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Immobilisation of a fibrillin-1 fragment enhances the biocompatibility of PTFE

Hamid Hajian[a,b,1], Steven G. Wise[a,c,d,1], Daniel V. Bax[d,e], Alexey Kondyurin[e], Anna Waterhouse[f], Louise L. Dunn[a,c], Cay M. Kielty[g], Young Yu[a], Anthony S. Weiss[d,h,i,2], Marcela M.M. Bilek[e,2], Paul G. Bannon[b,c,2], Martin K.C. Ng[a,j,*,2]

[a] Heart Research Institute, Sydney, NSW 2042, Australia
[b] The Baird Institute, Sydney, NSW 2042, Australia
[c] Sydney Medical School, University of Sydney, NSW 2006, Australia
[d] School of Molecular Bioscience, University of Sydney, NSW 2006, Australia
[e] Applied & Plasma Physics Group, Physics School, University of Sydney, NSW 2006, Australia
[f] Wyss Institute, Harvard, Boston, MA 02115, USA
[g] Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
[h] Bosch Institute, University of Sydney, NSW 2006, Australia
[i] Charles Perkins Centre, University of Sydney, NSW 2006, Australia
[j] Department of Cardiology, Royal Prince Alfred Hospital, NSW 2050, Australia

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ABSTRACT

Current vascular biomaterials exhibit poor biocompatibility characterised by failure to promote endothelialisation, predisposition to neoinitmal hyperplasia and excessive thrombogenicity. Fibrillin-1, a major constituent of microfibrils is associated with elastic fibres in the arterial wall. Fibrillin-1 binds to endothelial cells through an RGD cell adhesion motif in the fourth TB module. The RGD motif is present in PF8, a recombinant fibrillin-1 fragment. We investigated the potential of PF8 to improve the biocompatibility of PTFE. PF8 enhanced endothelial cell attachment and cell proliferation to a greater extent than fibronectin ($p < 0.01$). PF8 immobilised on PTFE using plasma immersion ion implantation (PIII), retained these favourable cell interactive properties, again promoting endothelial cell attachment and proliferation. The thrombogenicity of covalently bound PF8 on PTFE was assessed in both static and dynamic conditions. In static conditions, uncoated PIII treated PTFE was more thrombogenic than untreated PTFE, while PF8 coating reduced thrombogenicity. Under flow, there was no difference in the thrombogenicity of PF8 coated PTFE and untreated PTFE. Immobilised PF8 shows a striking ability to promote attachment and growth of endothelial cells on PTFE, while providing a non-thrombogenic surface. These features make PF8 a promising candidate to improve the biocompatibility of current synthetic vascular grafts.

1. Introduction

Coronary and peripheral vascular bypass are common surgical procedures used in the treatment of vascular disease to restore adequate blood supply to ischemic tissues in the heart and limbs. In the long-term, an ideal conduit capable of successful revascularisation would match the mechanical characteristics and biologic functions of native arteries to maximise functionality and patency. The internal mammary artery remains the preferred autologous replacement, though radial arteries and saphenous vein are also routinely used. However, such native conduits are either unsuitable or unavailable in up to one third of patients requiring revascularisation [1].

The development of a synthetic graft with comparable mechanical and biological properties to those of native arteries has been an ongoing challenge since the introduction of prosthetic vascular conduits in 1952. The endothelial cell monolayer lining the luminal surface of blood vessels plays a pivotal role in maintaining vascular health and patency. The endothelium provides dynamic mechanisms to prevent thrombosis, covering underlying

* Corresponding author: at Department of Cardiology, Royal Prince Alfred Hospital, Missenden Rd, University of Sydney, NSW 2050, Australia. Tel.: +61 2 95156111; fax: +61 2 95506262.
E-mail address: mkcng@med.usyd.edu.au (M.K.C. Ng).
[1] These authors contributed equally to the manuscript.
[2] Joint senior authors.

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thrombogenic matrix components, regulating vascular tone and inhibiting intimal hyperplasia [2]. In humans, current commercial synthetic conduit materials such as polyethylene terephthalate (Dacron®) and expanded polytetrafluoroethylene (ePTFE) do not provide a suitable surface for endothelialisation even decades after implantation [3,4]. This is particularly problematic for small diameter grafts with low flow rates. Therefore, synthetic grafts often fail clinically in bypassing small diameter blood vessels (<6 mm) due to poor endothelialisation and intrinsic thrombogenicity [5,6].

Seeding ePTFE grafts with endothelial cells before implantation improves the long-term patency of these grafts [7,8]. However, lengthy, cumbersome and costly graft preparation deters routine use of such methodologies in clinical practice. Other methods aim to enhance re-endothelialisation *in vivo* following implantation by using extracellular matrix (ECM) components from native blood vessels to endow biomaterials with improved vascular biologic properties [9].

Passive attachment and physisorption of biomolecules to biomaterials is often associated with variable adherence and unreliable persistence. This depends on surface energy, chemistry and hydrophobicity [10–12]. In contrast, covalent binding provides a consistent, reliable and persistent covering of the substrate with biomolecules. Different chemical linkers have been used to covalently anchor biomolecules on biomaterials [13,14]. However, these methods often involve arduous and multi-step wet chemical processes and employ linkers that can cause toxicity, and affect cellular interactions *in vivo*. In contrast, plasma immersion ion implantation (PIII) is a well-characterised substrate-independent surface treatment, which creates free radicals in the surfaces of polymeric materials [15]. These highly reactive radicals migrate to the surface and covalently bind biomolecules without the need for chemical linkers [16,17].

We have identified PF8, a recombinant fibrillin-1 fragment as a candidate biomolecule with the potential to improve the biocompatibility of ePTFE grafts. Fibrillin-1 is a 350 kDa cysteine-rich glycoprotein. It is the major constituent of microfibrils, and has a crucial role in the assembly and polymerisation of elastic fibres [18,19]. Fibrillin-1, has strong cell adhesion properties, attributed to a single Arg-Gly-Asp (RGD) cell-adhesion motif that strongly binds to endothelial cells, fibroblasts and smooth muscle cells through integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$ [20]. This cell-binding activity is contained within a recombinant fragment of fibrillin-1, termed PF8. We therefore propose that PF8 will contribute to a useful surface coating that improves endothelial cell interactions and the overall biocompatibility of ePTFE.

2. Methods

2.1. Expression and purification of PF8 from transfected 293-EBNA cells

The histidine tagged recombinant human fibrillin-1 fragment, PF8, encoded by exons 30–38 was expressed and purified from transfected HEK293-EBNA cells, as previously described [21,22]. The purity and mass of PF8 was validated by SDS-PAGE and Western blot using monoclonal anti-poly histidine (Sigma) as the primary antibody and anti-mouse IgG-peroxidase (Sigma) as the secondary antibody.

2.2. ELISA detection of PF8

Increasing concentrations up to 50 μg/ml of PF8 diluted in PBS were added in triplicate to a 96-well plate and incubated at 4 °C overnight. Unbound PF8 was removed with PBS washing and non-specific polymer binding was blocked with 3% (w/v) bovine serum albumin (BSA) in PBS for 1 h at room temperature. Bound PF8 was detected using a primary monoclonal anti-poly histidine antibody at 1:3000 dilution for 60 min followed by incubation with anti-mouse IgG-peroxidase secondary antibody at 1:5000 dilution for 60 min. ABTS solution was added to the samples and the absorbance was measured at 405 nm after incubation for 45 min. ELISA detection of PF8 bound to PTFE was performed on 0.8 cm × 1.2 cm samples in a 24-well plate. The samples were coated with increasing concentrations of PF8 at 4 °C overnight. Samples were washed, BSA blocked and PF8 was detected with primary and secondary antibodies as described above. After the secondary antibody detection the samples were transferred to a new plate before ABTS incubation.

2.3. PIII of PTFE

Polytetrafluoroethylene (PTFE), sheets (Goodfellow, 0.1 mm thickness) were treated by plasma ion implantation (PIII), as previously described [23–25]. Briefly, plasma was generated in high purity nitrogen at 2×10^{-3} torr with 100 W radio frequency (rf) power. For PIII, 20 kV pulses lasting for 20 μs with a repetition rate of 50 Hz were applied to the substrate holder. Samples were treated for 400 s.

2.4. Radiolabelling of PF8 with ^{125}I

PF8 (1.66 mg/ml) in PBS was labelled with ^{125}I using iodination beads (Pierce). One iodobead/mg protein was washed twice with PBS and air-dried before incubation with 50 μl of PBS/bead. One mCi Na ^{125}I was added to the iodobeads and agitated intermittently for 5 min at room temperature. After incubation, 1 mg protein/bead was added to the beads and agitated intermittently for 15 min. The labelled protein solution was passed through a PD-10 desalting tube equilibrated with PBS to remove unbound ^{125}I. The radioactivity of samples before and after desalting was measured using a scintillation counter. Fractions with highest radioactivity were combined. A BCA assay (Sigma) was used to confirm the concentration of radiolabelled protein in the final solution as previously described [26].

2.5. Covalent binding of PF8 to PIII PTFE

Triplicate 1 cm × 1 cm squares of untreated PTFE and PIII PTFE were coated with 0.5 ml of ^{125}I labelled PF8 in PBS (3 μg/ml) at 4 °C overnight. To remove non-covalently bound PF8 the samples were stringently washed with 5% SDS at 80 °C as previously described [27,28]. The quantity of retained ^{125}I labelled protein was determined using a scintillation counter. The radioactivity readings were converted to number of molecules using a standard curve produced using radiolabelled defined PF8 concentrations.

2.6. Endothelial cell attachment and proliferation on tissue culture plastic

Cell attachment and proliferation assays were performed in 24-well plates, in triplicate, as previously reported [29]. Human coronary artery endothelial cells (HCAEC, Cell Applications) from passage 2 to 5 were used in all cell experiments. Attachment and proliferation were determined in comparison to tissue culture plastic alone and to control wells coated with human plasma fibronectin or 10 mg/ml heat denatured (80 °C for 10 min) bovine serum albumin (BSA). All protein-coated wells were blocked with heat denatured BSA for 60 min at room temperature to block non-specific binding. For attachment studies, HCAEC (5×10^5 cells/well) were incubated with test wells for 60 min. For proliferation assays, HCAEC (1×10^4 cells/well) were incubated for 3 and 5 days. At