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In vitro and *in vivo* biotinylation of endothelial cell surface proteins in the pursuit of targets for molecular therapies for brain AVMs

Margaret Simonian[1]*, Mark P Molloy[2] and Marcus A Stoodley[1]

[1]*Australian School of Advanced Medicine, Macquarie University, North Ryde, NSW, 2109, Australia*
[2]*Australian Proteomics Analysis Facility (APAF), Department of Chemistry and Biomolecular Sciences, Macquarie University, North Ryde, NSW, 2109, Australia*

Abstract

Identification of membrane proteins that are expressed on the endothelium after radiosurgery is of fundamental importance in developing a new treatment for brain vascular formations. We optimized then employed *in vitro* and *in vivo* biotinylation methodology to label membrane proteins in a murine cerebral endothelial cell cultures (bEnd.3) and a rat model of arteriovenous malformation (AVM). Membrane proteins were then captured on streptavidin resin and identified using proteomics analysis.

Keywords: Biotinylation; Membrane proteins; Murine cerebral endothelial cells

Introduction

Arteriovenous malformations (AVMs) consist of a tangle of abnormal arteries and veins linked by one or more fistulae [1,2]. AVMs in the brain can occur in any region, and range in size from small (< 3cm) to large (> 6cm). Patients with AVMs present with headaches (migraines), seizures and most commonly haemorrhages. The first haemorrhage is most likely to occur between the ages of 20-40 years [3,4].

Treatment of AVMs depends on their location (eloquent or non-eloquent brain) and size [1,5,6]. Small AVMs located at the surface of the brain are suitable for direct surgery [2]. Large AVMs are usually wedge-shaped and extend deeper into the brain, these are more difficulty to treat with surgical removal [2].

Embolization involves occluding the blood flow to an AVM using endovascular catheters and can be effective for rare lesions that are less than 1cm in diameter and fed by a single artery [7,8]. Radiosurgery is a treatment option for lesions < 3cm in diameter and located in eloquent areas where surgery can cause neurological deficits [1]. Compared to other treatments, the immediate risk at the time of the radiosurgery is very low. However, vascular occlusion after radiosurgery can take up to 3 years to complete, and patients remain at risk of haemorrhage during this time [9,10].

Approximately one third of AVMs are unsuitable for current treatment methods. Therefore there is a need for a new treatment, that is safer and more effective than current treatment methods for these large and deep lesions

In 2007 a study by Storer et al [11]. demonstrated induction of thromboses in an animal model of AVM treated with radiosurgery by administering lipopolysaccharide (LPS) and tissue factors which is a non-ligand type of vascular targeting. However this approach wasn't successful in the large vessels and there are safety concerns regarding injecting humans with LPS [11,12].

A ligand-based vascular targeting approach has the potential to overcome these problems, but requires a luminal surface molecule that discriminates AVM vessels from normal vessels. We propose that radiosurgery can stimulate the cell surface expression of discriminating proteins. In this study we aim to identify potential protein targets in AVM endothelium after radiosurgery. These protein candidates could then be investigated for ligand-directed treatment to promote rapid thrombosis in AVM vessels. To achieve this goal, a successful labelling of cell surface proteins is crucial.

Here we describe the *in vitro* and *in vivo* biotinylation method that we employed to label membrane proteins in the murine endothelial cell culture (bEnd.3) and in an animal model of AVM. Surface biotinylation with biotin derivatives was followed by purification on streptavidin resin. This approach has been shown to be successful in recovering membrane proteins both in *in vitro* and *in vivo* studies [13-15]. Membrane proteins were then identified by ESI LS MS/MS analysis.

Materials and Methods

In vitro biotinylation

Cell culture: Murine bEnd3 endothelia cells (American Type Culture Collection, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g /L D-glucose (Invitrogen Gibco, CA, USA), 4mM L-glutamine, and 0.11 g/L sodium pyruvate containing 10% fetal bovine serum (Invitrogen Gibco), HEPES and streptomycine (Invitrogen Gibco) in a 5% CO_2 atmosphere at 37°C. Cells were seeded in 75 CM^3 tissue culture flasks until 80% confluent.

Surface biotinylation was performed on the endothelial cell cultures using a modified protocol [13,14].

Each 75 cm^2 flask containing approximately 1×10^6 cells was washed four times with PBS pH 7.4. Twenty millilitres of PBS containing 67 µM EZ-link (Sulfo-NHS-LC-Biotin) (Pierce, IL, USA) were added to the flasks and incubated for 5 min at room temperature. The biotinylation reaction was terminated by adding Tris-Hcl pH 7.5 to a final concentration of 670 µM. After 5 min incubation the cells

***Corresponding author:** Margaret Simonian, Australian School of Advanced Medicine, Macquarie University, Sydney, 2109, Australia, Tel: +61-2-9812 3608; Fax: +61-2-9812 3610; E-mail: Margaret.simonian@mq.edu.au

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were washed four times with PBS and harvested with 2-3 mL of lysis buffer containing [2% w/v NP40, 0.2% w/v SDS and protease inhibitor (Complete, EDTA-free from Roche, Switzerland)] and kept on ice for 30 min.

Capture of biotinylated proteins: Biotinylated proteins were captured on streptavidin sepharose high performance (GE health care, Australia). Five hundred microlitres of streptavidin sepharose were washed three times with buffer **A** containing (1% w/v NP40, 0.5% w/v SDS in PBS) before adding to cell lysates. Samples then were incubated with washed streptavidin sepharose for 2h in room temperature. Streptavidin sepharose was pelleted by centrifugation at 1600 g for 5min. Unbound proteins were removed by washing 3 times with buffer **A**, once with buffer **B** (0.1% w/v NP40, 0.5 M NaCl in PBS) and once with digestion buffer (0.25 mM AMB).

Tryptic digestion of biotinylated proteins and Nano-LC ESI MS/MS: Streptavidin sepharose was re-suspended in 200 µL of digestion buffer. Twenty microlitres of trypsin were then added and incubated overnight at 37°C. The samples were centrifuged at 14,100g for 2 minutes at room temperature. The supernatant was recovered and dried. Samples were then fractionated by strong cation exchange liquid chromatography (SCX) and nanoLC/MS/MS carried out with a Qstar Elite mass spectrometry (AB Sciex, Foster City, CA) as described [16].

In vivo biotinylation of the rat model of AVM

A rat model of AVM was developed that closely resembles human AVMs [17]. Six weeks after fistula creation, *in vivo* biotinylation perfusion was carried out on the rat model of AVM as described briefly below.

The rat was narcotized with a subcutaneous injection of combined anaesthesia of (ketamine 100 mg/mL, xylazine 20 mg/mL and acepromazine 10 mg/mL). Using blunt scissors, the skin was cut from the abdomen to the thorax, then dissect to open the peritoneum . The chest was opened through a median sternotomy. The heart was turned around quickly by holding it with forceps at the apex, and an injection needle was inserted into the left ventricle and then to the aorta. A small cut in the right atrium was made with Student Vannas Spring Scissors to allow blood and perfusion solutions to flow out. Using the Gilson Minipuls 3 perfusion pump attached to a tube and needle, the rat was perfused with 1L of saline (NaCl) to wash away the blood, then immediately followed with 100 mL of freshly prepared biotinylation solusion [1 mg/mL of EZ-Link, Sulfo-NHS-LC-Biotin in pre warmed PBS at 37°C + 10% Dextran 40] by pressing the syringe plug with a flow rate of 30 ml/min while monitoring the pressure and keeping it constant at ~100 mm Hg.

After 5 min of perfusing the biotinylation solution , the rat was injected with 100 mL of (50mM Tris-Hcl in PBS + 10% Dextran 40) with a flow rate of 30 mL/min to wash out excessive biotinylation reagent, then was perfused with 200 ml saline at 30mL/min to wash away Dextran. The fistula tissue then was excised and the surrounding fat and muscle tissue was removed. The vascular tissue was placed in a 1 mL Eppendorf tube and transferred to a -80°C freezer immediately.

Membrane extraction and nano-LC ESI MS/MS

The tissue sample was pulverized in liquid nitrogen and resuspended in 1mL of lysis buffer (20mM HEPES, 150mM NaCl, 10mM NaF, 1mM Na-EDTA, 1mM Na-EGTA, pH 7.5, pH adjusted with NaOH) + protease inhibitor (4µL per mL of HEPES buffer, Sigma P-2714). Each sample was probe sonicated for 3 x 15 sec in ice using the probe sonicator (Branson sonifier 450, John Morris Scientific) and centrifuged in a pre-cooled rotor at 1,500 x *g* for 15 min (4°C). The supernatant was collected and pellet was re-lysed with 0.5mL of HEPES buffer (same as above steps). The supernatant was collected and pooled with previous supernautant, the final volume of supernatant was ~1.5mL. Sodium Bicarbonate solution (0.1M, pH 11) was added to pooled supernatant (up to 5mL) and incubated 1 hr at 4°C on rocking platform. After incubation, the sample was centrifuged at 120,000 g for 1h (4°C) using a S80-AT3 rotor.

The pellet was dissolved with 200 µL of 100mM Amonium Bicarbonate containing 10mM DTT (freshly prepared) in water bath sonication (Transsonic 700/H, John Morris Scientific) for 20 minutes and then incubated for 1hr at 37°C to reduce the sample. To alkalize the sample, 5 µL of 1M idoacetamide stock was added to make final concentration to 20mM idoacetamide and incubate in dark at room tempreature for 30 min. The sample volume was then brought up to 5ml with 100mM Amonium Bicarbonate and centrifuged at 120,000 x g for 1 hr (4°C). The pellet was dissolved with 400 µL of 100mM Amonium Bicarbonate in water bath sonication then 600ul of methanol was added. Membrane proteins were captured on streptavidin beads followed by on-beads proteins digestion with trypsin overnight. Peptides then separated by SCX followed by ESI LC MS/MS as described above.

Figure 1 is a flow diagram of the *in vivo* rat perfusion and the subsequent sample preparation for proteomics analysis.

Data analysis

The nanoLC ESI MS/MS data were submitted to Mascot for protein identification using the SwissProt database containing *Mus musculus* protein entries. Biotinylated lysine and amino terminus were considered as static modifications. Peptide ion scores above 35 were reported giving a probability of correct identification (P<0.05).

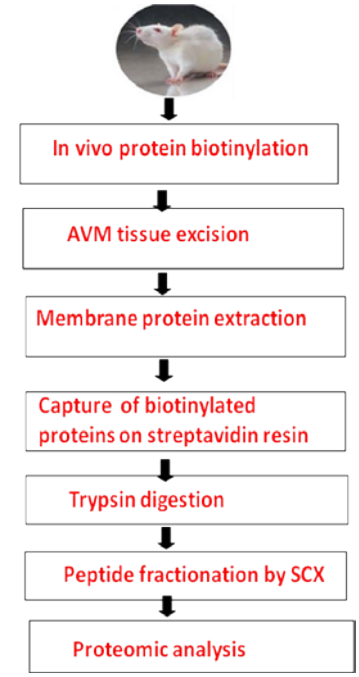

Figure 1: Flow diagram of *in vivo* perfusion in the rat model of AVM and the subsequent steps.

Results

We developed a strategy to derivatize and recover cell surface membrane proteins from cell cultures and a rat AVM animal model and analyses them using mass spectrometry (Figure 1). Recovered, biotinylated peptides from bEnd3 mouse endothelial cells were identified by mass spectrometry (Table 1). Two hundred and thirteen proteins were detected using ProteinPilot. Search of the literature confirmed that 56 of these proteins are annotated as cell membrane proteins. Future work involves determining the expression level changes of membrane proteins in response to irradiation treatment over a time course.

The proteomics data from the AVM rat model detected 135 proteins, 29 were annotated as membrane proteins. Table 2 shows selected membrane proteins.

This data demonstrates the viability of derivatising endothelial cell surface membrane proteins in situ, recovering them and determining their identity using mass spectrometry. We carried further *in vivo* biotinylation perfusion optimization on another 8 rats, using different biotin concentrations, perfusion rate and time. Harvested AVM tissues are currently being analysed using proteomics analysis. Future work will involve targeted radiosurgery using AVM rat model followed by the proteomics workflow we described. This will lead to the identification of vascular targets for treatment.

Protein name	Mascot Protein Ion	Number of matched peptides
Platelets endothelial cell adhesion molecule	218	4
Lamin-B1	219	12
Spectrin beta chain, brain-1	591	21
Tight junction protein ZO-2	32	1
Sodium/potassium-transporting ATPase	275	4
Ras-related protein Rab-1A	172	4
Transmembrane emp24 domain	52	1
Lamin-B2	91	3
Tight junction protein ZO-1	365	8
Glucose-6-phosphate isomerase	45	1
Alpha-internexin	40	3
Cadherin 5	179	3
Cadherin 13	90	1
Endothelial cell-selective adhesion molecule	152	2
Integrin alpha 3	149	3
Ras-related protein Rab-1B	138	3
Integrin alpha 6	86	1
Cell surface glycoprotein (Muc18)	141	3
Integrine beta 1	123	3
Intercellular adhesion molecule 2 (ICAM2)	68	3
Annexin	96	2
Leukocyte surface antigen CD47	97	1
Protein disulfide-isomerase	130	3
Ras related Rab-10	121	2
Catenin alpha-1	102	3
Ras related Rap-1A	102	2
Calnexin	88	2
Glyceraldehyde-3-phosphate dehydrogenase	160	4
Voltage-dependent anion-selective prot.	155	3
Dysferlin	87	3
Beta-2-syntrophin	69	1
Ras-interacting protein 1	139	3

Table 1: Selected bEnd3 membrane proteins identified by mass spectrometry analysis, using mascot search engine, their scores and number of matched peptides.

Protein name	Mascot Protein Ion	Number of matched peptides
Epithelial cell adhesion molecule	68	2
Cadherin 13	76	1
Platelet glycoprotein	17	1
V-set domain-containing T-cell activation inhibitor	48	3
Complement C3	166	6
Sodium/potassium-transporting ATPase subunit alpha-	68	1
Serine protease inhibitor A3K	70	2
Angiotensin-converting enzyme	42	2
Lumican	55	1
Complement C4	238	7
Membrane primary amine oxidase	131	3
Integrin alpha 6	96	1
Mast cell protease	84	3
Biglycan	71	1
Integrin beta 1	87	5
Alpha-1-antiproteinase	156	3
Sodium/potassium-transporting ATPase	44	1
Serine protease inhibitor	137	3
Integrin beta 4	74	1
Lactadherin	65	1

Table 2: Selected membrane proteins of AVM rat model identified by mass spectrometry analysis using mascot search engine, their scores and number of matched peptides.

Conclusion

Cell surface protein biotinylation and mass spectrometry successfully identified membrane proteins from endothelial cell models and vasculature in a rat model of AVM. Our future validation will also include primary endothelial cell cultures from resected AVMs. We will study radiation-induced changes in human AVM endothelial cells using proteomic analysis. Candidate proteins then will be investigated for use in ligand-directed human vascular targeting trials.

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