Identification of novel biomarkers of Abdominal Aortic Aneurysms (AAA) by 2D-DIGE and MALDI-MS from AAA-thrombus conditioned media

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Abstract

In the search for novel biomarkers, non-candidate based-proteomic strategies open-up new opportunities to gain a deeper insight into disease processes regarding their molecular mechanisms, the risk factors involved and the monitoring of disease progression (1, 2). To carry out these complex analyses, the combined use of gel electrophoresis with mass spectrometry (MS) represents a powerful choice. In addition, the introduction of protein dye-labeling has notably improved the reliability of differential expression studies by increasing the statistical significance of the protein candidates. Here we describe a strategy where different layers (luminal/abluminal) from the intraluminal thrombus (ILT) of human abdominal aortic aneurysm (AAA) patients were incubated in protein-free medium. Then, the levels of the proteins released were compared by two-dimensional differential in-gel electrophoresis (2D-DIGE), and the proteins of interest identified by MS. We consider that the use of tissue conditioned media could offer a substantial advantage in the analytical study of biological fluids, as they provide a source of proteins to be released to the bloodstream, which could serve as potential circulating biomarkers.

1. Introduction

The scarce correlation between mRNA and protein levels, together with the complexity added by alternative splicing mechanisms and post-translational modifications, points out that genome alterations alone fail to reflect the whole functional complexity of an organism. In this regard, proteomic approaches can provide essential information in different physiological scenarios, generally combining electrophoretic or chromatographic separations with mass spectrometry for protein identification (3). The past few years have witnessed tremendous

advances in the development of proteomic tools, especially those related to MS-based approaches. Nevertheless, gel-based strategies continue to offer outstanding capabilities for quantitative proteomic studies. Thus, the 2D-DIGE system enables to separate differentially dye-labeled proteins in the same gel, therefore minimizing technical variability. The overlay of the corresponding images enables an accurate comparison of protein components among the samples, which provides a reliable quantitation of differential protein expression.

The complexity of vascular lesions, as well as that of biological fluids involved (e.g. plasma), hinders proteomic approaches. We hypothesized that the blood compartment could reflect what was observed in the arterial conditioned medium and thus, that proteins potentially released into circulation could serve as disease biomarkers. Our study, performed on the thrombus conditioned medium, using 2D-DIGE labeling followed by MS, permitted the identification of several proteins associated to key mechanisms involved in AAA pathogenesis (4).

2. Materials

2.1. AAA-tissue conditioned media preparation

1. Wash buffer: Sterile saline buffer.
2. Culture medium: RPMI 1640 without fetal bovine serum, containing 1% antibiotics and antimycotic (Gibco).

2.2. Sample preparation for 2D-DIGE

1. 2D clean-up kit for protein precipitation (GE Healthcare).

2. Lysis buffer: 30mM Tris-HCl pH 8.5, 7M urea, 2M thiourea, 4% (w/v) CHAPS.

3. pH indicator paper strips pH 8.0-9.7 (Whatman International Ltd.).

4. RC-DC protein assay kit (Bio-Rad).

2.3. Protein labeling for 2D-DIGE

1. Ten millimolar of L-Lysine monohydro-chloride min 98% (MW 182.6, Sigma).

2. Ultrapure anhydrous dimethylformamide (DMF) 99.8% (DMF, Sigma). Stable for 3 months at room temperature.

3. Five nanomoles of CyDye DIGE Fluor Cy2 minimal dye, 5 nmol CyDye DIGE Fluor Cy3 minimal dye and 5 nmol CyDye DIGE Fluor Cy5 minimal dye (GE Healthcare). Store at -80°C.

2.4. Sample preparation and isoelectric focusing (IEF)

1. Rehydratation buffer for sample loading: 7M urea, 2M thiourea, 4% (w/v) CHAPS, 0.8% (v/v) IPG Buffer 3-11NL, 50mM DTT and bromophenol blue.

2. Rehydration buffer for Immobiline DryStrips: 7M urea, 2M thiourea, 4% (w/v) CHAPS, 0.8% (v/v) IPG Buffer 3-11NL, 97 mM DeStreak reagent (GE Healthcare) and bromophenol blue.

3. Immobiline DryStrip pH 3–11 NL, 24 cm and Immobiline DryStrip reswelling tray (GE Healthcare).

4. Paper wicks.

5. PlusOne^{TM} DryStrip Cover Fluid (GE Healthcare).

6. IPGphor II IEF system (GE Healthcare).

2.5. Second dimension

1. SDS equilibration buffer stock solution: 50 mM Tris-HCl, pH 8.0, 6 M urea, 40% (w/v) glycerol and 1% (w/v) SDS. This stock solution must be stored at −20°C.

2. Reducing Equilibration solution: 1% (w/v) DTT in 10 mL SDS equilibration buffer stock solution. Used immediately after preparation.

3. Alkylating Equilibration solution: 3.5% (w/v) iodoacetamide in10 mL SDS equilibration buffer stock solution. Used immediately after preparation.

4. Ettan DALT six Large Vertical System for 24-cm strips, low-flourescence plates with 1-mm integral spacers for Ettan DALT, Ettan DALT Cassette Rack and Ettan DALT Gel Caster (all from GE Healthcare).

5. Water-saturated butanol. Use to overlay gels. Store at room temperature.

6. Two-dimensional DIGE SDS-PAGE (sodium-dodecyl sulphate-polyacrylamide gel electrophoresis), 12.5% gel composition (500 mL for 6 gels): 200 mL 30% (w/v) acrylamide/bis, 186.5 mL 1M Tris-HCL pH 8.8, and 5 mL 10% SDS; make up to 500 mL with distilled water. Sonicate complete solution and add 2.5 mL 10% ammonium persulphate (APS) and 0.25 mL N,N,N,N′-tetramethyl-ethylenediamine (TEMED) before pouring the solution.

7. SDS electrophoresis running buffer: 25 mM Tris, 192 mM glycine and 0.2% SDS. Store at room temperature.

2.6. Staining of gels and analysis of gel images

1. Fixation solution: absolute methanol (12%) and acetic acid (7%).

2. Silver Staining: PlusOne Silver staining kit, protein (GE Healthcare), containing Sensitizing Solution (30% EtOH (v/v), 0.2% $Na_2S_2O_3$ (w/v), 6.8% CH_3COONa (w/v)), Silver solution (0.25% $AgNO_3$), Developing Solution (2.5% Na_2CO_3, 0.015% HCOH (v/v) and Stopping Solution (1.46% EDTA (w/v)).

3. For visualization of CyDye fluorescently labeled proteins, Typhoon 9400 (GE Healthcare).

4. For image analysis, the DeCyder v7.0 (GE Healthcare).

2.7. Spot digestion

The following buffers should be freshly prepared, as they degrade very quickly:

1. Reduction buffer: 10 mM DTT (GE Healthcare) in 50 mM NH_4HCO_3 (99.5% purity; Sigma Chemical).

2. Alkylation buffer: 55 mM iodoacetamide (Sigma Chemical) in 50 mM NH_4HCO_3.

3. Trypsin (Sequencing grade; Promega) at a final concentration of 7.5 ng/μl in 50 mM NH_4HCO_3.

4. 0.2% trifluoroacetic acid (TFA) (99.5% purity, Sigma Chemical)

5. Pierced V-bottom 96-well polypropylene microplate (Bruker Daltonik).

6. V-bottom 96-well polypropylene microplates (Greiner Bio-One).

7. Proteineer DP protein digestion station (Bruker Daltonic).

2.8. Sample preparation for MALDI-MS

1. Matrix solution: 0.2 g/l α-cyano-4-hydroxycinnamic acid (Bruker Daltonik) in 50% aqueous acetonitrile and 0.2% trifluoroacetic acid.

2. 600 μm AnchorChip prestructured MALDI probe (Bruker Daltonik).

3. Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik) with an automated analysis loop controlled by the flexControl 2.2 software (Bruker Daltonik).

3. Methods

3.1. Patient recruitment and samples

Recruit thrombus samples from AAA patients who have undergone surgical repair. Written consent must be given by the patients.

3.2. AAA-thrombus conditioned media isolation

1. Dissect the human AAA thrombus samples under sterile conditions into luminal and abluminal parts, respectively at the interface with circulating blood and with the remaining media (Figure1) (see Note 1).

2. Cut luminal and abluminal layers into small pieces (5 mm^3) and incubate them separately in RPMI 1640 medium containing antibiotics and an antimycotic (Gibco) for 24 hours at 37°C (6 mL/g of wet tissue) (see Note 2).

3. Centrifuge conditioned media (supernatant containing proteins released by the tissue sample) at 3,000 xg for 10 minutes at 20°C to eliminate particulates.

3.3. Experimental design and DIGE protein labeling

1. Isolate supernatants from luminal and abluminal layers of ILT human samples from SIX individual patients.

2. Precipitate proteins using the 2D clean-up kit and resuspend them in lysis buffer. Make sure that proteins are fully resuspended. Measure protein concentration by RC-DC protein assay (see Note 3).

3. Prepare stock CyDye reagents by adding 5µL of dimethyl formamide (DMF) to each of three CyDye vials. Spin and store at -80°C until use (see Note 4).

4. Freshly prepare working CyDye solutions. For 6 gels (12 protein samples), a final volume of 6µL per CyDye vial is required. For each CyDye use the following volumes:

    a. Volume needed from stock solution: 6 µL/µmol (CyDye) x 0,4 µmol = 2,4 µL

    b. Volume needed of DMF: 6 µL - 2,4 µL = 3,6 µL

    c. Working solution preparation: 2,4 µL (Stock solution) + 3,6 µL (DMF) = 6µL

5. Spin-down samples and prepare individual samples with 50 µg of protein extract (see Notes 5 and 6).

6. Label individual samples with Cy3 or Cy5 dyes by adding 1 µL of working solution and spin-down. Incubate on ice for 30 minutes in the darkness (see Note 7).

7. Quench the reaction with 1 µL of 10 mM lysine for 10 minutes in the dark on ice.

8. For the internal standard, prepare a pool with 25 µg of protein extract from each individual sample. Label with 6 µL of Cy2 dye on ice for 30 minutes in the darkness and quench the reaction with 6 µL of 10mM lysine for 10 minutes.

3.4. Two-dimensional electrophoresis and image acquisition

1. Rehydrate six 24 cm-long IPG Strips pH3-11non linear (NL) gradient with 450µL each of the aforementioned Rehydration buffer containing 97mM DeStreak (GE Healthcare) (see Note 8).

2. Sample preparation for the first dimension: Mix the six-paired samples of Cy3 and Cy5-labeled proteins with 50 µg of Cy2-labeled internal standard. Dilute the mixture in Rehydration buffer containing 50mM DTT, and resolve them by applying the sample mixtures via cup loading to the previously rehydrated IPG Strips (see Note 9).

3. Isoelectric focusing (IEF): The isoelectric focusing can be carried out on an IPGphor II IEF system (GE Healthcare) up to a total of 42 kVh (see Note 10).

4. IPG strips equilibration: Equilibrate the strips in 10 mL of Reducing SDS equilibration buffer during 15 minutes at room temperature with agitation.Then, eliminate the Reducing solution and incubate with Alkylating SDS equilibration buffer for 15 additional min (see Notes 11 and 12).

5. Second dimension: SDS-PAGE is carried out on 12% polyacrylamide gels at 2w/gel (for overnight running) or after sample entry in the running gel (40 min), increase to 20w/gel (max. 100w) for in-day gel running (see Notes 13, 14 and 15).

6. Image acquisition: For each gel acquire the Cy2-, Cy3-, and Cy5-labeled channel image at excitation/emission wavelength values of 488/520, 523/580, and 633/670 nm, respectively, using a Typhoon 9400 laser scanner (see Note 16).

3.5. DIGE image analysis

Analyse the images using the DeCyder v7.0 software for spot detection and quantification and inter-gel matching and statistics:

1. DIA (Differential In-gel Analysis): This is the DeCyder module for the initial spot detection, which compares all three fluorescence images obtained from each gel and performs co-detection, background removal and normalization. The outcome is the volume of each spot detected in Cy3- and Cy5-labeled samples after normalization with respect to the corresponding Cy2 signal measured from the pooled internal standard sample. The DIA data sets from each individual gel were collectively analyzed using the biological variation analysis (BVA).

2. BVA (Biological Variation Analysis): This is the second DeCyder module used for matching multiple 2D-DIGE gels, which provides statistical analysis of protein abundance changes. Consider spots present in all of the 12 images (Cy2-, Cy3-, and Cy5-channel images for each gel) with statistical significance at 95% confidence level for standardized average spot volume ratios over 1.5.

3. EDA (Extended Data Analysis): The third DeCyder module for advanced statistical analysis generates the final list of candidate spots, Principal Component Analyses (PCAs) and their hierarchical clustering, for the characterization and classification of biological samples based on protein expression data (see Note 17).

3.6. Gel silver staining

Stain the gels using PlusOne Silver Staining kit from GEHealthcare, with some modifications to the manufacturer´s instructions (see Note 18). Briefly:

1. Incubate gels individually with Fixation solution at least for one hour at 20 ºC for protein fixing and gel cleaning (see Note 19).
2. Incubate gels individually with cold sensitizing solution for 20-30 min (see Note 20).
3. Wash with distilled water, 3 times 10 min each.
4. Incubate with 10% silver solution (silver nitrate) for 20 min for silver impregnation.
5. Wash with distilled water, twice, 1 min each (see Note 21).
6. Add developing solution containing 0.4% formaldehyde freshly supplemented for silver staining. Incubate gels until spots are visible (usually less than 5 min) with vigorously shaking.
7. Incubate gels with stopping solution for at least 15 min to stop silver reduction.
8. Wash three times, 15 min each, with distilled water, and change the gel container.

3.7. In-gel tryptic digestion

Digest samples automatically using a Proteineer DP protein digestion station following the protocol of Schevchenko et al. (5) with minor variations:

1. Spot picking: Match silver-stained gels against DIGE images and excise selected spots manually. Transfer them to pierced V-bottom 96-well microplates loaded with ultrapure water.

2. Submit gel plugs to reduction with 10 mM DTT (GE Healthcare) in 50 mM NH_4HCO_3 (99.5% purity; Sigma)

3. Submit to alkylation with 55mM iodoacetamide (Sigma Chemical) in 50mM NH_4HCO_3.

4. Rinse gel pieces with 50 mM NH_4HCO_3 and acetonitrile (gradient grade; Merck) and dry them under a nitrogen stream.

5. Protein digestion: Add modified porcine trypsin at a final concentration of 7.5ng/μl in 50mM NH_4HCO_3 to the dried gel pieces and allow the digestion to proceed at 37 ºC for 10h.

6. Transfer the resulting digestion solutions by centrifugation to V-bottom 96-well polypropylene microplates, vacuum-dry them and keep at 4ºC for later MS analysis.

3.8. MALDI Mass spectrometry

1. Re-dissolve the dried samples in 10μl of matrix solution.

2. Deposite 0.6μl of this solution onto the 600μm AnchorChip prestructured MALDI probe (6).

3. Allow the sample-matrix drops to dry at room temperature.

4. Analyze samples on an Ultraflex MALDI-TOF/TOF mass spectrometer (7) using an automated analysis loop controlled by the flexControl 2.2 software.

5. First step: Acquire MALDI-MS spectra by averaging 400 individual spectra in the positive ion reflector mode at 50 Hz laser frequency in the 800 to 3500 m/z range.

Perform internal calibration of MALDI-MS mass spectra using the two trypsin autolysis ions showing at 842.510 and 2211.105 m/z (see Note 22).

6. Second step: Acquire MALDI-MS/MS spectra by averaging 800 individual spectra from precursor ions showing with intensity > 1500 in the above MALDI-MS spectra.

7. Perform an automated analysis of mass data using the flexAnalysis 2.2 software to obtain peptide (MALDI-MS) and precursor (MALDI-MS/MS) m/z values.

3.9. MALDI-MS database searching

1. Combine MALDI-MS and MS/MS data through the BioTools 3.0 program to search a nonredundant protein database (NCBInr 20091015, ~ 10^7 entries, National Center for Biotechnology Information, Bethesda US), using the Mascot software v2.2 (Matrix Science, London, UK; http://www.matrixscience.com) (8).

2. Set other relevant search parameters as follows: enzyme, trypsin; fixed modifications, carbamidomethyl; allow up to 1 missed cleavage; peptide tolerance ±20 ppm; MS/MS tolerance ±0.5 Da.

3. Consider protein scores significant when greater than 82 ($p<0.05$).

4. Notes

1. Tissue samples must be washed with saline buffer before their incubation in the culture medium, to minimize blood remains.

2. Sterile material is required for tissue dissection.

3. Supernatants or conditioned media must be precipitated before IEF to remove salts and other ionic components that could interfere in both the CyDye labeling reaction, and later in the isoelectroforetic separation. To facilitate complete resuspension of protein pellets shake vigorously for at least 1h at room temperature.

4. After reconstitution, dye reagents (stocks solutions) are stable for three months at -70 ºC /-80 ºC, while working solutions are stable for just one week at -70 ºC /-80 ºC.

5. Check that pH of protein extracts is *ca.* 8.5 to ensure appropriate Fluor dye labeling. The pH must be checked on ice after protein precipitation, and when necessary carefully modified by addition of small volumes of Tris-HCl (1M; pH9.0) or aqueous sodium hydroxide.

6. Under minimal labeling conditions, 400 pmol Fluor CyDye labels 50 µg of protein.

7. To ensure adequate and sustained protein labeling not only the labeling reaction must be performed on ice and in the dark, but also the labeled samples must be strictly kept under these conditions.

8. It is recommended that the rehydration of IPG strips be completed overnight (or at least 10-12 h) at around 20 ºC. The recommended volume must be used to ensure a homogeneous strip rehydration and conditioning.

9. For optimal labeling, the volumes must be adjusted in the 3-5 mg/ml range. Furthermore, it is advisable to use comparable loading volumes for all the strips (120 µL).

10. IEF conditions recommended for 24cm, pH3-11 IPG strips are as follows: Step 1, 300V, 3h, gradient; Step 2, 1000V, 5h, gradient; Step 3, 8000V, 2h, gradient; Step 4,

8000V, constant up to a total of 42kV h. A final step of 500V is also recommended to avoid protein diffusion after focusing.

11. In the alkylating SDS Equilibration buffer 3.5-4% iodoacetamide (IAA) is recommended. During the second equilibration step, IAA must be in excess with respect to DTT to guarantee that Cys residues will remain protected by alkylation. This excess IAA not only avoids reformation of sulfur–sulfur bonds, but also prevents DTT point streaking in gels.

12. If the second dimension in not run immediately, IPG strips can be stored at -20 ºC for a few weeks. Do not store the strips once they have been equilibrated.

13. Gels must be polymerized overnight at *ca.* 20 ºC to reduce acrylamide-modified artifacts. No stacking gel is necessary as IPG strips works as a stacking gel.

14. 0.2% SDS-running buffer is recommended instead of the usual 0.1% SDS buffer.

15. The second dimension can be run at 2w/gel and higher potential values, up to a maximum of 17w/gel. Control temperature (*e.g.*, 20 ºC) for optimal protein separation.

16. Gel pre-scanning is advised before acquiring the final image for DeCyder analysis. Scanner photomultiplier values must be adjusted in the three channels (Cy2, Cy3 and Cy5) to avoid saturation of the spot signals. It is also recommended to use comparable maximum values of pixel intensities for the three images.

17. Results derived from EDA module show data quality, in order to delete outlyers values if needed.

18. To ensure compatibility with later MS analyses, glutardialdehyde and formaldehyde must not be added to sensitizing and silver solutions, respectively. To compensate for the slightly decreased sensitivity of the as-prepared silver nitrate solution, the developing solution is supplemented with twice the usual concentration of formaldehyde.

19. As a gel fixing solution, methanol is preferred rather that ethanol, as its lower reactivity with acetic acid reduces unwanted protein esterification.

20. The use of a cold sensitizing solution is recommended to minimize gel background.

21. The gel must be completely submerged into water during the washing steps. Make sure that the finals washing steps before the developing process, last strictly one minute each to avoid removal of silver ions.

22. For MALDI-MS/MS, the calibrations must be performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region.

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FIGURE LEGENDS

Figure1. Detailed inspection of an abdominal aortic aneurysm fragment, showing a section of the intraluminal thrombus. Abluminal and luminal layers are delimited by different cellular composition. Adapted from Fontaine V, Touat Z, Mtairag el M, Vranckx R, Louedec L, Houard X, Andreassian B, Sebbag U, Palombi T, Jacob MP, Meilhac O, Michel JB. Role of leukocyte elastase in preventing cellular re-colonization of the mural thrombus. Am J Pathol, 2004, 164:2077–2087