Nanosized blood microparticles

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CHAPTER 7

Use of immuno-magnetic beads for direct capture of nanosized blood microparticles from plasma

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Abstract

The mechanism of cancer-associated thrombosis is still unclear. Recent data indicate that blood microparticles (MPs) carrying active tissue factor (TF) may play a role in cancer-associated thrombosis. Methods such as flow cytometry and impedance based-flow cytometry allow the analysis of MP subsets but give no information on which MPs carry active TF. Conversely, the MP-TF activity itself provides no information, on the cellular origin of the MPs carrying the active TF.

For this reason, we developed an immuno-magnetic beads method to capture subsets of MPs directly from plasma. The method was optimized for the capture of platelet-derived MPs (PMPs) from plasma. Only 100 µL platelet poor plasma (PPP) was needed in combination with 135 µL (27 µg) of biotinylated anti-human CD41 monoclonal antibody (MoAb) and 200 µL of streptavidin beads to achieve complete separation of PMPs from plasma. As a control biotinylated mouse IgG1 isotype control MoAb was used instead of the anti-CD41 MoAb.

Using biotinylated anti-CD14 MoAb, CD14-positive MPs were captured from normal plasma spiked with MPs isolated from the supernatant of LPS-stimulated monocytes (MoMPs). TF activity was found both in the positive (selected) and negative (depleted) fractions indicating that both CD14-positive and -negative MoMPs have active TF.

We propose that this method can be used in the future to investigate the source of MPs carrying active TF in plasma of patients with cancer and other diseases.

Introduction

There appears to be a two-way association between cancer and thrombosis. Cancer is associated with a 4-7 fold increased risk for venous thrombosis (VTE) (1;2), while on the other hand about 10% of patients with an episode of idiopathic VTE will develop cancer within 2 years after the event (3;4). Cancer patients who developed thrombosis also have a poor survival compared to those who did not develop thrombosis (5). Some studies indicate that in cancer patients blood microparticles (MPs) play a role both in the development of VTE and in the progression of disease. MPs, also named microvesicles, are nanosized vesicles which are released from virtually all cells of the body upon activation or apoptosis (6;7) and still express proteins derived from their parental cells (8;9). Some MPs also may carry active tissue factor (TF), a 47 kDa transmembrane glycoprotein, which is the main activator of blood coagulation (10-12). Tesselaar et al (11) reported that cancer patients with disseminated breast and pancreatic cancer, who presented with acute VTE, had higher levels of MP-associated TF activity (MP-TF activity) than healthy subjects, cancer patients without VTE and subjects with idiopathic VTE. Interestingly, in some of these patients a small fraction of their blood MP expressed MUC-1 antigen which raises the question whether a subset of MPs with TF activity is derived from tumour cells themselves. Increased MP procoagulant activity is not only found in cancer patients, but also in patients with cardiovascular and inflammatory diseases (7;13;14)

Blood MPs used to be known as ‘platelet dust’ which was obtained after ultracentrifugation of platelet free plasma (15). Most MPs have sizes below 1 µm and the detection of MPs with sizes less than 0.5 µm is not sensitive when using a commonly applied method such as flow cytometry or bright field microscopy (16;17). Recently, our group reported the use of atomic force microscopy (AFM) operated in fluid-tapping mode to detect platelet-derived MPs (PMPs) and showed that AFM enables sensitive detection of MPs with diameter ranging from 10-475 nm (mean: 67.5 ± 26.5 nm), far below the lower limit of what can be detected by conventional flow cytometry (18).

However, up till now there is no method available to identify the origin of the subsets of plasma MPs carrying active TF. Current methods using flow cytometry (8) and impedance based-flow cytometry (19) only allow the identification of subsets of MPs carrying TF antigen in plasma but provide no information with regard to which MPs carry active TF. In this context it is important to mention that most of the TF antigen in plasma is encrypted and not biologically active (20;21).

For the measurement of MP-TF activity MPs need to be separated from the plasma proteins (11;12;22;23), after which the TF activity of the isolated MPs is measured as the FVII-dependent factor Xa (FXa) generation. However, there is no easy way to measure TF activity in selected subsets of MPs.

For this reason, we developed an immuno-magnetic beads method to directly capture a subset of plasma MPs. The method was optimized for the separation of PMPs, which form about 80% of the total blood MP population (7;9;11), and further tested for capturing monocyte-derived MPs (MoMPs). Biotinylated anti-human CD41 and CD14 monoclonal antibodies were coupled to streptavidin-magnetic beads to separate CD41- and CD14-positive MPs from plasma. Subsequently, the separated fractions were used to isolate and analyze MPs for specific antigens and TF activity.

In this study we demonstrate that the use of immuno-magnetic beads can specifically and efficiently separate a subset of MPs from plasma. We propose to apply this method for further use in the identification of the MP subset(s) carrying active TF in patients.

Materials and Methods

Reagents

The µMACS™ streptavidin kit and CD14 MACS MicroBeads were purchased from Miltenyi Biotec Inc (CA, USA). Biotinylated anti-human CD41 monoclonal antibody (MoAb) clone P2 and biotinylated mouse IgG1 isotype control MoAb were from Beckman Coulter (Marseille, France). Biotinylated anti-human CD14 MoAb clone M5E2 and biotinylated mouse IgG2a, κ isotype control MoAb were from Biolegend (CA, USA). For PMP staining, anti-human CD41- phycoerythin (PE) MoAb clone P2 and anti-human CD61- fluorescein isothiocyanates (FITC) MoAb clone SZ21 from Beckman Coulter were used. MoMPs were stained by anti-human CD14-PE MoAb clone M5E2 from BD Pharmingen (CA, USA) and anti-human tissue factor (TF)-FITC MoAb clone CLB/TF-5 from Sanquin (Amsterdam, The Netherlands). For controls, mouse IgG1 isotype control-PE and -FITC MoAbs clone X40 from Becton Dickinson (CA, USA) and mouse IgG2a, κ isotype control-PE MoAb clone G155-178 from BD Pharmingen were used. Recombinant human annexin V-allophycocyanin (APC) from Caltag Laboratories (CA, USA) was used to stain MPs bearing phosphatidylserine. Megamix beads were purchased from Biocytex (Marseille, France) to calibrate the FACS Calibur flow cytometer. For counting the absolute number of MPs in samples, flow cytometry absolute count standard beads from Bang Laboratories (IN, USA) were used. These beads have a mean diameter

of 7.58 µm per bead and a known concentration. Hepes buffer consists of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (Merck, Darmstad, Germany), 137 mM NaCl (Merck), 4 mM KCl (Merck) and 0.1 mM Pefabloc SC (Fluka, Munich, Germany). Hepes-citrate buffer was prepared by adding 10.8 mM sodium citrate (Merck) to the Hepes buffer without Pefabloc. All buffers were set to pH 7.4. For the isolation of human monocytes, phosphate buffered saline (PBS) pH 7.2 was used (LUMC Pharmacy, Leiden, The Netherlands). For some experiments PBS was supplemented with 0.5% bovine serum albumine (BSA) (Sigma-Aldrich, MO, USA) and 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) (pH 7.2) (PBS-BE). Ficoll-amidotrizoate was from LUMC Pharmacy. Lipopolysaccharide (LPS) from Sigma-Aldrich was used to stimulate human monocytes. For monocyte culture, RPMI (Invitrogen, Breda, The Netherlands) was supplemented with 1% L-Glutamine (L-Glu) from Lonza (Cologne, Germany) and 1% Penicillin/Streptomycin (Pen/Strep) from Invitrogen.

Blood collection and plasma preparation

After giving informed consent venous blood was collected from three healthy volunteers by using a 21-gauge needle (BD Vacutainer; Becton Dickinson) with minimal stasis. After discarding the first 4 mL of blood, blood was collected in 1/10 volume of sodium citrate (3.2%, 0.105 M) using a 4.5-mL BD Vacutainer tube (Becton Dickinson). Within 10-15 minutes (min) of withdrawal, blood was centrifuged at 2,000g for 10 min at 20°C, without brake. Plasma was carefully collected and centrifuged again at 2,000g for 10 min at 20°C, without brake, to obtain platelet-poor plasma (PPP). PPP was aliquoted in 250-µL portions, snap frozen in liquid N_2, and stored at -80°C. Before use, plasma was thawed quickly with a gentle swirl at 37°C.

Eighteen mL of venous blood was collected in 4.5 ml K2 EDTA tubes (3.6 mg EDTA) (BD Vacutainer, Becton Dickinson). This blood was 3-fold diluted with PBS before use for monocyte isolation.

Human monocyte isolation and stimulation

Fifteen mL of Ficoll-amidotrizoate was pipetted to a Leucosep tube (Greiner Bio-One GmbH; Frickenhausen, Germany) and centrifuged in a swinging bucket rotor for 30 seconds (sec) at 1,000g, room temperature (RT), with brake. Diluted (3-fold) EDTA anticoagulated blood (15-30 mL) was carefully pipetted onto the filled Leucosep tube and centrifuged for 15 min at 800g, RT, without brake. The upper layer of plasma including the interphase (enriched in lymphocytes/mononuclear

cells) was harvested and placed into another centrifugation tube. PBS was added to a total volume of 50 mL and subsequently the cell suspension was centrifuged for 10 min at 300g, RT, with brake. The supernatant was removed and the cell pellet was resuspended in PBS and centrifuged for 10 min at 300g, RT, with brake. This washing step was repeated once. Finally, the cell pellet was resuspended in 5 mL PBS-BE. Cells were stained with Trypan blue (Sigma-Aldrich) to exclude dead cells counted by using a Bürker haemacytometer (Superior, Marienfeld, Germany).

CD14-positive cells were isolated using CD14 MicroBeads (Miltenyi Biotech) following the protocol of the manufacturer. Briefly, 80 µL of anti-human CD14 MACS MicroBeads (diameter: ~50 nm) were mixed with 320 µL of cell suspension ($4x10^7$ cells) and incubated for 15 min in the refrigerator (2-8°C). In the meantime, the MS column was placed on the miniMACS separator (both from Miltenyi Biotech). The column was rinsed with 500 µL of PBS-BE buffer. Then, the cell suspension was applied to the column and the flow through was collected. This flow through was applied again to the column and then discarded. The column was removed from the separator and placed on a collection tube. One mL of PBS-BE was pipetted into the column and immediately a plunger was applied to flush out the magnetically labelled cells.

For monocyte culture, cells were centrifuged for 10 min at 300g, RT, with brake. The cell pellet was resuspendend, counted, and distributed into a 6-wells plate at a concentration of $2x10^6$ cells per well in RPMI medium containing 1% L-Glu and 1% Pen/Strep, and 10 µg/mL LPS. After incubation for 0, 6, 18, and 24 hours (h), the cell supernatant was collected and centrifuged for 20 min at 2000g at RT, with brake to remove cell debris. The supernatant was aliquotted in 500-µL portions, snap frozen in liquid N_2, and stored at -80°C.

Immuno-magnetic beads capture

Hundred µL of PPP was mixed with 135 µL (27 µg) of biotinylated anti-human CD41-/CD14 MoAb and gently rotated for 30 min at RT, shielded from light. As negative controls, PPP was mixed with 135 µL (27 µg) of biotinylated mouse IgG1/IgG2a, κ isotype control MoAb. Next, 200 µL of streptavidin beads were added to the mixture and rotated for 5 min at RT, shielded from light. In the meantime, the µ Column was placed on the µMACS magnet separator (both from Miltenyi Biotech) and rinsed with 100 µL of the equilibration buffer included in the µMACS™ streptavidin kit. Afterwards, the µ Column was rinsed twice with 250 µL Hepes-citrate buffer. The mixture of PPP with antibody and beads was added to the µ Column and the flow through was collected. This flow through was added again into the µ Column to ensure that all the captured MPs were bound to the

magnet and then the flow through was collected (negative fraction). The µ Column was rinsed twice with 500 µL Hepes-citrate buffer. To collect the bound MPs, the µ Column was detached from the magnet after which quickly 75 µL Hepes-citrate buffer was added to the µ Column. The first eluate was collected. The µ Column was rinsed again with 75 µL Hepes-citrate buffer and a syringe plunger (5 mL) was applied to the µ Column to increase elution efficiency. Finally, the second eluate was collected and combined with the first eluate (positive fraction).

MP isolation

To isolate MoMPs, frozen supernatant of LPS stimulated-monocytes was thawed quickly with a gentle swirl at 37°C. The supernatant (500 µL) was centrifuged for 30 min at 18,890g, 20°C, with minimum brake, to isolate MPs and the supernatant was carefully removed, leaving a volume of 50 µL containing the MP pellet. To wash the MPs, 450 µL of Hepes buffer was added, and this mixture was then centrifuged again for 30 min at 18,890g, 20°C, with minimum brake. The supernatant was removed and the residual 50 µL was diluted to 200 µL with Hepes buffer. This MP fraction was directly used for the measurements.

MPs were isolated from plasma as previously described (22;24). Frozen PPP samples (250 µL) were thawed quickly with a gentle swirl at 37°C. Then, they were centrifuged three times at 18,890g for 30 min at 20℃ with minimum brake, to pellet the MPs. MPs were extensively washed between the centrifugation steps with Hepes-citrate buffer to reduce the concentration of plasma proteins to less than 0.5%. To isolate MPs from positive and negative fractions obtained from immuno-magnetic beads capture, the procedure (centrifugation and washing steps) was the same as the procedure to isolate MPs from plasma. At the third centrifugation step, the supernatant was carefully removed, leaving 25 µL of MP suspension. The MP suspensions obtained from plasma and positive/negative fractions were diluted to 100 µL before used for measurement.

Flow cytometry analysis

Flow cytometry analysis was performed using a FACS Calibur flow cytometer with CELLQUEST PRO software (Becton Dickinson). Before measurement the FACS Calibur was calibrated by using Megamix beads (25). Staining plasma MPs for CD41/CD61 after beads separation was done by adding 10 µL of anti-human CD41-PE MoAb and 10 µL of anti-human CD61-FITC MoAb into 15 µL of the MP fraction, and then diluting up to a volume of 50 µL with Hepes-citrate buffer. This mixture is incubated for 30 min at RT in the dark. As negative controls, mouse IgG1

isotype control-PE MoAb and mouse IgG1 isotype control-FITC MoAb were used at the same concentration as the antibodies.

When staining plasma MPs after beads isolation for CD14, 10 µL of anti-human CD14-PE MoAb was added into 15 µL of the MP fraction and diluted with Hepes-citrate buffer up to a volume of 50 µL. This mixture was incubated for 30 min at RT in the dark. As negative controls, mouse IgG2a, κ isotype control-PE MoAb was used at the same concentration as the antibody.

For staining MoMPs isolated from supernatant, 10 µL MPs was mixed with 5 µL anti-human CD14-PE MoAb, 5 µL anti-human TF-FITC MoAb, 5 µL annexin V-APC, and 25 µL Hepes buffer containing 2.5 mM $CaCl_2$. Anti-human CD14-PE MoAb and anti-human TF-FITC MoAb were 20 times diluted, whereas annexin V-APC was 300 times diluted in the final mixture. This mixture was incubated for 30 min, in the dark, at RT. As negative controls, mouse IgG2a, κ isotype control-PE MoAb and mouse IgG1-FITC MoAb were used at the same concentration as the antibodies. As a control of annexin V staining, Hepes buffer without $CaCl_2$ was used.

Before flow cytometry, 500 µL of Hepes-citrate buffer was added to the mixture containing labelled MPs. For counting absolute numbers of MPs per µL plasma or MoMPs per 500 µL supernatant, 200 µL of flow cytometry absolute count standard beads was mixed with Hepes buffer to a volume of 600 µL and then measured in triplicate. For measurement of counting beads as well as MPs, the flow cytometer was programmed to count events in 1 min.

MP-TF activity assay

Freshly isolated MPs were directly used for the measurement of MP-TF activity as previously described (22). In this assay the factor VII (FVII)-dependent factor Xa (FXa) generation was measured. MP-TF activity is expressed as fM FXa/min. The FXa generation was measured in the presence and absence of excess (33 µg/mL) polyclonal sheep anti-human TF IgG (Affinity Biologicals INC, Canada) and (1 nM) human FVII (Kordia, Leiden, The Netherlands). Human factor X (50 nM) (Kordia) was added to start FXa formation. The MP-TF activity (TF-activity in isolated MPs) was FVII and TF dependent confirming the specificity of the assay.

Results

The beads separation method was based on the capture of a specific subset of MPs from plasma by using biotinylated antibody and streptavidin magnetic beads.

We first tested the procedure to capture PMPs. The biotinylated anti-human CD41 MoAb was incubated with the plasma before the streptavidin magnetic beads were added. After application of this mixture to the column, positive and negative fractions were collected (Figure 1). Subsequently, the separated fractions were used to isolate and analyze MPs for specific antigens and TF activity.

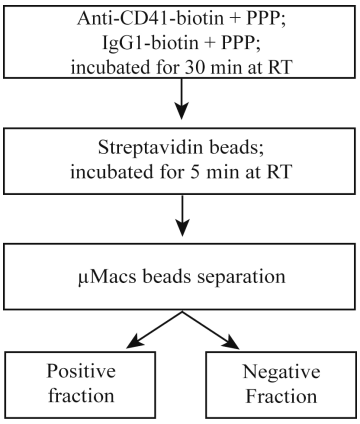

Figure 1. Scheme for the immuno-magnetic beads capture procedure using biotinylated anti-human CD41 MoAb and biotinylated mouse IgG1 isotype control MoAb. After beads separation using biotinylated anti-human CD41 MoAb, MPs in positive and negative fractions were collected.

Optimizing of the concentrations of anti-CD41 antibody and streptavidin magnetic beads

For PMP capture from plasma by antibody-coated magnetic beads both the concentration of antibodies and of streptavidin magnetic beads need to be optimized. To check whether the antibody saturated the surface antigen CD41 of PMPs, PPP (100 µL) was first reacted with different concentrations of biotinylated anti-human CD41 MoAb. Subsequently MPs were labelled by fluorescent antibodies, anti-human CD41-PE MoAb and anti-human CD61-FITC MoAb or the fluorescently labelled isotype controls, and analysed by flow cytometry. These experiments showed that 135 µL (27 µg) biotinylated anti-human CD41 MoAb specifically binds and saturates the surface antigen CD41 of PMPs in 100 µL plasma. Figure 2A and B show flow cytometry analysis of plasma MPs without biotinylated anti-human CD41 MoAb. The PMPs express both CD41 and CD61 (Figure 1A). When biotinylated anti-human CD41 MoAb (27 µg/135 µL) was added, plasma PMPs show only CD61 and no CD41 expression (Figure 2C). Figure 2E shows that when using biotinylated mouse IgG1 isotype control MoAb instead of

biotinylated anti-human CD41 MoAb, the surface antigens CD41 and CD61 could still be detected on PMPs by anti-CD41 and anti-CD61 antibodies.

Streptavidin magnetic beads should be added in excess to bind all biotinylated antibodies present in the plasma. We found that 200 µL streptavidin magnetic beads were needed and that the streptavidin magnetic beads did not interfere with the binding of anti-CD41 and anti-CD61 antibodies to PMPs (Figure 2G). Fluorescently labelled IgG1 were used as controls at the same concentrations as the staining antibodies (Figure 2 B, D, F, and H).

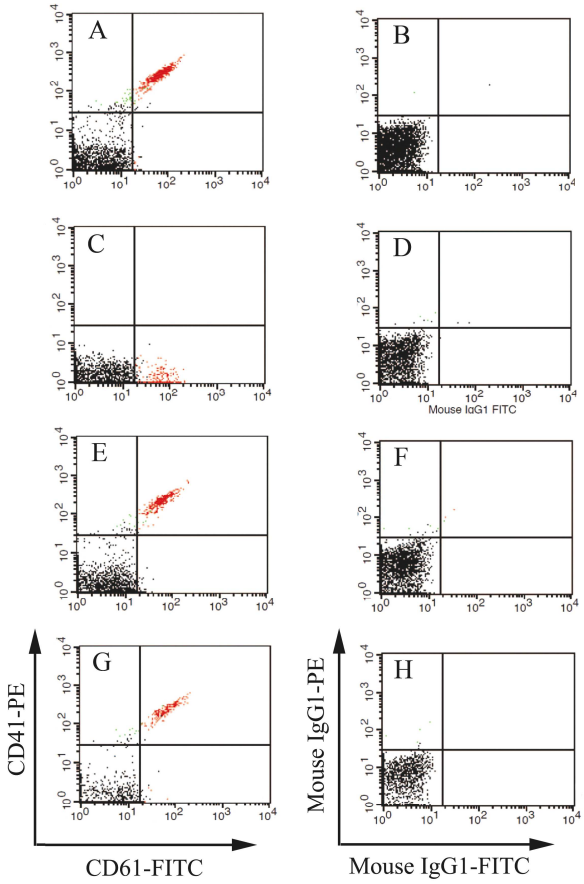

Figure 2. Optimizing the concentration of antibodies, streptavidin magnetic beads, and plasma for the immuno-magnetic beads method. Stainings of samples were performed by using anti-human CD41-PE and anti-human CD61-FITC MoAbs (A, C, E, G). As controls, mouse IgG1-PE and mouse IgG1-FITC MoAbs were used at the same concentrations (B, D, F, H). After staining, PPP (A, B) and PPP incubated with biotinylated anti-human CD41 MoAb (C, D), biotinylated mouse IgG1 isotype control MoAb (E, F), or streptavidin beads (G, H) were measured by flow cytometry. Experiments were done in duplicate.

In conclusion, 135 µL (27 µg) biotinylated anti-human CD41 MoAb and 200 µL streptavidin magnetic beads can be used for optimal capturing of CD41-positive MPs from 100 µL PPP. As PMPs bearing CD41/CD61 are the most abundant MPs in plasma, these optimized conditions can also be applied to capture other subsets of MPs from plasma.

Use of immuno-magnetic beads to capture PMPs from plasma.

After beads separation for PMPs bearing CD41, positive and negative fractions were collected (Figure 1) and stained by using fluorescently labelled anti-human CD41 and anti-human CD61 MoAbs or their isotype controls as described in the methods section. Flow cytometry analysis (Figure 3A) showed that there is no fluorescence signal of the anti-human CD41 MoAb, but only of the anti-human CD61 MoAb on MPs in the positive fraction. This indicated that the surface antigen CD41 of PMPs in this positive fraction still was saturated by the biotinylated anti-human CD41 MoAb. In the negative fraction no CD41- and CD61-positive MPs were detected (Figure 3C). Figure 3B and D show fluorescently labelled isotype controls staining of these fractions.

To check for non-specific binding of biotinylated anti-human CD41 MoAb, biotinylated mouse IgG1 isotype control MoAb was used (Figure 3E-H). In the positive fraction no CD41- and CD61-positive MPs were detected (Figure 3E). These were only present in the negative fraction (Figure 3G) indicating that CD41-positive MPs are specifically captured from plasma by using this procedure. Figure 3F and H show fluorescently labelled isotype controls staining of these fractions.

Flow cytometry quantification of CD41 and CD61-positive MPs

Frozen-thawed PPP from three healthy volunteers was used to perform the immuno-magnetic beads capture of PMP by using biotinylated anti-human CD41 MoAb. Figure 4 shows that no CD41-positive MPs were detected in the negative fractions. In these fractions about 1, 0, and 6% of CD61-positive MPs were found in volunteer 1, 2, and 3 respectively. This indicated that almost all PMPs (CD41-positive MPs) were captured. As the anti-CD41 coated-beads saturated the surface antigen CD41 of MPs, there were no CD41-positive MPs detected in the positive fraction. However, there were also few CD61-positive MPs present in this positive fraction. Possibly the magnetic beads which still present on the surface of MPs in the positive fraction interfere with the fluorescence staining with anti-CD61 antibody causing inaccurate quantification of CD61-positive MPs by flow cytometry.

When biotinylated mouse IgG1 isotype control MoAb was used instead of biotinylated anti-human CD41 MoAb, we found that about 5% and 80% of CD41/CD61-positive MPs were recovered in the positive and negative fractions of the biotinylated IgG1-beads separation.

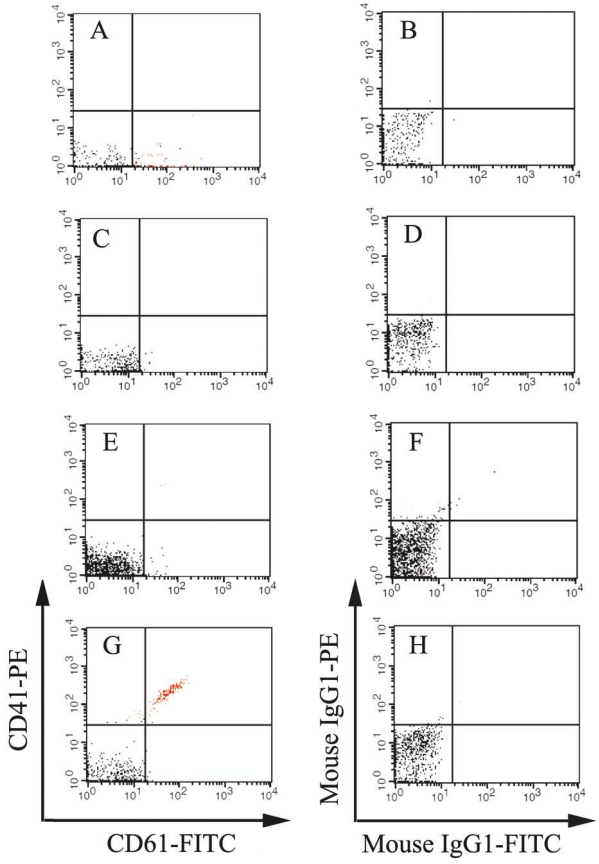

Figure 3. Flow cytometry analysis of positive and negative fractions after beads separation for PMPs bearing CD41. After beads separation using biotinylated anti-human CD41 MoAb, MPs in positive (A, B) and negative fractions (C, D) were collected, stained by anti-human CD41-PE and anti-human CD61-FITC MoAbs (A, C) or isotype controls (B, D), and measured by flow cytometry. Staining was also done for MPs in the positive (E, F) and negative (G, H) fractions collected from beads separation using biotinylated mouse IgG1 isotype control MoAb by using anti-human CD41-PE and anti-human CD61-FITC MoAbs (E, G) or isotype controls (F, H). All measurements were performed in duplicate.

Flow cytometry and MP-TF activity measurements of in vitro generated MoMPs

Monocytes were isolated from human blood and stimulated with LPS for 0, 6, 18, and 24 h to produce MoMPs. MoMPs were isolated from the supernatant, stained

with fluorescently labelled annexin V, anti-human CD14 and anti-human TF MoAbs and analysed by flow cytometry. Figure 5A shows that MoMPs expressed annexin V, CD14 antigen, and TF antigen. However, not all MoMPs were CD14 positive as measured by flow cytometry. After 6, 18 and 24 h stimulation with LPS, there were more TF- and annexin V-positive MoMPs than without LPS stimulation.

MP-TF activity measurements were performed to investigate whether MoMPs derived from LPS-stimulated monocytes carry active TF. The results are summarized in Figure 5B and show that MoMPs isolated from the supernatant of monocytes stimulated with LPS for 6, 18, and 24 h carried active TF and this TF activity could be completely inhibited by an anti-human TF antibody.

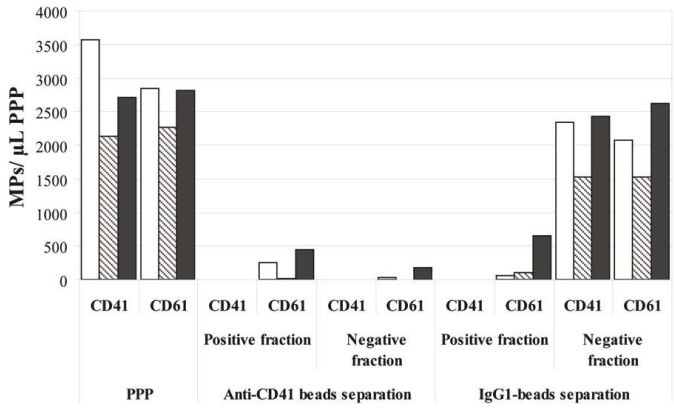

Figure 4. Flow cytometry quantification of CD41/CD61-positive MPs in the positive and negative fractions obtained from immuno-magnetic beads separation using biotinylated anti-human CD41 or mouse IgG1 isotype control MoAb. PPP used in these experiments was prepared from blood of 3 healthy volunteers (white bar: volunteer 1, black bar: volunteer 2, hatched bar: volunteer 3). Measurements were performed in duplicate.

Use of immuno-magnetic beads to capture MoMPs from plasma spiked with in vitro generated MoMP

We added MoMPs carrying active TF (isolated from the supernatant of monocytes stimulated with LPS for 0, 6, 18, or 24 h) to frozen-thawed PPP of a healthy volunteer in a 1:1 ratio (v/v); no visible clot was formed after 30 and 60 minutes at RT. The procedure of immuno-magnetic beads to capture MoMPs was the same as the procedure used to capture PMPs from plasma. Plasma spiked with MoMPs (100 µL) were incubated with biotinylated anti-human CD14 MoAb and streptavidin magnetic beads were added before the mixture was subjected to the column.

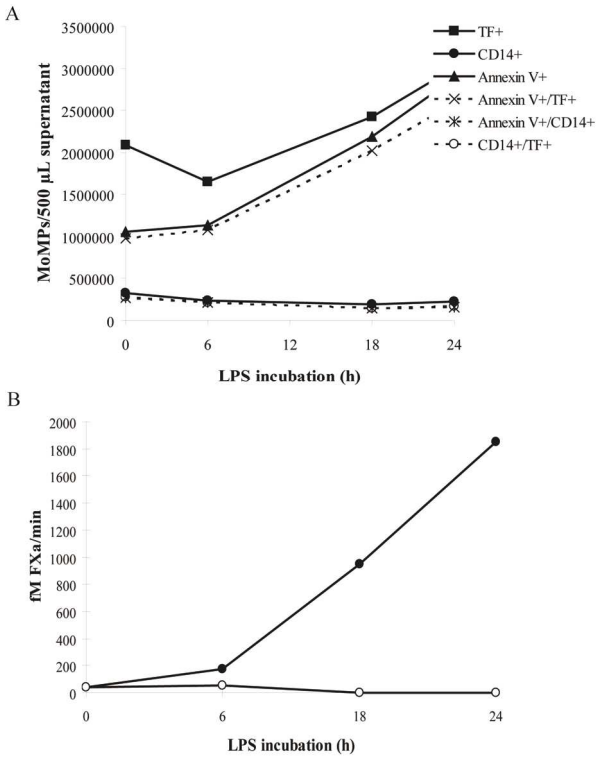

Figure 5. Flow cytometry analysis and MP-TF activity measurements of MoMPs. MoMPs isolated from the supernatant of monocytes stimulated with LPS for 0, 6, 18, and 24 h were stained by using annexin V APC, anti-TF FITC, and anti-CD14 PE antibodies and quantified by flow cytometry (A). The same batch of MoMPs was used for the measurement of TF activity (B) in the presence (○) or absence (●) of an anti-TF antibody.

The concentration of biotinylated anti-human CD14 MoAb used was the same as that of biotinylated anti-human CD41 MoAb in the PMP separations. The positive and negative fractions were collected and subjected to MP-TF activity analysis. For baseline measurements, PPP, MoMPs, and PPP spiked with MoMPs before beads separation were used.

The results of the MP-TF activity measurements are shown in Figure 6 and Table 1. In Figure 6 it is shown that MPs isolated from PPP have no active TF, while as expected MoMPs isolated from supernatant of monocytes stimulated with LPS for 6, 18, and 24 h have active TF, which is completely inhibited by an anti-TF antibody. TF activity was also present in the MPs isolated from PPP spiked with MoMPs; however, the TF activity was 2-fold lower than expected on the basis of the TF activity of the isolated MoMPs added, which might be due to loss of MPs during the isolation of MPs from PPP. After the column separation we found TF activity both in the positive and negative fractions of PPP spiked with MoMPs

isolated from the supernatant of monocytes stimulated with LPS for 0, 6, 18, and 24h. This indicated that both CD14-positive and negative MoMPs have active TF. When the MP-TF activities (fM FXa/min) of PPP spiked with MoMPs before and after beads separation were compared, we found that after beads separation the combined MP-TF activities of the positive and negative fractions were about 1.2-fold lower than those before beads separation (Table 1).

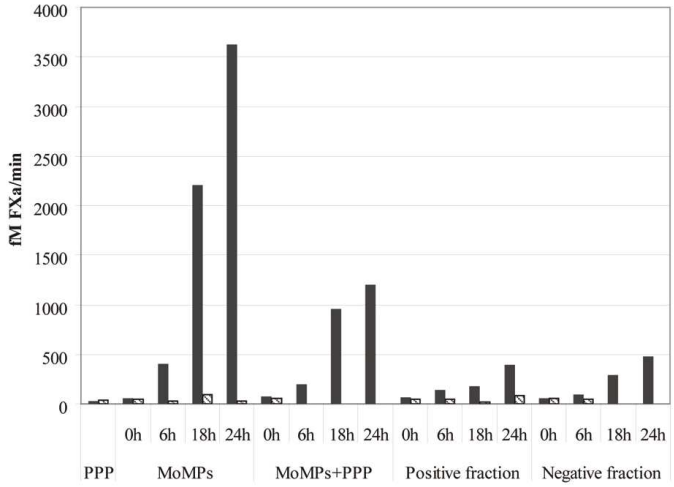

Figure 6. MP-TF activity of PPP spiked with MoMPs before and after beads separation. PPP spiked with MoMPs were incubated with biotinylated anti-human CD14 MoAb and streptavidin magnetic beads. This mixture was subjected to a column for magnetic beads separation. Positive and negative fractions from this beads separation were collected and measured by MP-TF activity assay in the presence (hatched bar) or absence (black bar) of an anti-TF antibody. PPP without MoMPs, MoMPs alone, and PPP spiked with MoMPs before beads separation were used as baseline measurements.

Table 1. TF activity levels (expressed as fM FXa/min) of PPP spiked with MoMPs before and after beads separation are compared.

Samples	**fM FXa/min**
Before beads separation	
MoMPs (0h) + PPP	78.70
MoMPs (6h) + PPP	197.91
MoMPs (18h) + PPP	953.43
MoMPs (24h) + PPP	1202.43
After beads separation	
Positive + negative fractions (0h)	123.67
Positive + negative fractions (6h)	236.78
Positive + negative fractions (18h)	462.34
Positive + negative fractions (24h)	869.42

Discussion

In this study we present a method to use a combination of a biotinylated antibody and streptavidin magnetic beads to capture a specific subset of MPs from plasma. A positive fraction (containing selected subset of MPs) bound to the beads was separated from the negative fraction (containing the depleted plasma) and these fractions can be analysed for specific markers like for instance TF activity.

This immuno-magnetic beads method was optimized to capture PMPs. We used biotinylated anti-human CD41 MoAb at a concentration that saturated the surface antigen CD41 of MPs. The streptavidin beads were added in excess to bind all biotinylated antibodies present in the plasma. To obtain a specific interaction between the antibody and the surface antigen of MPs, it is important to first incubate plasma with the biotinylated antibody before the streptavidin magnetic beads are added. The biotin-streptavidin interaction was chosen for this procedure because the non-covalent interaction between biotin and streptavidin is the strongest interaction known with a dissociation constant, K(d), in the order of $4x10^{-14}$ M (26). The bond also forms very rapidly and is stable over a wide range of pH and temperature (27).

The capture of the CD41-positive MP subset from plasma was successful as flow cytometry analysis demonstrated that these MPs were barely found in the negative (depleted) fraction obtained after column separation (Figure 3). The recovery of the CD41-positive MPs in the positive fraction could not accurately be quantified by flow cytometry. The magnetic beads (diameter: ~50 nm) which were still attached to CD41-positive MPs after the column separation apparently hindered the detection of MPs by using fluorescently labelled anti-human CD61 MoAb and flow cytometry. To capture CD41-positive PMPs, PPP of healthy volunteers were used. As these plasma contained very low MP-TF activity (~20 fM Xa/min), it was not possible to obtain accurate results for the MP-TF activity measurements in the positive and negative fractions.

We also tested the immuno-magnetic beads method to capture CD14-positive MPs from plasma and analysed the positive and negative fractions for MP-TF activity. However, the concentration of MoMPs in plasma of healthy individuals is low (about 2% of the total blood MP population) (8;11). Thus, MoMPs isolated from LPS-stimulated monocytes were used as a model of monocyte derived active TF bearing MPs and spiked to plasma of a healthy volunteer. We found TF activity both in the positive (CD14-positive) and negative (CD14-negative) fractions of plasma spiked with these MoMPs. This is in agreement with our finding that not all

active TF carrying MoMPs isolated from the supernatant of LPS stimulated monocytes were CD14 positive.

In conclusion, the immuno-magnetic beads method can be used for capture specific subsets of MPs directly from plasma. For future application, other specific biotinylated antibodies can be used to capture different subsets of MP from plasma. Ultimately this approach will help us to identify the cellular sources of MP bearing active TF in plasma of patients with cancer or other diseases.

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