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Chapter 2

PHENOTYPING OF SMALL EXTRACELLULAR VESICLES FROM CLINICALLY IMPORTANT BODY FLUIDS USING PROTEIN MICROARRAY

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ABSTRACT

The Extracellular Vesicle (EV) Array is a novel microarray platform, which facilitates the ability to detect and profile EVs for the presence of multiple surface-exposed antigens simultaneously (Jørgensen et al. 2013). The EV Array uses only small amounts (1-100 µL) of starting material and can be processed in a high-throughput manner.

The diagnostic and prognostic possibilities of this technology have been explored in various technical and clinical correlations. In this study, several body fluids have successfully been tested including, urine, saliva, bone marrow, ascites, bronchoalveolar lavage, synovial and cerebrospinal fluids. For each sample type, unpurified materials have been analyzed with respect to their contents of a panel of EV related markers (CD9, CD63, CD81, CD82, TNF RI, Flotilin-1, TSG101 and Alix). A pronounced variety was seen between the body fluids with respect to the contents of EVs. This study illustrates the numerous applications of the EV Array and the possibilities to extensively phenotype small EVs from various unpurified body fluids.

Keywords: small EVs, protein microarray, phenotyping, EV Array, urine, saliva, ascites, bone marrow, ceresponial fluid, synovial fluid, broncheoalveolar lavage fluid

INTRODUCTION

The interest in extracellular vesicles (EVs) has increased immensely and several studies have revealed the potential of utilizing them in a clinical setting, both diagnostic, prognostic and as therapeutic agents as reviewed by György et al. (György et al. 2015) and Revenfeld et al. (Revenfeld et al. 2014). EVs are small membrane-derived entities produced from a diverse range of cell types throughout the human body and, therefore, they are accessible in various body fluids such as plasma (Caby et al. 2005), urine (Pisitkun et al. 2004; Gonzales et al. 2009; Mitchell et al. 2009), bronchoalveolar lavage fluid (Admyre et al. 2003; Qazi et al. 2010; Peinado et al. 2013; Rodriguez et al. 2014), saliva (Ogawa et al. 2008; Gonzalez-Begne et al. 2009; Keller et al. 2011), ascites (Runz et al. 2007; Keller et al. 2009; Andre et al. 2002), synovial fluid (Berckmans et al. 2005; Boilard et al. 2016), and cerebrospinal fluid (Akers et al. 2013; Street et al. 2012; Stuendl et al. 2015).

To determine the phenotypes of small EVs (sEVs) a novel protein microarray-based analysis was established. This analysis platform, termed EV Array and described by Jørgensen et al. (Jørgensen et al. 2013), is optimized to capture and detect the smaller types of EVs, such as exosomes and exosome-like vesicles, with diameters up to 150 nm. The detection may be performed by utilizing a cocktail of antibodies against the EV hallmark tetraspanins CD9, CD63 and CD81. The detection antibodies are easily exchangeable and the analysis is performed in a 96 well setup and consumes only low amount of sampling material, which makes the platform very cost-efficient, multiplexed and high-throughput (Jørgensen et al. 2015). The EV Array has been optimized for the analysis of EVs in cell culture supernatant and plasma and has already demonstrated great diagnostic potential regarding non-small cell lung cancer (NSCLC) (Jakobsen et al. 2015).

The aim of this study was to test whether the EV Array similarly can be utilized to phenotype unpurified sEVs from other body fluids such as urine, saliva, synovial fluid, cerebrospinal fluid (CSF), ascites, bone marrow (BM) and bronchoalveolar lavage fluid (BALF).

MATERIAL AND METHODS

Sampling

All research involving samples from human subjects were approved by the respective local ethics legislations. Each person has signed a written consent form allowing for the use of the samples in research purposes.

Bone marrow (BM) from the posterior iliac crest of healthy donors was aspirated into syringes containing 2 mL Heparin 1000 IE pr. 10 mL sample. BM plasma was obtained after centrifugation at 1,000 rpm at 5 min. CSF (from multiple sclerosis patients) and synovial fluid (from swollen joints due to rheumatoid arthritis) were processed with a centrifugation at 10,000 g for 30 min prior to storage. Saliva (from healthy donors), ascites (from ovarian cancerous women) and urine (from healthy donors, collected in Monovette, Urine Z tubes, Sarstedt, DE) were centrifuged at 3,000 rpm for 6 min prior to storage. Bronchoalveolar lavage fluid from healthy donors was conducted with 100 mL of saline at 37°C and centrifuged at 3,000 g at 4°C for 15 min prior to storage.

Printing of Protein Microarrays

Printing of the protein microarray slides to the EV Array analysis was performed on a SpotBot® Extreme Protein Edition Microarray Printer using a 946MP4 pin (ArrayIt Corporation, CA, USA). As a positive control 100µg/mL of biotinylated human IgG was printed and PBS with 5% glycerol was applied as negative control. Antibodies were printed in triplicate on epoxy-coated glass slides (75.6 × 25.0 mm; SCHOTT Nexterion, Germany), which were left to dry at RT overnight prior to further analysis.

The anti-human antibodies included were: Flotilin-1 and Tumor susceptibility gene 101 (TSG101, clone 5B7) from Abcam, MA, USA; CD9 and CD81 from LifeSpan BioSciences, WA, USA; CD63 (clone MEM-259) and Alix (clone 3A9) from Cell Signaling, MA, USA; Tumor necrosis factor receptor 1 (TNF RI) and CD82 (clone 423) from R&D Systems, MN, USA. All antibodies were diluted in PBS with 5% glycerol and printed at 200µg/mL.

EV Array Analysis

The EV Array analyses were performed as described by Jørgesen et al. (Jørgensen et al. 2015). In short, the printed slides were initially blocked (50 mM ethanolamine, 100 mM Tris, 0.1% SDS, pH 9.0) prior to incubation with various amounts of body fluids (2-100 µL) diluted in wash-buffer (0.05% Tween®20 in PBS). The incubation was performed in Multi-Well Hybridization Cassettes (ArrayIt Corporation, CA, USA) at RT for 2h using an orbital shaker (450 rpm) and subsequently overnight at 4°C (no shaking). Following a short wash, the slides were incubated for 2h with a cocktail of biotinylated detection antibodies (anti-human CD9, CD63 and CD81, LifeSpan BioSciences, WA, USA) diluted 1:1500 in wash-buffer. After washing, a subsequent 30-minute incubation step with Cy5-labelled streptavidin (Life Technologies, CA, USA) diluted 1:1500 in wash-buffer was carried out for detection. Prior to scanning, the slides were washed first in wash-buffer and then in deionized water followed by drying using a Microarray High-Speed Centrifuge (ArrayIt Corporation, CA, USA). Scanning at 635 nm and subsequent detection were performed as previously described (Jørgensen et al. 2015).

Data Analysis

The statistical analyses and generation of graphs were performed using Excel 2013 (Microsoft, WA, USA) and Genesis ver. 1.7.6 (IGB TU Graz, Austria). Mean signal intensities of the triplicate antibody spots were used and corrected for unspecific binding from the detection antibodies by subtracting the mean signal intensities from a blank well containing no sample. For each capturing antibody, the corrected intensities were expressed in relation to the mean signal of negative spots (containing PBS). The antibody signals were log2 transformed prior to visualization. Signal-to-noise values were calculated as:

$$\frac{positive\ control - negative\ control}{positive\ control}$$

RESULTS AND DISCUSSION

The technology of protein microarray was used to capture sEVs from various body fluids. Spots of capturing antibodies against eight known vesicular surface- and surface-related antigens were printed in a customized array. Two to 100 µL of unpurified sample were applied depending on the type of body fluid and the results are visualized in Figure 1 (red colors). The disadvantage of using protein microarray to phenotype unpurified sEVs is, that other molecules present in the samples (protein, lipids, etc.) can bind unspecifically to the microarray slides and thereby induce an unwanted background noise. The background noise is sample dependent and the signal-to-noise (S/N) values were calculated for each sample (Figure 1 (blue colors)). When analyzing sEVs with the EV Array the amount of sample should be considered carefully as applying more samples also tends to increase the background noise, whereas loading less will decrease the signals obtained from the sEVs.

For all the tested types of body fluids, it was possible to obtain a signal from unpurified material (Figure 1) although saliva was only observed positive (weakly) for the presence of CD82. For the majority of the samples, applying more sample increased the detected amount of sEVs although a decrease in the S/N values were also seen. In particular, the bone marrow samples from donor Bm1 were observed to have a low S/N value, but then again, also a high amount of sEVs.

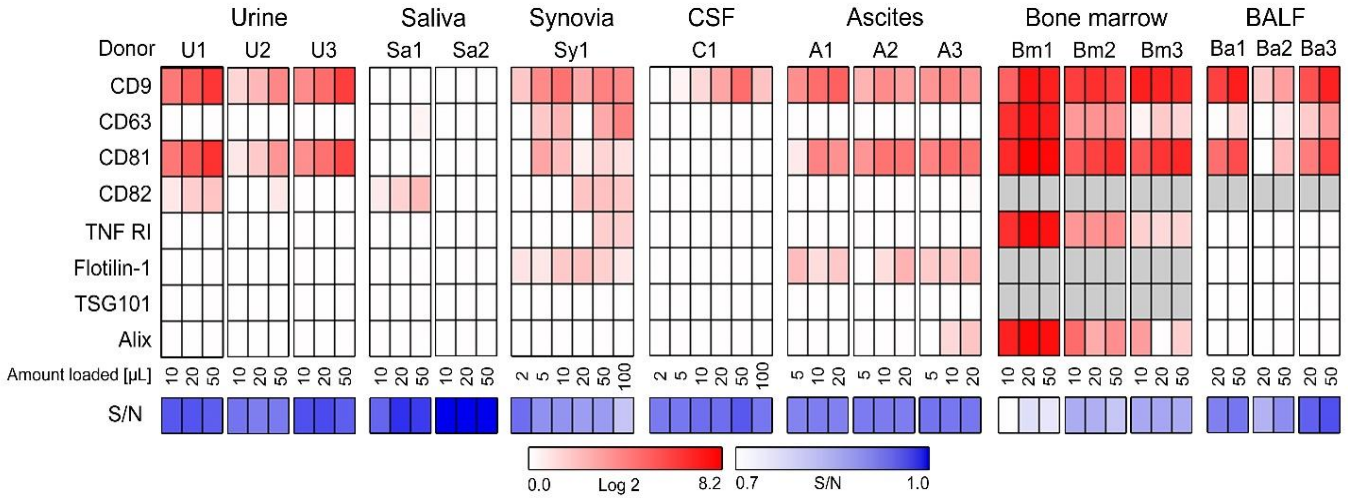

Figure 1. Heatmap of signal intensities (Log2 transformed) obtained from the EV Array analysis of several body fluids. A various number of donors and loaded amounts were tested. The sEVs from unpurified body fluids were captured with antibodies against CD9, CD63, CD81, CD82, TNF RI, Flotilin-1, TSG101 and Alix and afterwards detected with a cocktail of anti-CD9, CD63 and CD81. In blue scale, the calculated signal-to-noise (S/N) values are illustrated. Gray; not determined.

The urine from the healthy donors tested in this study revealed the presence of sEVs with CD9 and CD81 for all three donors and additionally CD82 for donor U1. Loading of 10 µL of unpurified urine was sufficient to obtain signals, although 50 µL are beneficial as the S/N values reveals no influence on the background signal. Mitchell et al. analyzed urine from prostate cancer patients by western blotting and were able to detect CD9, CD81 and TSG101 after sucrose cushion purification of 200 mL of urine (Mitchell et al. 2009). In this study, we were not able to detect TSG101 in the urine samples.

CD82 was the only protein detected in the saliva samples. Although no signals were detected when using CD9, CD63 or CD81 for capturing the sEVs, their presence are shown indirectly, as the detection cocktail consists of antibodies against these markers. In 2011, Keller and co-workers isolated exosomes from urine and saliva and their FACS analysis of several antigens (including CD9) also showed that the signal from saliva EVs was weaker compared to urine EV signal (Keller et al. 2011; Keller et al. 2007), which confirms our findings.

Only one sample of synovial fluid and one sample of CSF were tested in the current study. With regards to the different amounts loaded, both sample types showed the best signals using 50 µL, as increasing to 100 µL gave an evident decrease in the S/N values. CSF was found only to be positive for CD9, whereas synovia was positive for CD63, CD81, CD82, TNF RI, and Flotilin-1 as well. In comparison, Stuendl et al. and Street et al. (Stuendl et al. 2015; Street et al. 2012) analyzed EVs purified from CSF and found Flotilin-1 and TSG101 to be represented, whereas CD9 was not tested.

Ascites from three ovarian cancerous women were analyzed and all three samples were observed to have CD9, CD81, and Flotilin-1 positive sEVs. Whereas the EVs from donor A3 also carried Alix. CD9 has previously been identified to be present on EVs purified from ascites (Runz et al. 2007). Three amounts (5, 10 and 20 µL) of unpurified ascites were tested and the S/N values were at a similar level for all three amounts. Hence, for ascites the proposed amount are 10-20 µL as low expressed antigens are only seen here.

To our knowledge, this is the first time sEVs from BM have been analyzed with regard to their protein profiles. Samples from three healthy donors were analyzed using three different amounts. The analysis showed some variation between the donors as seen for the other samples types and plasma (Jørgensen et al. 2013). In relation to the other body fluids tested in this study, the BM samples showed the highest signals from sEVs, but they

also tended to influence the S/N values to the highest degree. For analysis of BM we suggest to use 10 µL.

For BALF only two different amounts were tested (20 and 50 µL). Using 50 µL revealed a better signal for the lower expressed CD63. For all three donors the sEVs were observed to be positive for CD9, CD63 and CD81 although CD63 was found in a low amount. Other studies of EVs purified from BALF also found CD63 to be present (Admyre et al. 2003; Rodriguez et al. 2014).

The obtained protein profiles of the sEVs (defined positive for CD9, CD63 and CD81) revealed that only synovial fluid, bone marrow and BALF were positive for CD63.

Table 1 summarises the amounts of sample that are suggested for protein profiling with the EV Array. These recommendations are based on the currently tested setup of the EV Array with standard experimental conditions optimized for plasma samples. For each of the body fluids, further optimization of the conditions such as incubation times, buffer compositions, etc. could probably gain an even more sensitive array. However, it should be noted that there was only one sample from synovial and cerebrospinal fluid, making the results for these two body fluids hypothesis-generating.

In this study, we have shown that the protein microarray technology, the EV Array, can be utilized to phenotype sEVs from various unpurified body fluids. Our results were compared to findings in other studies although their analyses have been performed on samples exposed to various purification procedures, as only a few other technologies are based on unpurified material.

Table 1. Summary of suggested amount of unpurified samples

Sample Type	*Suggested Amount*
Urine	50 µL
Saliva	100 µL
Synovial fluid	50 µL
Cerebrospinal fluid	50 µL
Ascites	10-20 µL
Bone marrow	10 µL
Bronchoalveolar fluid	50 µL
Plasma*	10 µL

* From (Jørgensen et al. 2015).

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