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Proteomic analysis of intra-arterial thrombus secretions reveals a negative association of clusterin and thrombospondin-1 with abdominal aortic aneurysm

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ABSTRACT

Objective: Abdominal aortic aneurysm (AAA) is usually accompanied by the formation of a large volume of intra-luminal thrombus (ILT). ILT-derived proteins have been suggested as circulating markers for AAA. We conducted a proteomic study screening whole and hexapeptide ligand library (HLL) treated ILT explant secretions to identify potential ILT-derived markers for AAA.
Methods: Unfractionated and HLL-treated ILT secretions from 3 AAA patients were analysed in parallel using liquid chromatography tandem mass spectrometry (LC–MS/MS). *In silico* analyses were employed to identify proteins with biomarker potential. Proteomic findings were validated by measuring serum concentrations of 2 representative ILT proteins in 313 AAA patients and 690 controls.
Results: A total of 150 proteins were identified from thrombus conditioned media; HLL treatment enabled the detection of 53 previously unseen polypeptides. Gene ontology analysis revealed high representation of platelet-secreted proteins. Thrombospondin-1 (TSP-1) and clusterin were selected for further assessment. Serum TSP-1 and clusterin were negatively associated with AAA after adjusting for other risk factors. Odds ratio and 95% confidence intervals were 0.62, 0.41–0.94, and 0.50, 0.33–0.75, for men with serum TSP-1 and clusterin in the fourth compared to first quartiles, respectively.
Conclusion: This proteomic analysis has identified a group of proteins concentrated in AAA ILT. Assessment of circulating concentrations of two representative polypeptides suggests for the first time that the ILT selectively sequesters proteins rather than actively releasing them. Further work is required to assess the mechanisms underpinning this observation and the associated clinical implications.

1. Introduction

Abdominal aortic aneurysm (AAA) is commonly implicated in mortality in older adults due to aortic rupture, complications of surgical repair or associated cardiovascular events [1]. Blood-borne markers for AAA could potentially contribute to screening for AAA in at-risk subjects and provide a means to target new therapies. A non-occlusive intraluminal thrombus (ILT) forms within the aneurysm sac in most AAAs. ILT volume is highly correlated with AAA size, and has been associated with AAA growth and the incidence of cardiovascular events in AAA patients [2,3]. Previous studies suggest that the ILT may contribute to AAA pathogenesis [4,5]. High circulating concentrations of a number of thrombus associated proteins, such as D-dimer, have been previously associated with AAA [1,6]. Characterisation of ILT-associated proteins could provide further biomarkers or improved insight into AAA pathogenesis of clinical value. Furthermore, in contrast to aortic biopsies which are often recovered in relatively small quantities, large amounts of thrombus can be harvested during AAA surgery. In this way, it is possible to partially overcome limitations regarding access to experimental tissue, a common bottleneck in many vascular investigations. Thus the ILT represents an attractive tissue to probe for potential markers of AAA.

Recently, proteomic methodologies have been employed to identify novel AAA-related proteins within the ILT [7,8]. These investigations confirm the presence of abundant plasma proteins which limit the detection of more weakly expressed polypeptides and reduce the sensitivity of proteomic analyses [9]. This diffi-

Abbreviations: AAA, abdominal aortic aneurysm; ILT, intraluminal thrombus; FBS, foetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; HLL, hexapeptide ligand library; TSP-1, thrombospondin-1.

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culty is encountered in proteomic analysis of most blood associated samples. It has been suggested that this hurdle may be overcome by depleting abundant proteins to increase detection of weakly expressed components [10]. The aim of the current study was to identify novel AAA associated biomarkers through proteomic analysis of ILT secretions. A non-specific hexapeptide ligand library (HLL) was employed to deplete abundant proteins [10]. Proteomic data were analysed *in silico* to identify proteins of likely biomarker potential, and the validity of these findings was further assessed by measuring serum concentrations of two putative thrombus markers in a large population of men screened for AAA.

2. Materials and methods

2.1. Thrombus explant culture

ILT specimens were collected from informed consenting patients during open surgical repairs for AAA under institutional ethics approval. ILT tissues were rinsed in sterile PBS (pH 7.4), finely sliced and incubated in DMEM supplemented with penicillin, streptomycin, glutamine, 5% (v/v) foetal bovine serum (FBS) and 2 μg/mL Fungizone at 37 °C in a humidified 5% CO_2 atmosphere. After 1 h culture media was refreshed. Explants were cultured for a further 20.5 h to provide conditioned media which was decanted and centrifuged at 5000 × *g* for 10 min to pellet large debris. Supernatants containing secreted ILT proteins were stored at −80 °C.

2.2. Extraction of non-equalized ILT secretions

One third of the volume of ILT conditioned media was decanted for crude (non-equalized) protein analysis – the remainder was stored for HLL-equalization. Culture media was concentrated to ~500 μL via centrifugal filtration using filters with a 3 kDa cut-off (Amicon Ultra) as directed by the manufacturer. Samples were desalted by buffer-exchange into 40 mM Tris–HCl (pH 7.4), 0.2% (v/v) Triton X 100, 5 mM dithiothreitol (DTT) and a cocktail of protease inhibitors (Roche, Australia). Desalted proteins were precipitated overnight in 20 volumes acetone containing 20% (w/v) citric acid at −20 °C. Precipitated proteins were recovered by centrifugation at 5000 × *g* for 20 min at 4 °C, before washing in acetone with centrifugation to pellet as before. Proteins were air dried at room temperature, solubilised in a buffer containing 40 mM Tris, 7 M Urea, 2 M Thiourea, 1% (w/v) C7BzO (Sigma–Aldrich, Australia) and reduced and alkylated by adding 40 mM Tris, 5 mM tributylphosphine, and 10 mM acrylamide. After 2 h, the reaction was quenched by adding 10 mM DTT.

2.3. HLL-equalization of ILT secretions

Abundant protein depletion was conducted using a commercially available HLL (Proteominer, Bio-Rad Laboratories, Australia). Conditioned media containing >100 mg protein was pH-corrected to 7.5 ± 0.3 and made up to a final volume of 50 mL through addition of PBS (pH 7.5) prior to incubation for a total of 4 h with 100 μL hexapeptide ligand beads (Proteominer, BioRad). Equalized proteins were eluted by boiling in Laemmli buffer (4% SDS, 0.1 M Tris–HCl pH 8.9, 2 mM EDTA, 20% (v/v) glycerol) after the published elution methods were unsuccessful, and precipitated in excess acetone containing 3% (w/v) citric acid at room temperature. SDS-eluted proteins were pelleted, washed, solubilised and reduced and alkylated as described above.

2.4. One dimensional electrophoresis

Aliquots containing 70 μg protein were mixed with Laemmli buffer boiled at 95 °C for 5 min prior to separation in a 4–12% Bis–Tris polyacrylamide gradient gel (Bio-Rad, Australia). Gels were fixed and stained with Flamingo fluorescent protein stain (Bio-Rad, Australia), according to manufacturer's instructions. Gels were imaged using a Chemidoc XRS molecular imager (Bio-Rad, Australia) with the Quantity One software package (Bio-Rad, Australia) using UV transillumination.

2.5. Protein excision and in-gel tryptic digestion

Gels were over-stained with Coomassie G250 (Bio-Rad, Australia) and cut into 1 cm slices spanning the length of the protein lane. Gel slices were diced and destained in 2 washes with 50% acetonitrile/50 mM NH_4HCO_3 pH 9. Destained gel slices were dehydrated in 100% acetonitrile for 10 min and rehydrated in 30 μL of NH_4HCO_3 pH 9 containing 12.5 ng/μL trypsin (Promega) at 4 °C. After 30 min, 50 μL 50 mM NH_4HCO_3 pH 9 was added and the samples incubated overnight at 37 °C. The digest solution was decanted to a new tube and 50 μL of 50% acetonitrile, 2% formic acid was added to the gel slices followed by 10 min incubation in an ultrasonic water bath. This solution was removed, pooled with the digest solution, lyophilised to 15 μL for LC–MS/MS.

2.6. LC–MS/MS

Using an AS-1 autosampler connected to a Tempo nanoLC system (Eksigent, USA), 10 μL peptide sample was loaded at 20 μl/min with MS buffer A (2% acetonitrile + 0.2% formic acid) onto a C8 trap column (Michrom, USA). The trap was washed for 3 min before peptides were eluted 300 nL/min onto a IntegraFrit column (75 μm × 100 mm) packed with ProteoPep II C18 resin (New Objective, Woburn, MA). Peptides were eluted into the source of a QSTAR Elite hybrid quadrupole-time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex) using the following program: 5–50% MS buffer B (98% acetonitrile + 0.2% formic acid) over 15 min, 50–80% MS buffer B over 5 min, 80% MS buffer B for 2 min, 80–5% for 3 min. Peptides were ionised with a 75–15 μm ID emitter tip (New Objective) at 2300 V. An intelligent data acquisition experiment was performed, continually scanning a mass range of 375–1500 Da for charged peptides (2+ to 5+) with an intensity >30 counts/s. Selected peptides were fragmented and the product ion masses measured over 100–1500 Da. The mass of the precursor peptide was then excluded for 60 s.

2.7. MS data analysis

MS/MS data files were analysed using Mascot Daemon (v2.2.2, provided by the Australian Proteomics Computational Facility, http://www.apcf.edu.au/. Perkins, D.N. 1999) to search against the LudwigNR database (vQ209. 8785680 sequences, 3087386706 residues). Search parameters were: species: human; fixed modifications: none; variable modifications: propionamide, oxidised methionine; enzyme: semi-trypsin; number of allowed missed cleavages: 3; peptide mass tolerance: 100 ppm; MS/MS mass tolerance: 0.2 Da; Charge state: 2+ and 3+. Protein hits with ≥1 unique peptide, and a *P*-value < 0.05 were included in this study. MS/MS spectra were manually inspected to ensure sufficient b- and y-ion series for accurate peptide identification. Peptide and protein identifications were validated using the Peptide Prophet algorithm within the Scaffold V 3.00.03 software package (Proteome Software Inc., Portland, OR, USA) [11,12]. Peptide identifications established at >95% were accepted. Proteins identified at ≥ 95% probability with ≥1 identified peptide were included. Proteins containing similar peptides which could not be differentiated using MS/MS analysis alone were grouped to satisfy the principles of parsimony.