz 95: 221–223.

Disclosures
The authors have no financial conflicts of interest.

References


