Report by Janice Chew-Harris

Aim 1: Examine major circulating suPAR form

June 2020-October 2021

Samples were selected from the Christchurch Heart Institute's (CHI) biorepository for adults who had undergone clinically indicated cardiac catheterisation. The measurement of soluble urokinase plasminogen activator receptor (suPAR) was undertaken using the commercially available ELISA (Virogates suPARnostic ®) in 46 patients who have complete set of site bloods from the following regions; femoral, renal hepatic vein, inferior vena cava, jugular vein, coronary sinus, pulmonary artery with matching arterial samples (9 sites per patient). In this part of the project, we found that coronary sinus (vein across the heart) concentrations are significantly lower than inferior and superior vena cava veins (P=0.03). This novel data shows a possible uptake or clearance of suPAR across the heart. Renal vein suPAR concentrations are also lower than its femoral artery concentrations (P=0.003), results that are to be expected, considering the kidneys are a major organ of suPAR clearance. These important results have provided us with pilot data to further investigate whether increased levels of suPAR may be harmful to the heart (and resulted in a successful Heart Foundation 3-year Research Fellowship).

November 2020-March 2021

During this period, I supervised a summer medical student to examine the major circulating form of suPAR in the circulation of patients with heart disease using Western Blotting methods. We were able to couple three immunoaffinity columns with different polyclonal antibodies targeted to Domain 1, Domain 2 and Domain 3 of suPAR separately. Prior to analyzing patient samples, multiple experimentation procedures were conducted to increase optimization of suPAR immunopurification and Western blotting procedures. As suPAR is present in circulation in (very low) concentrations of 2 ng/mL for healthy individuals, we discovered that a minimum of 100µL of plasma sample for each immunoaffinity column was required for band visualization. Although in the original project proposal our goal was to examine circulating forms of suPAR in the regional plasma samples of patients mentioned above, the requirements of large amounts of plasma for immunopurification (approximately 300 uL in total for the 3 suPAR domains) was deemed not feasible in these samples.

We therefore proceeded with analyzing venous plasma samples from patients from our other CHI cohorts including those with acute heart failure and from patients with acute myocardial infarction. Results from these patients were then compared with circulating suPAR forms from the samples of healthy subjects.

Figure 1 shows an example of the Western Blot for suPAR and its cleaved forms. Full-length suPAR is visualized as a band at 50 kDa, which was confirmed with a control sample (recombinant suPAR). The D2-D3 suPAR bands were observed around the 20-30 kDa mark.

Our initial results using this method (shown in Figure 1), shows that for healthy individuals and acutely breathless patients, both full-length and D2-D3 cleaved suPAR bands were present (Figure 1A and B). The cleaved bands were however not seen in acutely decompensated heart failure patients (example

shown in Figure 1C). We were unable to find the D1 form in our experiments, possibly because of its relatively small size (~10kDa), suggesting that it is either cleared relatively quickly via the kidneys or its concentration was too low to be detected by our current methods.

April-September 2021

Western Blot analyses to further determine circulating suPAR and its cleaved forms were undertaken in the plasma samples of healthy individuals (n=5), recombinant suPAR (n=1) acutely breathless patients (n=3), patients with acute decompensated heart failure (n=14), patients admitted to hospital with acute chest pain (n=3) and patients admitted to hospital with myocardial infarction (n=12) (total = 38) following immunopurification procedures.

Figure 2 shows an example of circulating suPAR and its fragments in patients who were admitted to hospital following an acute onset of myocardial infarction. Of note, the non-specific binding we originally obtained at the start of the Western Blot procedures (Figure 1) were now less visible and less frequent during this period.

However, we noted some heterogeneity in circulating suPAR and its fragments amongst patients presenting with different disease states. For example, in a few patients admitted to hospital with acute myocardial infarction and who died within 12 months after hospital admission, we saw some novel bands visualised at the cleaved suPAR fragment of D2-D3 (orange box in Figure 3). These bands were not observed in other patients (e.g. in acute decompensated heart failure and who died within 12 months since index admission).

To investigate whether these heterogenous suPAR bands were related to glycosylation aspects (please refer to Figure 3), we treated the patient samples above with deglycosylation enzymes following overnight incubation at 37°C and repeated the Western Blot procedures. We expected the bands to shift in molecular weight following deglycosylation treatment. However, we were unable to view these bands on Western Blot indicating either loss of protein during treatment procedures or detection of bands were not possible with current technique due to low levels of protein.

To further assess the glycosylation aspects of suPAR, we treated 13 samples with deglycosylation enzymes and incubated these overnight at 37°C. suPAR levels were then measured using the current clinical suPAR immunoassay in samples before treatment and after treatment with glycosylated enzymes. In 5 normal individuals, after treatment, we saw suPAR levels to increase, and these ranged between 12.5% to 81.8% increase in suPAR concentrations. In 2 patients with acute heart failure, suPAR levels also increased; 7% and 29.0% increment, respectively, in concentrations. In the remaining samples which came from patients with myocardial infarction, we also saw suPAR concentrations to increase following treatment, and these ranged from 18.2% to 63.0% increments. Although increases in suPAR concentrations were obtained for all the patients tested, values were not raised above our suPAR reference range for healthy individuals (*see reference: Chew-Harris J et al. Analytical, biochemical and clearance considerations of soluble urokinase plasminogen activator receptor (suPAR) in healthy individuals. Clin Biochem 2019. 69:36-44*). However, in patients with acute onset of myocardial infarction, the levels of suPAR for 3 individuals increase above the reference interval after treatment with glycosylated enzymes.

Aim 2: Characterise the relative abundance of glycoforms of suPAR in heart disease using mass-spectrometry (MS) technology

November 2020-March 2021

Optimisation experiments using the Velos Pro Ion Trap Mass Spectrophotometer, University of Otago Christchurch, were conducted during this period (in collaboration with Dr Louise Paton) to detect a commercially prepared recombinant suPAR protein (Sino Biological cat no:10925-H08H). This included experimenting on different sample preparations (chymotrypsin digestion, +/-deglycosylation procedures) and using different separation columns (HILIC, C18, and reverse phase). Using an ion pairing hydrophobic interaction liquid chromatography (IP-HILIC-MS) method, we identified a tryptic peptide for each domain of the recombinant suPAR (Figure 3).

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For this period, we tried to identify endogenous suPAR in patient samples using mass-spectrometry (MS). We identified potential peptides (amino acid sequence) from each domain that may be used to quantify suPAR cleaved forms using Multiple Reaction Monitoring (MRM) LC-MS. The results from our previous tryptic digests of recombinant suPAR (above) were used to select peptides that did not contain known posttranslational modifications and oxidizable side chains. As suPAR is highly cysteinylated (which may interfere with the MRM readings), two of the peptides that each contained only one cysteine residue were chosen (after a search in BlastP to ensure that our peptide was unique to human suPAR). An MRM method was set up using the prior Independent Data Acquisition (IDA) method to identify suPAR tryptic peptides. Collision energy parameters were calculated using the rolling collision energy table. A control plasma sample was acetonitrile precipitated and tryptic digested. It showed coelution in the MRM method of two different transitions for the domain two peptide chosen for quantitation (Figure 4). However, the peptides from domains 1 and 3 could not be detected. We will now optimise the MRM method by infusing synthetic versions of the chosen quantification peptides to maximise the collision energy and other MS source parameters. The method for protein extraction from plasma may also be improved to detect lower molecular weight peptides. Unfortunately, this part of the project proved challenging and time-limiting/consuming, but will remain as “on-going”.

Conclusion:

From this project, we have obtained several novel findings;
(i) There is possible uptake or clearance of suPAR across the heart which requires further investigations.
(ii) Although we have not been able to quantify and determine site of occupancy of glycosylated chains for suPAR, we have visualized cleaved suPAR bands which have not previously been reported in the cardiovascular disease setting.
(iii) The current commercialized clinical assay for suPAR is limited by glycosylation aspects. Therefore, this project has provided insights to potential new targets for suPAR measurements. For example, new antibodies could be raised against suPAR that is not impacted by the glycosylation sites (please refer to Figure 5)
(iii) The MRM LC-MS method for domain 2 of suPAR is unique and may be used to quantify cleaved suPAR forms.

(iv) There may be a need to differentiate circulating endogenous suPAR forms in patients with heart disease and this may elude to different disease states in these patients.

Future direction:

We have prepared electrophoretic SDS-PAGE of endogenous suPAR from patient samples that are awaiting MS analysis to identify the amino-acid sequence of suPAR, particularly in relation to the novel circulating suPAR fragments (shown in Figure 3). This will enable development of a new immunoassay targeting specific regions of suPAR, and to allow the measurements of different circulating suPAR forms (potential for commercial development).

Our summer studentship project will involve investigations into whether suPAR induces deleterious or protective mechanisms for heart function using isolated rat heart models.

Acknowledgements

I am extremely grateful for the funding provided by CMRF towards this project. The new skills and results obtained in this project have significantly benefited my career development and provided insights for research progression. The results of this project are exciting, novel and adds to the knowledge of suPAR biology in cardiovascular disease settings. Expertise and knowledge provided by Dr Louise Paton in relation to the mass-spectrometry work is greatly appreciated. Resources and support from the Christchurch Heart Institute and University of Otago (Christchurch) are also acknowledged.

Presentations:

Summer studentship 2020-2021: (presented by medical summer student; Fergus Allan. Summer studentship was funded by National Heart Foundation). *Title: Identification of suPAR cleaved products as potential new biomarkers of heart disease.*