Microvesicles of Women With Gestational Hypertension and Preeclampsia Affect Human Trophoblast Fate and Endothelial Function

Einat Shomer, Sarah Katzenell, Yaniv Zipori, Rami N. Sammour, Berend Isermann, Benjamin Brenner, Anat Aharon

Abstract—Microvesicles shedding from cell membrane affect inflammation, apoptosis, and angiogenesis. We hypothesize that microvesicles of women with gestational vascular complications reflect pathophysiological state of the patients and affect their endothelial and trophoblast cell function. Microvesicles of healthy pregnant women, women with gestational hypertension, mild, or severe preeclampsia/toxemia, were characterized, and their effects on early-stage or term trophoblasts and endothelial cells were evaluated using apoptosis, migration, and tube formation assays. Patient subgroups differed significantly only in proteinuria levels, therefore their microvesicles were assessed as 1 group, demonstrating higher levels of inflammatory and angiogenic proteins compared with those of healthy pregnant women. In endothelial cells, microvesicles of healthy pregnant women reduced caspase 3/7 activity, increased migration, and induced tube formation. These processes were suppressed by microvesicles of women with gestational vascular complications. In early-stage trophoblasts, microvesicles of healthy pregnant women decreased apoptosis compared with untreated cells (6±5% versus 13.8±5.8%; \( P < 0.001 \)) and caspase 3/7 activity and induced higher migration (39.7±10.1 versus 20.3±8.3 mm²; \( P < 0.001 \)). This effect was mediated through extracellular signal-regulated kinase pathway. Conversely, microvesicles of women with gestational vascular complications increased term trophoblast apoptosis compared with cells exposed to microvesicles of healthy pregnant women (15.1±3.3% versus 6.5±2.1%; \( P < 0.001 \)) and inhibited early-stage trophoblasts migration (21.4±18.5 versus 39.7±10.1 mm²; \( P < 0.001 \)). In conclusion, microvesicle content and effects on endothelial and trophoblast cells vary according to the physiological/pathological state of a pregnant woman. Microvesicles seem to play a pivotal role in the course of pregnancy, which could potentially result in gestational vascular complications. (Hypertension. 2013;62:893-898.)

Key Words: apoptosis ▪ cell movement ▪ endothelial cells ▪ hypertension ▪ preeclampsia ▪ pregnancy ▪ toxemia ▪ trophoblasts

Vascular complications, including gestational hypertension (HT) and preeclampsia/toxemia (PET), are major causes of maternal morbidity and fetal mortality. PET is characterized by poor placentaion and endothelial injury, which can lead to arterial hypertension, glomerular lesions, and hepatic failure. Distortion of angiogenic balance, a further increase in proinflammatory cytokines, deficiency in uteroplacental circulation, and impaired maternal endothelial function are key processes in this disease.

Trophoblast cells constitute the interface between maternal and embryonic vascular system. Their migration and invasion play a crucial role in placentaion, embryo implantation, and local hemostasis. Trophoblast differentiation and fusion to multinuclear cells require activation of caspases. However, antiapoptotic members of the Bcl-2 family upregulate the synctiotrophoblast after syncytial fusion, preventing execution of final apoptosis steps. Microvesicles (MVs) are membrane vesicles, found in blood circulation that can be divided to 2 subgroups: exosomes (<100 nm intracellular luminal vesicles that fuse with cell membrane and release their content) and microparticles (=1 µm vesicles), shed from cell surface membrane, which vary in their phospholipid and protein content and their antigens, reflecting their cellular origin. Although MVs are found in blood under normal physiological
conditions, their levels increase in vascular diseases. The number of erythrocyte and endothelial MVs increases in severe PET, and shedding of trophoblast MVs into maternal circulation is higher in early-onset than late-onset diseases. It is of interest that MVs of women with PET were found to promote vascular wall inflammation.

Our previous study, characterizing MVs of normal healthy pregnant (NHP) women and women with gestational vascular complications (GVCs), including HT and PET, showed that trophoblast MV levels were similar in these groups. However, endothelial CD144 MVs were elevated in the GVC groups compared with the NHP cohort, which may reflect the vascular injury typical of these pathologies. There are limited data on the alteration of MV content and function in GVCs. Regulatory mechanisms controlling human trophoblast differentiation, invasion, and endothelial dysfunction remain unclear.

We hypothesize that MVs of NHP women reflect their physiological state, whereas MVs of women with GVC correspond to their pathophysiological condition, and MVs of these populations differentially affect endothelial and trophoblast cell function. The current study aimed to explore the effects of MVs on endothelial and trophoblast cells in NHP and GVC, which could potentially contribute to the elucidation of the mechanisms involved in GVC development.

**Methods**

This study was conducted between 2006 and 2012. It was approved by the institutional review boards of the Rambam Health Care Campus (Approval No. 2030) and Bnaï-Zion Medical Center, Haifa, Israel (Israel Ministry of Health Approval No. 9200800), and all participants signed an informed consent form. Blood samples were collected during the third trimester from NHP women (n=112) and 3 subgroups of women with GVC classified in accordance with the Practice Guidelines of the American Congress of Obstetricians and Gynecologists:

- Group I, pregnant women with HT (n=29; blood pressure >140/90 mmHg without proteinuria after 20 weeks of gestation);
- Group II, pregnant women with mild PET (n=23; hypertension accompanied by proteinuria >300 mg per 24 hours);
- Group III, pregnant women with severe PET (n=19; blood pressure elevation ≥160/110 mmHg in presence of ≥1 of the following parameters: proteinuria ≥2.5 g in 24 hours, thrombocytopenia [<100 000 platelets/µL], pulmonary edema or cyanosis, symptoms of central nervous system dysfunction, hepatocellular injury, and HELLP syndrome (H) hemolysis, (E) elevated liver enzymes, (L) low platelet count).

**MV Isolation From Blood Samples**

Fifteen milliliters of peripheral venous blood were drawn from study participants into sodium citrate (3.2%) tubes; platelet-poor plasma (PPP) was obtained after 2 centrifugations (10 minutes, 1500 g) within 1 hour of collection and frozen at −80°C. MVs were isolated from thawed PPP by centrifugation (1 hour, 18 000g). Supernatant liquid was discarded, and MV pellets were used for cell culture stimulation. Only part of obtained samples was used in every assay.

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained at term of normal pregnancy, according to the previously described technique. Passages 4 to 8 were used for experiments.

Human term trophoblasts were obtained from term placenta of normal pregnancy (n=6). Cells were isolated and cultured as previously described.

Early-stage trophoblast (EST) cells isolated from second-trimester placenta were purchased from ScienCell (Carlsbad, CA). They were recultured in a modified medium containing 50% trophoblast medium with supplements (as provided by ScienCell), 22% DMEM, 22% F12, 4% FCS, 1% antibiotics (10 000 U/mL penicillin, 10 mg/mL streptomycin, 250 U/mL nystatin), and 0.0001% amphotericin B. Cells were incubated at 37°C, 5% CO2, and used for experiments at passages 4 to 15. ESTs were seeded in 6-well tissue culture plates and cultured for 1 to 2 days until 80% confluence. Because trophoblasts are the only cells producing the human placental lactogen (hPL) hormone, trophoblast cell cultures (EST and term) were labeled with anti-hPL to ensure quality control for their content. A culture where >90% of cells were labeled with anti-hPL was considered as a pure trophoblast culture.

**Antibodies and Dyes**

Cells and MVs were characterized using conjugated mouse antihuman antibodies: APC-Flt-1 (VEGFR-1), PE-KDR (VEGFR-2), APC-IgG1 Isotype control (R&D Systems, Minneapolis, MN), PE-CD41, PE-CD11a, PE-IgG1 Isotype controls (BD Biosciences; Pharmingen, CA), FITC tissue factor (American diagnostica, Stamford, CT), CD144 (R&D Systems, Minneapolis, MN), and NGDGI-IgM (Serotec, Bio-Rad Laboratories, Inc, United Kingdom). Additionally, rabbit anti-hPL (Epitomics, CA), anti-rabbit IgG, and anti-mouse IgG PE (Jackson, PA), antibodies against mitogen-activated protein kinases, extracellular signal-regulated kinases (Erk1/2), phosphor-p44/42 mitogen-activated protein kinases (Erk1/2) (Cell signaling technology, MA), ERK 2 (6G1) (Santa Cruz Biotechnology, California), anti-fibronectin (SIGMA, Missouri), and DRAQ5 (Biostatus Limited, United Kingdom) were used.

**Flow Cytometry Analysis**

MV size and granularity were evaluated by flow cytometer, CyAn ADP analyzer (Beckman Coulter). Forward scatter and side scatter parameters were set on logarithmic scales. The MV size was evaluated using standard Megamix (Biocytex, Marseille, France) and 0.78 µm beads (BD Biosciences; Figure S1A–S1C in the online-only Data Supplement).

MV concentrations were measured using 7.5 µm count beads (Flow Cytometer Absolute Count Standard, Bangs Laboratories Inc., Indiana), as described previously. MVs were labeled with antibodies for 30 minutes, suspended in PBS containing 0.02% formaldehyde, and scanned by the CyAn ADP analyzer. EST cells were fixed and permeabilized using BD Cytofix/Cytoperm Fixation/Permeabilization Kit according to the manufacturer’s instructions and were labeled with antibodies. The forward scatter and side scatter parameters were set on linear scales.

**Human Angiogenesis and Inflammatory Protein Content**

**Protein Array**

Purified MVs were resuspended in lysis buffer (20 mmol/L Tris pH=6.8, 150 mmol/L NAC, 1 mmol/L dithiothreitol, 10% glycerol, and 1% triton [final pH=7.5]). MV protein extract was obtained from a pool of 4 specimens within each study group and quantified using the BCA protein quantification kit. Twenty-five micrograms of cell proteins were loaded on the SDS-PAGE (10%) gel followed by transfer to the polyvinylidene fluoride membrane. The

- protein kinases (Erk1/2) (Cell signaling technology, MA), ERK 2 (6G1) (Santa Cruz Biotechnology, California), anti-fibronectin (SIGMA, Missouri), and DRAQ5 (Biostatus Limited, United Kingdom) were used.

**ELISA**

MVs were isolated from 1 mL of PPP and lysed by freeze-thaw cycles. The ELISA kits of MMP9, VEGF-A, and PDGF (Bender MedSystems, California) were used according to the manufacturer’s instructions and were labeled with antibodies. Membranes were analyzed using TotalLab software.

**Western Blot**

Cells were seeded in 6-well tissue culture plates and cultured for 1 to 2 days. For each study group, MV pellets from 4 mL of PPP (from individual samples and from a pool of samples) were added to the cells for 15-minute incubation. Then, cells were washed and incubated with 2.5% trypsin, which was neutralized with PBS+10% FCS. Cell pellet was lysed in the lysis buffer and incubated on ice for 40 minutes. Total protein was measured with the BCA protein quantification kit. Twenty-five micrograms of cell proteins were loaded on the SDS-PAGE (10%) gel followed by transfer to the polyvinylidene fluoride membrane. The
membrane was blocked and probed overnight at 4°C with primary antibodies (Erk1/2, phosphor-p44/42 Erk1/2, ERK 2, or anti-β-actin) and with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were detected by the enhanced chemiluminescence reagent (Thermo Fisher Scientific Inc, Illinois).

Interactions Between MVs and ESTs

ESTs were characterized using anti-hPL and CD41. MVs were labeled with antihuman PE-conjugated CD41 antibodies. Stained MVs were added to ESTs and incubated for 1 hour at 37°C. Finally, samples were fixed with 0.5% formaldehyde. Cells were scanned using the Amnis flow cytometry analysis device (Amnis, Seattle, WA). Cell fluorescence intensity was analyzed with Ideas 4.0, an ImageStream instrument. A gate was created to differentiate between cell’s membrane and cytosol areas in each image. MVs were quantified as the number of spots per area and also as total fluorescence per area.

Apoptosis

TUNEL Assay

Cells (HUVEC, EST, term trophoblasts) and an MV pellet (isolated from 2 mL of PPP obtained from several individual samples) were cocultured in 48-well tissue culture plates for 20 hours. Cells treated with 50 U of DNase (Sigma-Aldrich, Israel) for 10 minutes served as a positive control. Then, cells were washed, and the TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) assay (Roche Diagnostics, Mannheim, Germany) was performed according to the manufacturer’s instructions. Acquirement was performed using flow cytometry analysis device and by fluorescence microscopy. Results were expressed as percentage of TUNEL positive cells out of the total cell population in each well.

Caspase 3/7 Activity

Cells (HUVEC, EST, term trophoblasts) were seeded in 48-well tissue culture plates, and an MV pellet (isolated from 1 mL of PPP obtained from several individual samples) was added for 20-hour incubation. Cells treated with 1 μmol/L staurosporine served as a positive control. Then, cells were washed, and caspase 3/7 activity assay was performed according to the manufacturer’s instructions (CASPAS 3&7 FLICA KIT, ImmunoChemistry Technology, Minnesota). Acquirement was performed using flow cytometry analysis device and by fluorescence microscopy. Results were expressed as percentage of active caspase 3/7 positive cells out of the total cell population in each well.

Bcl-2 mRNA Expression

Cells (HUVEC, EST, term trophoblasts) were seeded in 24-well tissue culture plates with or without MVs pellet from 2 mL of PPP (obtained from individual and pool samples) and were cultured for 6 hours. Total RNA was extracted with a TRI-Reagent kit (Molecular Research Center, Cincinnati, OH). RNA concentration and purity were determined by UV absorption at 260 and 280 nm (NanoDrop). cDNA was constructed with the cDNA synthesis kit (Applied Biosystem). mRNA expressions of Bcl-2 gene were evaluated with ABI 7700TM quantitative real-time PCR system (Applied Biosysm) and compared the use of pool GVC samples. All reagents were obtained from Applied Biosystems. Results were expressed as relative quantification.

Migration Assay

Cell migration was measured using 24-transwell inserts (BD Biosciences) coated with Poly-L-Lysine. Cells (HUVEC, EST) were added to the upper chamber, whereas MVs (isolated from 1 mL of PPP obtained from individual samples in the serum-free medium) were added to the lower chamber (medium without MVs was used as control). After 24 hours, the inserts were fixed with 4% formaldehyde and stained with 0.5% crystal violet for 10 minutes each. Cells on the top of the membrane were removed, and remaining cells on the bottom side of the membrane were photographed using inverted microscopy. Additionally, several inhibitors of cell signal transduction were used to examine the involvement of MVs in trophoblast cell migration. The following inhibitors were assessed in the presence or absence of NHP-MVs: SB203580 (p38 inhibitor), SP600125 (c-Jun N-terminal kinase inhibitor), and PD98059 (MEK 1 inhibitor) and U0126 (MEK 1/2 inhibitor). The area occupied by migratory cells was calculated using the image J software.

Tube Formation Assay

HUVECs were seeded on matrigel (Sigma-Aldrich, Rehovot, Israel) in 48-well tissue culture plates with medium free of serum and growth factors. MVs were isolated from 1 mL of PPP obtained from individual samples. MVs were added to the wells for 20 hours. The wells were continuously photographed, using time lapse imaging (Zeiss, Standort Göttingen, Germany). The length of formed tubes was measured using Image-Pro Plus software (Leeds Precision Instruments, Minneapolis, MN) and was normalized to the initial cell number in each well.

Statistical Analysis

The data were analyzed using GraphPad 5 software. Results were assessed by 1-way ANOVA, Bonferroni multiple comparisons test. When only 2 groups were compared, t test was used. P<0.05 was considered statistically significant. The results were expressed as a mean±SD. The exact numbers of each performed experiment (n) appear at the bottom of each graph and in Tables (see also the Tables in the online-only Data Supplement).

Results

Study Population

Blood samples were collected from 183 pregnant women. All study cohorts were similar in age. The gestation period at sampling was shorter in the combined GVC group as well as each of the GVC subgroups (HT, mild or severe PET) compared with the NHP cohort, without significant differences between the GVC subgroups. There was a significant increase in systolic and diastolic blood pressure in the combined GVC and each of GVC subgroups compared with NHP, without significant differences between the GVC subgroups (Table). Proteinuria level significantly increased in severe PET compared with HT or mild PET. Fetal weight was found to be significantly lower in the combined GVC group compared with NHP, specifically when compared with mild or severe PET; however, the Apgar score 5 minutes after birth was similar across the study groups.

The fact that all parameters apart from proteinuria level did not significantly differ between the GVC subgroups justified the use of pool GVC samples.

MV Characterization

We found a large variation in the MV numbers among the individuals in each of the groups; therefore, the MV numbers did not significantly differ between the study groups. Characterization of membrane antigen aiming to define MV cell origin demonstrated similar levels of platelet MVs (CD41) and trophoblast MVs (NDOG1) in all the groups. However, levels of endothelial MVs (CD144) and leukocyte MVs (CD11a) appeared to be low in the NHP group compared with the GVC group, without significant difference between the GVC subgroups (Table S1, Figure S2A–S2D).

A comparing screen of MVs obtained from NHP and combined GVC group was done using the protein array, revealing differences in the content of inflammatory and angiogenic proteins. Of 43 proteins screened, 12 were not different, 2 were reduced, whereas the remaining 29 were higher in the GVC-MV compared with NHP-MV (Figure S1A and S1B). MVs expressed comparable levels of vascular endothelial growth factor receptors (Flt-1 and KDR) and similar levels
of matrix metalloproteinase-9, vascular endothelial growth factor A, and platelet-derived growth factor (Table S2).

**MV–Cell Interaction**

EST cells expressed high levels of the hormone hPL (93%) but did not express the platelet marker CD41 (Figure S2E–S2G). Coincubation of CD41 fluorescent-labeled NHP-MVs with unlabeled endothelial cells demonstrated that 45% of MVs were internalized into the cells whereas the rest of MVs bound to cell membranes. Coincubation of CD41 fluorescent-labeled NHP-MVs with unlabeled EST resulted in penetration of ≈90% of MVs into EST cells and changed their fluorescent intensity (Figure S2H and S2I). Exposure of term trophoblast cells to starvation conditions (serum-free medium) caused shedding of MVs from cell surface as depicted by confocal microscopy (Figure S3). Trophoblasts and shed MVs were covered with tissue factor (labeled in green) and contained nucleic acid (blue).

**Apoptotic Effect of MVs**

Exposure of endothelial, EST, and term trophoblast cells to circulating MVs demonstrated different effects. MVs obtained from study groups did not affect either HUVEC apoptosis or Bcl-2 gene expression. NHP- and GVC-MVs significantly decreased caspase 3/7 activity of HUVEC compared with untreated cells (9.36±2.42% or 10.09±1.55% versus 14.2±1.8%; *P*<0.001; Figure 1B). MV apoptotic effects on trophoblasts depended on pregnancy stage. NHP-MVs decreased EST apoptosis and caspase 3/7 activity compared with untreated cells (TUNEL: 6±5% versus 13.8±5.8%; *P*<0.001 and caspase 3/7 activity: 0.9±1.0% versus 4.1±0.9%; *P*<0.001, respectively; Figure 1A and 1B). In contrast, there was no significant reduction in apoptosis in response to GVC-MVs compared with untreated cells in both assays: TUNEL (9.7±6.9% versus 13.8±5.8%) and caspase 3/7 activity (2.6±1.9% versus 4.1±0.9%). Bcl-2 gene expression (Figure 1C) significantly decreased because of exposure of ESTs to GVC-MVs (1.3±2.4 relative quantification; *P*<0.05) compared with untreated cells (4.4±3.8 relative quantification). Conversely, NHP-MVs associated with a trend of nonsignificant increase in Bcl-2 expression. Remarkably, and in contrast to ESTs, we observed increased cell apoptosis and caspase activity in term trophoblasts exposed to GVC-MVs, compared with untreated cells or NHP-MVs treated cells.

**Table. Clinical Data of the Study Population**

<table>
<thead>
<tr>
<th>Characteristic Parameters</th>
<th>NHP</th>
<th>Total GVC</th>
<th>HT</th>
<th>Mild PET</th>
<th>Severe PET</th>
<th><em>P</em> Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>112</td>
<td>71</td>
<td>29</td>
<td>23</td>
<td>19</td>
<td>NS*†‡§</td>
</tr>
<tr>
<td>Age, y</td>
<td>31.81 (4.78)</td>
<td>31.48 (6.54)</td>
<td>31.86 (6.33)</td>
<td>31.26 (6.72)</td>
<td>31.16 (6.97)</td>
<td>NS*†‡§</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>112 (12.25)</td>
<td>148 (14.24)</td>
<td>149 (10.46)</td>
<td>141.3 (16.81)</td>
<td>154 (13.44)</td>
<td><em>P</em>&lt;0.001*†‡§ NS</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>66 (11.08)</td>
<td>91 (10.39)</td>
<td>91 (7.98)</td>
<td>88 (12.25)</td>
<td>95 (10.29)</td>
<td><em>P</em>&lt;0.001*†‡§ NS</td>
</tr>
<tr>
<td>Gestational age at sampling, wk</td>
<td>38.78 (1.50)</td>
<td>35.8 (3.09)</td>
<td>36.67 (2.78)</td>
<td>35.3 (2.57)</td>
<td>34.5 (3.52)</td>
<td><em>P</em>&lt;0.001*†‡§ NS</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>Negative</td>
<td>1018 (1375)</td>
<td>201.6 (75.16)</td>
<td>852.3 (626.5)</td>
<td>2411 (2191)</td>
<td>NS</td>
</tr>
<tr>
<td>Fetal weight, g</td>
<td>3304 (476.1)</td>
<td>2850 (798.3)</td>
<td>3181 (435.9)</td>
<td>2835 (784.6)</td>
<td>2328 (927.6)</td>
<td><em>P</em>&lt;0.001*, P&lt;0.01‡ NS</td>
</tr>
<tr>
<td>APGAR (5 min after birth)</td>
<td>9.756 (0.71)</td>
<td>9.662 (0.62)</td>
<td>9.731 (0.53)</td>
<td>9.818 (0.39)</td>
<td>9.353 (0.86)</td>
<td>NS*†‡§</td>
</tr>
</tbody>
</table>

The study population included women with normal healthy pregnancy (NHP), a combined group of pregnant women with gestational vascular complications (GVCs), and 3 subgroups of women with GVCs: pregnant women with hypertension (HT), pregnant women with mild preeclampsia/toxemia (PET), or severe PET. The data are presented as mean (SD). BP indicates blood pressure; and NS, not significant.

Statistical significance: *NHP vs total GVC; †NHP vs HT; ‡NHP vs mild PET; §NHP vs severe PET; ||HT vs mild PET; ¶HT vs severe PET; #mild PET vs severe PET.

**Figure 1.** Microvesicle (MV) effects on endothelial, early-stage trophoblast (EST), and term trophoblast cell apoptosis. Human umbilical vein endothelial cells (HUVECs), ESTs, and term trophoblasts were exposed to MVs obtained in study groups. MV effects on cell apoptosis were evaluated by the TUNEL assay (A), caspase 3/7 activity assays (B), and Bcl-2 gene expression (C). GVC-MVs indicates MVs of women with gestational vascular complication; and NHP-MVs, MVs of normal healthy pregnant women; HUVEC groups, *P*<0.001***; EST groups, *P*<0.05+, *P*<0.01++, *P*<0.001+++.
Microvesicle (MV) effects on trophoblast cell migration via extracellular signal-regulated kinase (ERK) pathway. Early-stage trophoblast (EST) cells were seeded on 24-transwell inserts. MVs obtained from the study groups were added to the lower chamber medium. PD98059 (MEK 1 inhibitor) and U0126 (MEK 1/2 inhibitor) were added to the ESTs 1 hour before incubation with MVs of normal healthy pregnant women (NHP-MVs). Migrated cells were revealed using inverting microscopy (Figure S6E). Migration rates were calculated using the ImageJ software. The areas covered by migrated cells are presented by the graph. GVC-MVs indicates MVs of women with gestational vascular complication.

versus 2.7±1.2% and 6.4±2.2%; *P <0.001; caspase 3/7 activity: 37.3±12.4% versus 16.5±10.3%; *P <0.001 and 23.2±11.2; *P <0.01, respectively; Figure 1A and 1B). In addition, exposure of term trophoblasts to GVC-MVs resulted in nonsignificant reduction in Bcl-2 gene expression compared with NHP-MVs.

Migration Effects of MVs
Exposure of endothelial cells to circulating NHP- and GVC-MVs induced significant cell migration without differences between the study groups (16.0±4.8 mm² and 15.6±2.9 mm²; *P<0.001, respectively) compared with untreated cells (3.4±0.6 mm²; Figure S4). NHP-MVs significantly increased EST migration (39.7±10.1 mm²; *P<0.001) compared with untreated cells (20.3±8.3 mm²), whereas GVC-MVs failed to induce EST migration (21.4±18.5 mm²) and their effects were similar to those of untreated cells (Figures 2 and S5A–S5E). In addition, we found increased ERK phosphorylation after exposure of ESTs to NHP-MVs and a lower rate of phosphorylation in the cells exposed to GVC-MVs (Figure S5F). To evaluate the MV involvement in cell signaling, several inhibitors of signal transduction were used. The combination of MEK1 inhibitor with NHP-MVs failed to block EST-ERK phosphorylation or cell migration (33.7±10.3%) and induced migration rate similar to that of NHP-MVs alone (Figures 2 and S5). However, the combination of MEK1/2 inhibitor with NHP-MVs significantly reduced cell migration (10.8±4.8%; *P<0.001) and demonstrated a trend to reduction in ERK phosphorylation. P38 inhibitor did not obstruct migration at any examined concentration (25–100 μmol/L). While low doses (5 μmol/L) of c-Jun N-terminal kinase inhibitor did not affect migration, its higher dose (25 μmol/L) affected cell viability and consequently prevented migration (data not shown).

Angiogenic Effect of MVs on Endothelial Cells
Figure S7 displays the diverse influence of NHP- and GVC-MVs on angiogenesis. In the serum-free medium without MVs (control), HUVECs formed a small unstable network that rapidly collapsed. When HUVECs were incubated with NHP-MVs, a branched network was formed within 1 hour (Figure S6A and S6B). HUVEC incubation with GVC-MVs resulted in a small amount of tubes (Figure S6C–S6E). The characteristics and life span of this network were very similar to those described for the control group (Tables S1–S3 and Figures S1–S6).

Discussion
PET is a major disorder of pregnancy characterized by an increased maternal inflammatory response and vascular dysfunction, affecting placentation, trophoblast proliferation, invasion, and apoptosis.

The current study demonstrated that NHP women and women with GVC displayed similar levels of circulating MVs, which is in accordance with our previous report and other studies. However, we found that the MV protein content differed between the groups. Particularly, the GVC-MVs presented higher levels of pro- and antiangiogenic proteins and proinflammatory cytokines that may be characteristic of these pathologies.

As GVCs can occur at various gestational stages, the present study evaluated MV effects on different trophoblast cultures obtained at early stage and term of gestation. ESTs and term trophoblasts were found to respond differently to MVs. Although NHP-MVs increased EST survival, they induced apoptosis in term trophoblasts. In contrast, GVC-MVs did not affect EST survival but caused massive apoptosis in term trophoblasts, which indicates the involvement of MVs in placental aging and demonstrates that MVs regulate trophoblast apoptosis. In general, apoptosis pathways have a dual nature in the placenta, affecting both trophoblast survival and differentiation. Normal pregnancies are characterized by a low rate of trophoblast apoptosis that increases as gestation proceeds, whereas in PET, the hallmark is a significantly higher rate of trophoblast apoptosis and abnormal placental invasion, which could ultimately result in early delivery and pregnancy loss.

What triggers human preterm or term labor is not entirely clear. The involvement of prostaglandins and proinflammatory cytokines has been suggested as a potential mechanism. It is possible that the elevation in the apoptosis rate induced by potent GVC-MVs bearing significant amounts of concentrated inflammatory cytokines, as revealed in our study, contribute in part to early delivery observed in women with GVCs.

Cell migration and invasion play a crucial part in the development of uteroplacental circulation. The present study showed that endothelial and EST cell migration was induced by NHP-MVs and inhibited by GVC-MVs. In our opinion, these results suggest a role of NHP-MVs in preserving endothelial and trophoblast function, whereas the GVC-MVs could be major players in the antiangiogenic milieu, potentially impairing endothelial function. This may explain a mechanism of placental implantation failure at early stages of pregnancy observed in women with PET.

The current study is the first to demonstrate that MVs affect trophoblast migration through altering ERK signal transduction. Mitogen-activated protein kinase pathways are known to participate in a wide range of biological processes, and ERKs are the major modulators of trophoblast invasion and migration.

The present study has some limitations. Gestational HT and preeclampsia are complex pathologies, and the study population was not homogeneous. Despite a relatively large number of participants, not every assay was performed in all patients.

In conclusion, our findings demonstrate that the MV content differs in healthy and pathological gestational states. MVs
play a crucial role in angiogenesis, inflammation, and apoptosis of endothelial and trophoblast cells, which affect their function and fate and can influence placental implantation and vascularization at an early gestational stage, potentially affecting pregnancy development and duration.

**Perspectives**

The current study, using human samples, has demonstrated that GVC-MV content reflects the perturbation in angiogenic and inflammatory proteins, characterizing this type of pathological pregnancies. MVs may affect placenta in a stage-specific pattern. At early stages of pregnancy, MVs decrease trophoblast apoptosis and enhance migration, both functions being crucial for proper placenta. Strikingly, this MV effect on ESts is lost in women with GVC. In contrast, GVC-MVs were found to promote apoptosis of term trophoblast cells, which may suggest their involvement in preterm delivery. MVs, both shed from the syncytial surface and circulating, are localized for relatively long periods in the intervillous space as a consequence of the low shear stress in this area, thereby affecting trophoblast and endothelial function. Although larger studies are warranted to confirm these results, the contribution of MVs to the development of NHPs and GVC may pave the way for relatively long periods in the intervillous space as a consequence of the low shear stress in this area, thereby affecting trophoblast and endothelial function. Although larger studies are warranted to confirm these results, the contribution of MVs to the development of NHPs and GVC may pave the way to better understanding of normal pregnancy physiology and improved management of these major pathologies.

**Acknowledgments**

We thank David Merkel Golan and Ariel Roytman of Merkel Technologies for their expert assistance in using the Amnis ImageStream Multispectral Imaging Flow Cytometer.

**Sources of Funding**

This study was supported by grants of the German-Israeli Foundation for Scientific Research and Development and Rappaport Family Institute for Research in the Medical Sciences,

**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is New?**

- Physiological effects of microvesicles (MVs) of normal healthy pregnant (NHP) women on endothelial and trophoblast cells can be partially diminished by MVs of women with gestational vascular complication (GVC-MVs).
- GVC-MVs vary according to gestational age and cell type.
- GVC-MVs present significantly higher levels of pro- and antiangiogenic proteins as well as proinflammatory proteins compared with NHP-MVs.
- NHP-MVs decrease early-stage trophoblast cell apoptosis, whereas GVC-MVs induce high level of term trophoblast apoptosis.
- NHP-MVs induce early-stage trophoblast migration involving extracellular signal-regulated kinase 2 signal transduction pathway, whereas GVC-MVs reduce early-stage trophoblast cell migration.
- NHP-MVs induce endothelial tube formation which is inhibited by GVC-MVs, pointing to their role in endothelial dysfunction related to preeclampsia.

**What Is Relevant?**

- Gestational hypertension and preeclampsia are major causes of maternal morbidity and fetus mortality.
- Hypertension/preeclampsia/eclampsia MVs significantly affect mechanisms related to trophoblast and endothelial dysfunction, contributing to hypertension.

**Summary**

These findings demonstrate the significant role of MVs in angiogenesis, inflammation, and apoptosis, determining endothelial and trophoblast cell function. MVs were found to have an impact on the development of normal pregnancies and GVCs.
Microvesicles of Women With Gestational Hypertension and Preeclampsia Affect Human Trophoblast Fate and Endothelial Function
Einat Shomer, Sarah Katzenell, Yaniv Zipori, Rami N. Sammour, Berend Isermann, Benjamin Brenner and Anat Aharon

Hypertension. 2013;62:893-898; originally published online September 30, 2013; doi: 10.1161/HYPERTENSIONAHA.113.01494

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http://hyper.ahajournals.org/content/suppl/2013/09/30/HYPERTENSIONAHA.113.01494.DC1
Online Supplement Data- Title page

MS ID: HYPE201301494
Microvesicles of women with gestational hypertension and preeclampsia affect human
trophoblast fate and endothelial function

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Short title: HT/PET-MPs affect trophoblast and endothelial function

Word count: 5319+600 (4 figures)=5919
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Table S1: Microvesicle (MV) count and cell origin characterization

MVs count/μl was evaluated using count beads (7μ). Cellular origins of MVs obtained from normal healthy pregnant (NHP) women and three subgroups of women with gestational vascular complications (GVC) [hypertension (HT), mild and severe preeclampsia/toxemia (PET)] were determined by labeling with cell-specific antibodies. Acquisition was then performed using a FACS device. Cellular origins are expressed as the proportions (%) of positively labeled MVs.

The data are presented as mean (Std. Deviation). Statistical significance: NHP vs. total GVC – *; NHP vs. HT – †; NHP vs. mild PET –‡; HP vs. severe PET –§; HT vs. mild PET –||; HT vs. severe PET –¶; mild PET vs. severe PET–#.
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**Table S2: Microvesicles (MV) angiogenic protein content**

MVIs were isolated from 1 ml of PPP and lysed by freeze-thaw cycles. The ELISA kits of MMP9, VEGF-A and PDGF (Bender MedSystems, CA, USA) were used according to the manufacturer's instructions.
Supplement figures

Figure S1: Angiogenic and inflammatory protein content of microvesicles

Protein content of 4 pooled specimens of NHP-MVs and GVC-MVs was screened using the Human Angiogenesis Protein Antibody Array. Levels of GVC-MVs are expressed as % of NHP-MVs. A) Pro- and anti-inflammatory MV proteins. B) Pro- and anti-angiogenic MV proteins.
Figure S2:

A. Megamix beads

B. 0.78µm beads

C. NHP MVs: IgG PE (blue) CD41 (red)

D. NHP MVs: IgG PE (blue) hPL (red)

E. EST cells

F. EST cells IgG PE (blue) hPL (red)

G. EST cells IgG PE (blue) CD41 (red)

H. HUVEC

Cells BF Cells and Overlay Membrane bound: Cytosol

I. EST- trophoblast 24 weeks
Figure S2: MV characterization and their interaction with endothelial and trophoblast cells

(A-B). MV size evaluation gate (R1) established using Megamix beads (0.5μm, 0.9μm, 3μm) and 0.78μm beads with side scatter (SS) and forward-scatter (FSC) in logarithmic scale. (C). HP-MVs population (SS and FSC, gate R1). (D). HP-MVs labeled with anti-CD41-PE antibody (red) compared to IgG PE as negative control (blue). (E). EST cells: SS and FSC are proportional to the cell-surface area or size in linear scale. (F). EST labeled with anti-hPL-PE antibody. (G). EST labeled with anti-CD41-PE antibody. (H). HP-MVs labeled with anti-CD41-PE (red) and added to EST. Images of cell MVs uptake were taken using the AMNIS FACS device [bright field (BF), column I, fluorescence (orange) columns II,III]. A gate was created to differentiate between the entire cell volume (column II) and the cell surface. PE-MV signal was overlaid on the membrane gate (column III). (I). Summarized data on cell fluorescence intensity: cytosol penetrating MVs (right from 0 X-axis) versus membrane bound MVs (left from 0 X-axis). Level of fluorescent signal in the membrane and cytosol area was measured. The membrane signal was considered a negative value, while the cytosol signal was considered a positive value.
Figure S3: Term trophoblast cells and MVs

Term trophoblast cells were labeled with anti-TF-FITC (green). DRAQ5 (blue) was used for nuclei staining. Cells were viewed using the Biorad confocal microscope at x 60 magnification. Arrows indicate shedding of MVs from cell surface.
Figure S4: MV effects on endothelial cells migration

HUVEC were seeded on 24-transwell inserts. MVs obtained from study groups were added to the lower chamber medium. After 20 hours the inserts were fixated and stained. Membranes were photographed using inverted microscopy. Migration rates were calculated using the image J software. (A) Cells without MVs. (B). Cells with NHP-MVs. (C). Cells with GVC-MVs. (D). Migration rates are summarized in graph.
Figure S5: MV effects on EST cells migration and EST- ERK phosphorylation

ESTs were seeded on 24- trans-well inserts. MVs obtained from the study groups were added to the lower chamber medium. Migrated cells were revealed using inverted microscopy (A-E). (A) Cells without MVs. (B). Cells with NHP-MVs. (C). Cells with GVC-MVs. (D, E). PD98059 (MEK 1 inhibitor) and U0126 (MEK 1/2 inhibitor) were added to the cells 1 hour before incubation with NHP-MVs. (F). MV effects on EST- ERK phosphorylation were evaluated by the Western blot analysis PD98059 (MEK 1 inhibitor) and U0126 (MEK 1/2 inhibitor) were added to the cells 1 hour before incubation with NHP-MVs.
Figure S6: MV effects on HUVEC tube formation

HUVEC were seeded on matrigel and photographed for 20 hours after MVs addition, using time lapse imaging. Magnification ×10. (A) Cells without MVs. (B). Cells with NHP-MVs. (C). Cells with GVC-MVs. (D). Total tube length was measured and normalized to the cell number in each well over 12-hour exposure. (E). Tube length after 2-hour exposure.