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Regular Article

Procoagulant activity and cellular origin of microparticles in human amniotic fluid

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ABSTRACT

Introduction: Amniotic fluid contains various procoagulant factors involved in intro-vascular amniotic fluid-induced coagulopathies. During the progression of normal pregnancy, microparticles would be shed off from cells and accumulate in amniotic fluid over time. In this study, our aims were to investigate the cellular origin and procoagulant entity of these microparticles.
Materials and methods: Twenty amniotic fluid samples from healthy parturient women were collected, and the microparticles were isolated and stained with phycoerythrin-labeled antibodies to CD138, CD41a, CD144 and CD11b to identify their cellular origin. Their phosphatidylserine and tissue factor expression levels were quantified with fluorescein isothiocyanate-labeled annexin V and anti-tissue factor antibody staining. Their procoagulant activity was tested with plasma coagulation assay and factor Xase and prothrombinase assays.
Results: Phenotypic analysis showed 36.8% and 33.8% of amniotic fluid microparticles positive for CD138 and CD11b, respectively, indicating their epithelial cell or leukocyte origin. Of these microparticles, 66.3 ± 5.9% expressed phosphatidylserine while 37.4 ± 4.1% expressed tissue factor. In addition, amniotic fluid microparticles could significantly shorten the plasma coagulation time and increase the production of factor Xa and thrombin. Inhibition assays with annexin V and anti-tissue factor antibody confirmed the coagulation effects of amniotic fluid microparticles.
Conclusion: The microparticles derived from epithelial and leukocytes may be a mechanism of amniotic fluid-induced coagulopathies.

Introduction

During pregnancy the hemostatic balance shifts to hypercoagulative state [1]. This physiological change can be partially explained by excessive procoagulants, such as coagulation factors and microparticles (MPs), released into maternal circulation [2,3]. Similarly, as pregnancy progresses, many amniotic fluid (AF) cells go through apoptosis and secrete MPs into AF. The fetal excreta may be another source for AFMPs. These AFMPs contain cell membrane compositions such as phosphatidylserine (PS) and tissue factor (TF) [4,5]. Both PS and TF are essential for factor Xa and thrombin formation [6,7].

AF contains procoagulant contents which can trigger and contribute to coagulation abnormalities in maternal circulation [8]. However the precise mechanism of this rare and fatal disorder remains unknown. Most of AF cells are exfoliated fetal epithelial cells [9]. Previously we have identified the procoagulant activity of apoptotic AF cells with PS and TF expression [10]. Interestingly, further investigations showed that the cell-free AF supernatant could significantly shorten plasma coagulation time in vitro. We speculate some factors, such as MPs, present in AF supernatant inducing coagulopathy cascade. The PS and TF expression of AFMPs may be evaluated by staining with different cell markers [11,12]. The coagulant activities of AFMPs may be further assessed by using functional coagulation assays.

This study aimed to determine the coagulant activity and cellular origin of AFMPs. We performed phenotypic analysis and functional assays of PS/TF-positive population of AFMPs in healthy full-term primiparas undergoing cesarean section. We found that, unlike the plasma microparticles (PMPs), most AFMPs were of epithelial and leukocytic origin. Furthermore, these microparticles were demonstrated in functional assays to have procoagulant activity.

Materials and Methods

Patients

AF samples from 20 healthy primiparas undergoing cesarean section at term (mean age: 25.6 years, range: 23–31 years) were harvested in this study from July 2010 to December 2012. Peripheral blood samples

Abbreviations: AF, amniotic fluid; MPs, microparticles; AFMPs, amniotic fluid microparticles; PMPs, plasma microparticles; PS, phosphatidylserine; TF, tissue factor; PE, phycoerythrin; FITC, isothiocyanate.

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from these primiparas and other 20 child-bearing aged healthy volunteers were obtained as controls. This study was performed with the approval of the hospital's Institutional Research Ethics Committee (HMUFH-RE 2010–11). Research samples were collected with permission from all parturient women with informed consent.

Sample Collection

AF was collected (30–50 ml from each patient) by amniocentesis during cesarean section, as described previously [10]. The samples were treated with 3.2% sodium citrate and centrifuged at 1,500 rpm for 15 min at 37 °C. The supernatant was centrifuged again at 18,000 rpm for 30 min, and then the precipitates were washed and resuspended with appropriate volume of Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.5 mM glucose, 5 mM Hepes, 0.35% bovine serum albumin (BSA), pH 7.4). Aliquots of AFMPs resuspension were snap-frozen in liquid nitrogen, and then stored at −80 °C. Control PMPs samples were isolated and treated the same as described above for AFMPs, and platelet-/microparticle-free plasma from non-pregnant volunteers was stored at −80 °C.

Flow Cytometry Aassays

These assays were performed to determine the cellular origin and the PS and TF expression of AFMPs. Five microliters of each microparticle samples were diluted with 35 μl of Tyrode's buffer. Events falling in the predefined microparticle gate (< 1.0 μm) that were fluorescein isothiocyanate (FITC)-annexin V (Beijing Biosynthesis Biotechnology Co., LTD., Beijing, China)-positive were defined and enumerated as PS-positive MPs [13]. The samples were then incubated separately with 5 μl of phycoerythrin (PE)-conjugated antibodies to CD41a, CD144, CD11b, CD138 and IgG (as control) (BD Becton Dickinson Biosciences, San Jose, CA, USA) in 200 μl of Tyrode's buffer for 30 min at room temperature in the dark to assess the cellular origin of MPs. The specific cell markers of platelet, endothelial cell, leukocyte and epithelial cell were selected in this study because the former three types of cells have significant coagulation function when activated or undergone apoptosis, and AF also contains certain amount of leukocytes and endothelial cells [14–17].

To detect the TF-positive MPs, FITC-anti-TF antibody (Beijing Biosynthesis Biotechnology Co., LTD., Beijing, China), CY5–annexin V and FITC-IgG (BD Becton Dickinson Biosciences, San Jose, CA, USA) (as a negative control) were also used in the same experimental conditions. To identify the cellular markers of TF-expressing MPs, the samples were incubated with CY5–annexin V, specific PE-conjugated phenotypic antibodies and FITC-anti-TF antibody. For each sample, control labeling was performed in parallel by incubating with FITC-IgG or PE-annexin V (BD Becton Dickinson Biosciences, San Jose, CA, USA). After incubation, all samples were analyzed on a flow cytometer (BD FACSAria; Becton, Dickinson and Company, NJ, USA).

The MP gate setting was calibrated by using Megamix beads of 0.5 and 0.9 um (BioCytex, Marseille, France). MPs of < 1.0 um were determined based on forward scatter (FS)/side scatter (SS) parameters according to the standard Megamix beads (Fig. 1A). Similarly, 1.0-μm latex beads (Invitrogen) were used for acquisition and analysis of MPs of < 1.0 um in all the samples. The MPs were identified on FS and SS, and binding of annexin V and cell-sepcific antibodies. The number of total MPs was calculated as Number/L = N × [1000 μl/(5 min × 12 μl/min)] × 10^3, as previously described[18].

Plasma Coagulation Time and Inhibition Assays

To primarily evaluate the coagulation function of AFMPs, their effect on plasma coagulation time was observed and determined. All reagents were preheated at 37 °C for the following experiments. Equal volume of stock AFMPs and control microparticle suspensions was diluted with Tyrode's buffer (vol./vol.) to 1:2, 1:4, 1:8, 1:16 and 1:32 (0.9% normal saline was used as placebo), and 70 μl of each sample was incubated with 70 μl of platelet-/microparticle-free plasma at 37 °C for 5 min. Subsequently 70 μl of 25 mM Ca^{++} was added into each reaction system to start clotting reaction and coagulation time was immediately measured by a KC4A-coagulometer (Amelung, Labcon, Heppenheim, Germany).

To separately define the procoagulant activity of PS and TF on MPs, each stock microparticle sample was incubated with 8, 16, 32, 64 and 128 nM annexin V or 12.5, 25, 50, 100 and 200 μg/ml anti-TF antibody (Beijing Biosynthesis Biotechnology Co., LTD., Beijing, China) in 70 μl of Tyrode's buffer for 10 min at room temperature. The mixtures were then incubated again with platelet-/microparticle-free plasma, and coagulation time measurement was started after the addition of 25 mM Ca^{++}, as described above.

Factor Xase and Prothrombinase Formation Assays

These assays were performed to further assess the effects of AFMPs on the activation of factor X and prothrombin, whereby microparticle samples were the only source of PS and TF. TBS (Tris-buffered saline) was prepared with 50 mM Tris (Sigma Chemical Co., St. Louis, MO, USA) and 150 mM NaCl (pH 7.4). Factor Xase buffer consisted of 1 ml of 10 × TBS, 200 μl of 10% BSA and 8.8 ml of dd-H_2O. Prothrombinase buffer was composed of 1 ml of 10 × TBS, 50 μl of 10% BSA and 8.95 ml of dd-H_2O. Stop buffer was prepared with 1 ml of 10 × TBS, 50 μl of 10% BSA, 320 μl of 0.5 M EDTA and 8.63 ml of dd-H_2O. In these assays, various amounts (10 μl, 5 μl, 2.5 μl, 1.25 μl, 0.625 μl and 0 μl) of stock microparticle samples were added into each reaction system. Each reaction was brought up to a final volume of 40 μl with Tyrode's buffer and 0.9% normal saline was used as the placebo for the following experiments.

Intrinsic factor Xase formation assay was started with the incubation of various amounts of each stock microparticle sample with 1 nM factor IXa, 130 nM factor X (Enzyme Research Laboratories, South Bend, IN, USA), 5 nM factor VIII, 0.8 nM thrombin (Haematologic Technologies Inc., Burlington, VT, USA) and 5 mM Ca^{++} in factor Xase buffer for 5 min at room temperature. The reaction was quenched by the addition of stop buffer. Subsequently 4 μl of 2 mM Chromogenix S-2765 (DiaPharma Group, West Chester, OH, USA) was added to each reaction system and factor Xa product was determined immediately at 75 sec interval for 15 min at 37 °C and 405 nm on a Molecular Devices ELISA plate reader. In prothrombinase formation assay, the samples were incubated with 1 nM factor Va, 1 μM prothrombin (Haematologic Technologies Inc., Burlington, VT, USA), 0.05 nM factor Xa (Enzyme Research Laboratories, South Bend, IN, USA) and 5 mM Ca^{++} in prothrombinase buffer for 5 min at room temperature. Stop buffer and then 4 μl of 2 mM Chromogenix S-2238 (DiaPharma Group, West Chester, OH, USA) were added into each microplate to evaluate the production of thrombin. Extrinsic factor Xase formation assay was performed in a reaction system containing 4 nM factor VIIa (Haematologic Technologies Inc., Burlington, VT, USA), 130 nM factor X, 5 mM Ca^{++} and factor Xase buffer, and factor Xa product was measured in the same way as above.

Inhibition Assays of Factor Xase and Prothrombinase Formation

To confirm the coagulation function of AFMPs with PS and TF expression, annexin V and anti-TF antibody were used as inhibitors in the following experiments. In inhibition assays of intrinsic factor Xase and prothrombinase formation, 10 μl of stock microparticle samples was incubated with varying amounts of annexin V for 10 min at room temperature. Then the mixtures were further incubated in intrinsic factor Xase and prothrombinase reaction systems, respectively, as described in factor Xase and prothrombinase formation assays. Subsequently stop buffer and reaction substrates were added into each reaction system, and the production of active enzymes were