Nanosized blood microparticles

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CHAPTER 4

The detection of tissue factor bearing-microparticles by atomic force microscopy

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Abstract

TF associated with cell-derived vesicles (MPs) in blood has been shown to be involved in (cardio) vascular disorders, inflammation, and cancer. However, such studies have been hampered by the lack of methods for the sensitive detection and accurate quantification of MPs. In a previous study, our group demonstrated that atomic force microscopy (AFM) can be used to sensitively detect and accurately quantify a specific subset of MPs.

In the current study, we investigated whether TF-bearing MPs could be captured on mica coated with anti-TF antibody and sensitively be detected by AFM operated in fluid tapping mode. MPs derived from the supernatant of MDA-MB231 cells stimulated with calcium ionophore were used as an experimental model. We showed that FITC-labelled anti-TF monoclonal antibody (MoAb) evenly coated the surface of modified mica. This coated surface captured TF-positive MPs which have diameters (d_{sph}) ranging from 6-296 nm (median: 40.4 nm; mean: 46.4 ± 26.0 nm). These MPs were virtually absent on the mica surface coated with FITC-labelled mouse IgG1 isotype control MoAb. Numbers of TF-positive MPs were 2,000-fold higher than those measured by flow cytometry (8×10^6 per µL supernatant vs. 4×10^3 per µL supernatant).

In conclusion, AFM provides a sensitive detection of TF-positive MPs. We propose that this method may be used for the detection of TF-positive MPs in patient samples.

Introduction

The role of tissue factor (TF) as the major player in initiation of blood coagulation has been known for more than 100 years (1). TF is a 47 kDa transmembrane glycoprotein consisting of 263 amino acids: 219 amino acids on the extracellular domain, 23 amino acids on the transmembrane domain, and 21 amino acids on the cytoplasmic tail.

TF is normally not expressed by cells within the vasculature; rather, it is found predominantly in the adventitial and much less within the medial layers around blood vessels (2). Later, fibroblasts, adipocytes (3) and pericytes (4) were also shown to constitutively express TF. Smooth muscle cells, endothelial cells, and peripheral blood mononuclear cells (e.g. monocytes, neutrophils) do not express TF constitutively, but TF expression can be induced in these cells *in vitro* and *in vivo* by various agonists (5;6). Most of the TF antigen in plasma is encrypted and not biologically active (7;8). In 1999 Giessen et al (9) demonstrated the presence of TF antigen and activity on monocytes, neutrophils, and cell-derived vesicles (also named 'blood-borne TF') in blood and plasma of healthy volunteers. In the mouse model TF-bearing vesicles have been shown to bind to the developing thrombus and play a role in the propagation of the fibrin clot (10;11). These vesicles, later known as microparticles (MPs) based on their submicron diameter, express protein surface antigens from the parent cells from which they are derived (12;13).

In recent years, blood MPs have increasingly received attention as potential biomarkers in the diagnosis and prognosis of disease (12-14). However, such studies have been hampered by the lack of methods for the sensitive detection and accurate quantification of MPs and also by variations in pre-analytical conditions related to plasma and MPs preparation (15;16).

New methods to provide sensitive detection of MPs have been proposed (17). By using an impedance-based flow cytometer, Zwicker et al (18) characterized TF-bearing MPs in plasma of cancer patients. Harrison et al (19) used a nanoparticle tracking analysis to measure MPs size distribution and quantify MPs in isolated MPs fraction and platelet-poor plasma (PPP). Our group (20) has chosen atomic force microscopy (AFM) operated in fluid tapping mode to analyse a defined subset of MPs (platelet MPs) in the MPs isolated from PPP. We demonstrated that AFM detects 1,000-fold more CD41-positive MPs than conventional flow cytometry, a commonly used method for MPs detection, and that the size of the majority of these MPs (mean d_{sph}: 67.5 ± 26.5 nm) is well below the detection limit of conventional flow cytometry.

In the current study, we investigated whether TF-bearing MPs could be captured on mica coated with anti-TF antibody and detected by AFM operated in fluid tapping mode. The number of TF-bearing MPs in plasma of healthy subject is low (18;21). Therefore, as an experimental model we used TF-bearing MPs isolated from the supernatant of cultured breast carcinoma cells (MDA-MB-231), which constitutively express TF, and were stimulated with calcium ionophore.

Assuming a spherical shape of unbound MPs, we found that the calculated diameter (d_{sph}) of TF-positive MPs ranged from 6-296 nm (median: 40.4 nm; mean: 46.4 ± 26.0 nm) in the supernatant of MDA-MB231 cells stimulated with calcium ionophore. These MPs were barely found attached on IgG1 isotype control-coated mica. We propose AFM as a suitable method for the detection and analysis of TF-positive MPs.

Materials and methods

Cell culture

Breast carcinoma cell line, MDA-MB-231, was cultured in DMEM (Invitrogen, Breda, The Netherlands) with 10% fetal bovine serum (Invitrogen), 1% L-glutamine (Lonza, Cologne, Germany) and 1% Penicillin/Streptomycin (Invitrogen) at 37ºC in an incubator with humidified atmosphere under 5% CO_2. Cells at 80% confluence were subcultured twice a week.

Generation and isolation of microparticles

Cells grown at 80% confluence in a T75 flask (Greiner Bio One, Alphen a/d Rijn, The Netherlands) were used for the experiment. To stimulate cells with calcium ionophore (A23187, VWR International, Amsterdam, The Netherlands), the culture medium was removed from the cells and cells were washed twice with Hank's buffered salt solution (HBSS) without $CaCl_2$ (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). Next, 10 mL of Dulbecco's modified eagle medium (DMEM) containing 10 µM calcium ionophore was added to the cells. Cells were placed at 37°C in the incubator for 30 minutes.

After stimulation, the culture medium was collected and centrifuged for 20 minutes at 2,000g, at room temperature (RT) without brake to remove dead cells and debris. The supernatant was collected, aliquoted in 1 mL-portions and centrifuged for 30 minutes at 18,890g at RT with minimum brake. The supernatant was discarded except for the 25 µL containing the MPs pellet. The MPs pellets isolated from 3 mL supernatant were pooled, resuspended in 1 mL Hepes buffer [10 mM

Hepes (Merck, Darmstad, Germany), 137 mM NaCl (Merck), 4 mM KCl (Merck), 0.1 mM Pefabloc SC (Fluka, Munich, Germany), pH 7.4], and centrifuged for 30 minutes at 18,890g at RT with minimum brake. The supernatant was discarded except for the 25 µL containing the MPs pellet. These preparations were used immediately or snap-frozen in liquid nitrogen and stored at –80ºC.

Flow cytometry analysis of TF-positive microparticles

Flow cytometry analysis was performed using a FACS Calibur flow cytometer with CELLQUEST PRO software (Becton Dickinson, CA, USA). Five µL of MPs fraction was incubated with 5 µL of fluorescein (FITC)-labelled mouse anti-human TF monoclonal antibody (MoAb) (clone CLB/TF-5, Sanquin, Amsterdam, The Netherlands) and 5 µL of allophycocyanin (APC)-labelled annexin V (Caltag Laboratories,CA, USA) in 35 µL of Hepes buffer containing 2.5 mM $CaCl_2$ (Sigma Aldrich) for 30 minutes, in the dark, at RT. Anti-human TF-FITC MoAb was 20 times diluted, whereas annexin V-APC was 300 times diluted in the final mixture. As negative controls, FITC-labelled mouse IgG1 isotype control MoAb (clone X40, Becton Dickinson) was used at the same concentration as FITC-labelled anti-TF MoAb. As a control of annexin V staining, Hepes buffer without $CaCl_2$ was used.

Before flow cytometry measurement, the labelled MPs were diluted with 300 µL of Hepes-citrate buffer. For counting absolute numbers of MPs per µL supernatant, 200 µL of flow cytometry absolute count standard beads (Bang Laboratories, IN, USA) 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 minute.

Modification of mica for immobilization of mouse anti-human TF antibody

The surface of mica was modified as described by Yuana et al (20). Fifty µL of FITC-labelled mouse anti-human TF MoAb (clone CLB/TF-5, Sanquin) (0.1 mg/mL) was applied onto the modified mica and incubated for 30 minutes at RT. Excess of FITC-labelled anti-human TF MoAb was removed by washing with Hepes buffer. As a negative control, FITC-labelled mouse IgG1 isotype control MoAb (clone X40, Becton Dickinson) was used at the same concentration of 0.1 mg/mL.

AFM analysis of TF-positive microparticles

Frozen MPs isolated from the supernatant of MDA-MB231 cells stimulated with calcium ionophore were quickly thawed at 37ºC, diluted 2 times with Hepes buffer, and used immediately for AFM measurement. Twenty µL of the MPs fraction was

applied to the FITC labelled-anti-human TF and IgG1 isotype control-coated mica surfaces and incubated for 30 minutes. To remove unbound MPs, the mica was washed twice with Hepes buffer. During the incubation step and before imaging, the mica surface was kept in a closed container. AFM imaging, image analysis, and calculation of diameter of MPs were performed as described by Yuana et al (20). For each sample, the mica surface was scanned at different positions to obtain 10 images with a scan size of 100 μm^2.

Results and discussions

Flow cytometry

Using flow cytometry we compared the number of MPs and the expression of TF and annexin V on MPs in fresh and frozen-thawed MPs fractions isolated from the supernatant of MDA-MB231 stimulated with calcium ionophore. MPs detected in fresh and frozen-thawed fractions expressed both TF and annexin V (Table 1). We observed about a 2-fold decrease in the total number of MPs, TF-positive MPs, and annexin V-positive MPs after one freeze-thaw cycle. In contrast to what is observed in plasma, depending on the centrifugation speed used for plasma isolation, some groups observed an increase in the number of platelet MPs and annexin V-positive MPs caused by freezing and thawing the plasma (22-24).

Table 1. Flow cytometry analysis of MPs from fresh and frozen-thawed MP fractions isolated from supernatant of MDA-MB231 cells stimulated with calcium ionophore.

Flow cytometry analysis	**Fresh**		**Frozen-thawed**	
	Per µL supernatant	**%**	**Per µL supernatant**	**%**
Total MPs	1,1725		7,479	
TF-positive MPs	10,293	88	4,076	55
Annexin V-positive MPs	8,794	75	3,416	46
TF and annexin V-positive MPs	5,870	50	3,248	43

In fresh and frozen-thawed MPs fractions, flow cyotmetry measured the total number of MPs, TF and annexin V-positive MPs per µL supernatant of MDA-MB231 cells after stimulation with 10 µM calcium ionophore for 30 minutes and also the percentage of TF- and annexin V-positive MPs. Experiments were performed in duplicate.

AFM

Before antibody was immobilized to the modified mica surface, we first scanned the surfaces of the cleaved mica and the modified mica by AFM. The surface topography of cleaved mica was flat (height (z): ~0.1 nm) and after modification, the mica surface was still flat, with an apparent height of less than 0.5 nm (Figure 1A).

Immobilization of the anti-human TF MoAb on the modified mica surface should be optimal and evenly distributed over the surface. In our previous study (20), we found that for immobilizing the anti-human CD41 MoAb a concentration of 0.01 mg/mL was needed for the optimal capture of CD41-positive MPs on the mica surface (100 μm^2). Therefore, we tested different concentrations of unlabelled mouse anti-human TF MoAb (clone CLB/TF-5, Sanquin), 0.01, 0.02, 0.1, and 0.2 mg/mL, to coat the modified mica surface. It appeared that at 0.01 and 0.02 mg/mL anti-human TF the antibody did not distribute homogenously on the mica surface (100 μm^2). There were also small protein aggregates found and the number of these aggregates increased with increasing concentration of anti-TF (0.1 and 0.2 mg/mL) used. Apparently this particular anti TF was not suitable for the production of homogeneously coated mica surfaces.

Subsequently we compared the unlabelled anti-human TF MoAb with the FITC-labelled anti-human TF MoAb from the same manufacturer. Similar to our previous observation, unlabelled anti-human TF MoAb (0.1 mg/mL) formed aggregates on the mica surface (Figure 1B). The surface topography of aggregates was 20 nm (Figure 1B, z_1), whereas that of a single molecule of anti-TF was 5 nm (Figure 1B, z_2). In contrast, FITC-labelled anti-human TF MoAb (0.1 mg/mL) coated the mica surface evenly without any aggregates and has an apparent height of 4.9 nm (Figure 1C). As a negative control, FITC-labelled mouse IgG1 isotype control MoAb (0.1 mg/mL) was immobilized on the modified mica (Figure 1D, z= 1 nm). Possibly, the FITC molecules attached to the anti-TF IgG create a spacer between anti-TF molecules in the solution which prevents aggregation.

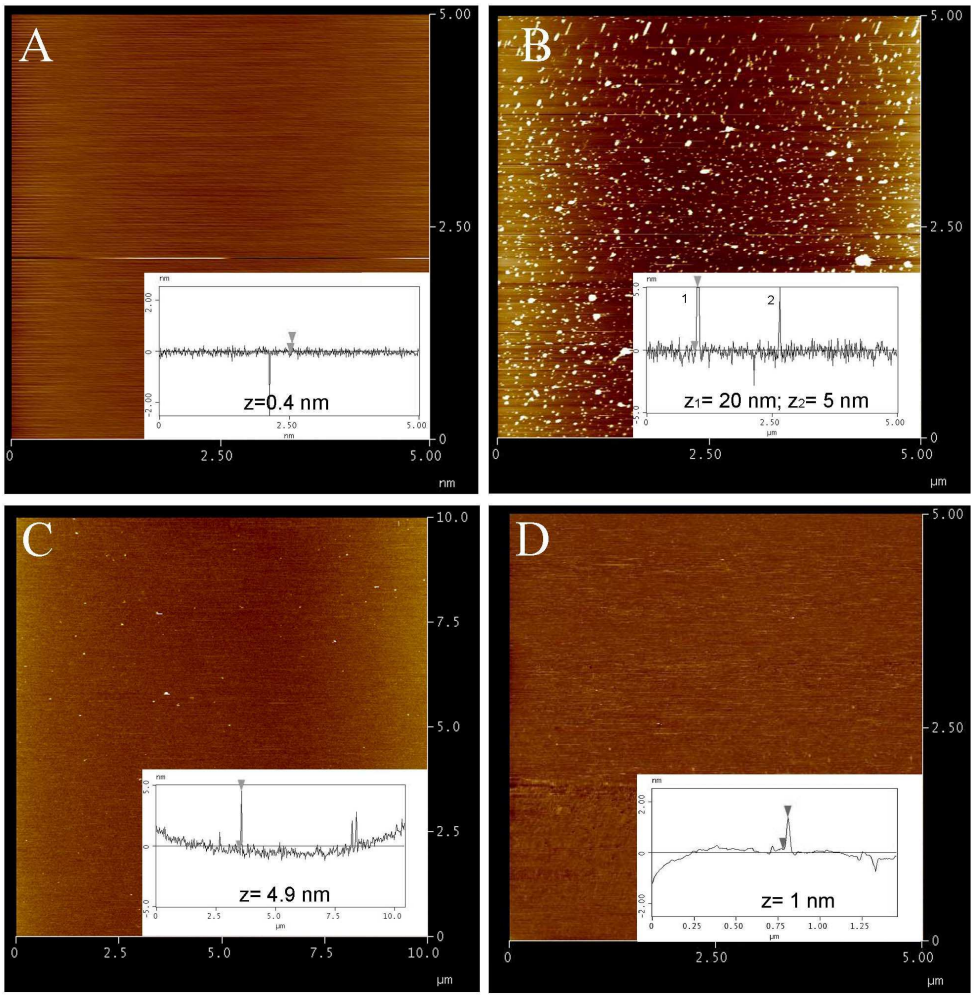

Figure. 1. AFM images showing the three-dimensional surface topography of (A) modified mica, (B) unlabelled anti-TF MoAb-coated mica (0.1 mg/mL), (C) FITC-labelled anti-TF MoAb-coated mica (0.1 mg/mL), and (D) FITC-labelled IgG1 isotype control MoAb-coated mica (0.1 mg/mL). Inserts show the results of the height analysis (z) of the surfaces.

After the mica surface was coated with FITC-labelled anti-human TF MoAb, frozen-thawed MPs were applied. FACS analysis showed that about 50% of the MPs in the frozen-thawed fraction still express TF (Table 1). As expected, three-dimensional AFM topography also showed that there were MPs captured on the mica surface coated with FITC-labelled anti-human TF MoAb (Figure 2A), whereas these were virtually absent on the mica surface coated with FITC-labelled mouse IgG1 isotype control (Figure 2B).

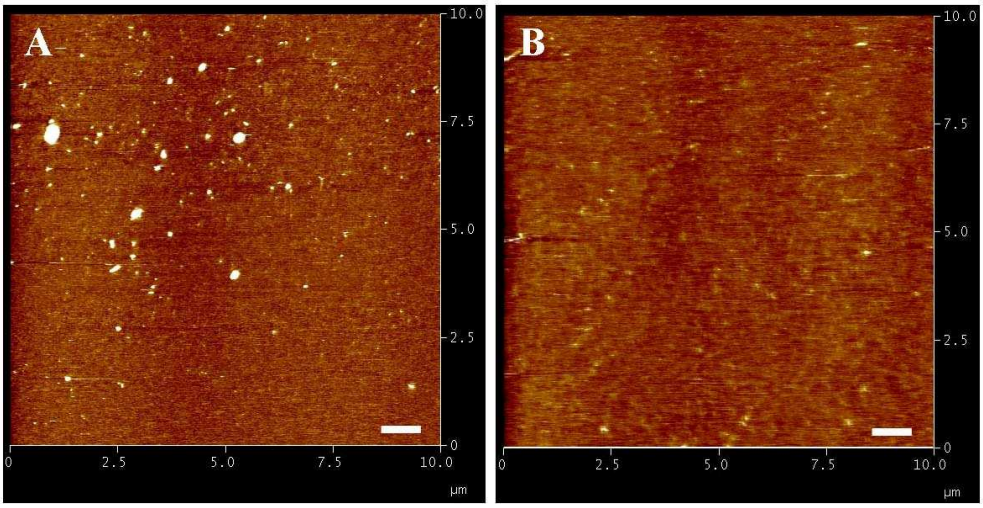

Figure 2. Three-dimensional AFM topography of MPs bound to (A) FITC-labelled anti-TF coated mica and (B) FITC-labelled isotype control (IgG1)-coated mica. The scale bar in these images is 1 µm.

The size of the TF-positive MPs calculated from the AFM images ranged from 6 to 296 nm (median d_{sph}: 40.4 nm; mean d_{sph}: 46.4 ± 26.0 nm) (Figure 3). About 113 TF-positive MPs were detected by AFM per 100 µm^2 of FITC-labelled anti-TF-coated mica surface, whereas about 11 MPs per 100 µm^2 attached non-specifically to FITC-labelled IgG1 isotype control-coated mica (Figure 3). After correction for the number of MPs found on FITC-labelled-IgG1 control-coated mica, the total number of TF-positive MPs detected by AFM is 8 x 10^6 per µL supernatant. This number is 2,000-fold higher than the number of TF-positive MPs from the same MPs preparation detected by flow cytometry (4 x 10^3 per µL supernatant, Table 1). Interestingly, we previously reported that AFM detects 1,000-fold more CD41-positive MPs than flow cytometry (20).

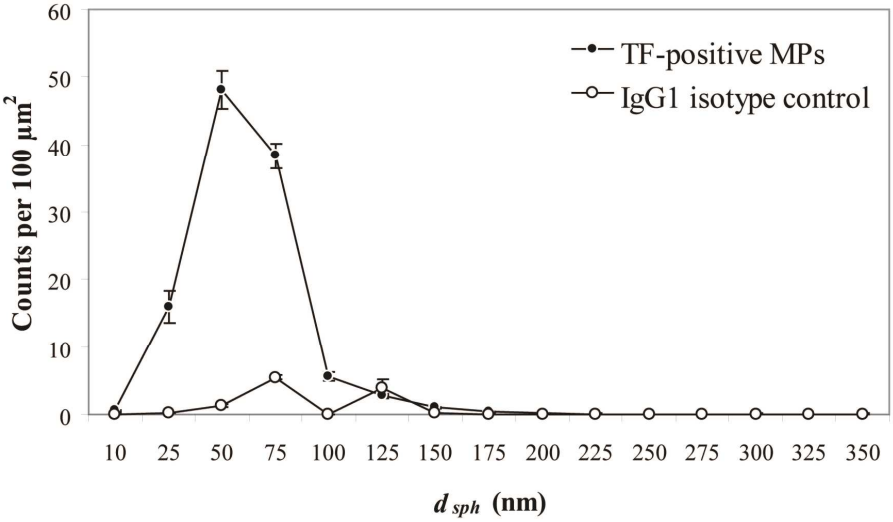

Figure 3. Size distribution (d_{sph}) and counts of TF-positive MPs per 100 µm^2 surface area.

Conclusions

The results of this study show that FITC-labelled mouse anti-human TF MoAb at a concentration of 0.1 mg/mL coated the modified mica surface homogenously. This coated surface could capture TF-positive MPs isolated from the supernatant of MDA-MB231 stimulated with calcium ionophore. AFM operated in fluid tapping mode sensitively detected MPs with sizes ranging from 6 to 296 nm (median d_{sph}: 40.4 nm; mean d_{sph} 46.4 ± 26.0 nm). There were only about 11 MPs captured on the mica surface coated with FITC-labelled mouse IgG1 isotype control MoAb. Numbers of TF-positive MPs were 2,000-fold higher than those measured by flow cytometry (8 x 10^6 per µL supernatant vs. 4 x 10^3 per µL supernatant). In this study, we frozen-thawed MPs isolated from cell culture supernatant for the AFM measurement. However, when a precise quantification of the number of TF-positive MPs in the sample is needed, AFM measurement on freshly prepared samples is preferred.

Acknowledgments

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