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Markers for human brain pericytes and smooth muscle cells

Leon C.D. Smyth[a,b], Justin Rustenhoven[a,b], Emma L. Scotter[a,b], Patrick Schweder[b,d], Richard L.M. Faull[b,c], Thomas I.H. Park[a,b,c], Mike Dragunow[a,b,*]

[a] Department of Pharmacology and Clinical Pharmacology, Auckland, New Zealand
[b] Centre for Brain Research, Auckland, New Zealand
[c] Department of Anatomy and Medical Imaging, Auckland, New Zealand
[d] Auckland City Hospital, Auckland, New Zealand

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ABSTRACT

Brain pericytes and vascular smooth muscle cells (vSMCs) are a critical component of the neurovascular unit and are important in regulating cerebral blood flow and blood-brain barrier integrity. Identification of subtypes of mural cells in tissue and *in vitro* is important to any study of their function, therefore we identified distinct mural cell morphologies in neurologically normal post-mortem human brain. Further, the distribution of mural cell markers platelet-derived growth factor receptor-β (PDGFRβ), α-smooth muscle actin (αSMA), CD13, neural/glial antigen-2 (NG2), CD146 and desmin was examined. We determined that PDGFRβ, NG2, CD13, and CD146 were expressed in capillary-associated pericytes. NG2, and CD13 were also present on vSMCs in large vessels, however abundant CD146 and desmin staining was also detected in vSMCs on large vessels, co-labelling with αSMA. To determine whether cultures recapitulated observations from tissue, primary human brain pericytes derived from neurologically normal autopsies were analysed for the presence of pericyte markers by immunocytochemistry, western blotting and qPCR. The proteins observed in brain pericytes in tissue (PDGFRβ, αSMA, desmin, CD146, CD13, and NG2) were present *in vitro*, validating a panel of proteins that can be used to label brain pericytes and vSMCs in tissue and *in vitro*. Finally, we showed that the proteins CD146 and desmin that are expressed on large vessels *in situ*, are also selective markers of a smooth muscle cell phenotype *in vitro*.

1. Introduction

Pericytes are spatially isolated cells that surround capillaries. Together with vascular smooth muscle cells (vSMCs) that surround large vessels (arteries, arterioles, venules, and veins), they make up the mural cells that support blood vessels. Pericytes in the brain are unique in that they are derived from both mesenchymal and neuroectodermal lineages (Etchevers et al., 2001) and demonstrate the highest vessel coverage in the body, with a 1:4 ratio of pericytes to endothelial cells (Armulik et al., 2011; Bonkowski et al., 2011). vSMCs are also critical to proper brain function, as they cover arterioles and venules, stiffening them, and are responsible for vasoconstriction and dilation at pre-capillary arterioles, that are thought to be the principal site of blood flow regulation (Hill et al., 2015; Damisah et al., 2017). Mural cells are an integral component of the neurovascular unit, along with astrocytes, neurons and endothelia, however, until recently, the role of mural cells in the brain has been underappreciated. Over the last decade, the role of pericytes in regulating cerebral blood flow (Hall et al., 2014), angiogenesis (Hellström et al., 2001), the blood-brain barrier (BBB) during development (Daneman et al., 2010; Armulik et al., 2010) and ageing (Bell et al., 2010), as well as scarring (Göritz et al., 2011) and inflammation (Jansson et al., 2014; Rustenhoven et al., 2016a; Jansson et al., 2016) has been demonstrated.

It is important to the field of cerebrovascular biology to confirm biochemical markers of pericytes and vSMCs in human tissue and cells and to assess these systematically. In the brain, pericytes can be identified anatomically because they are embedded within the basement membrane, with round nuclei that protrude from the vessel in a classical 'bump-on-a-log' shape (Hill et al., 2015; Attwell et al., 2015). In the mouse brain, pericytes are typically identified with a transgenic NG2 and/or PDGFRβ reporter (Armulik et al., 2010; Hartmann et al., 2015; Andrae et al., 2008; Jung et al., 2017), but have also been identified with CD13 (Armulik et al., 2010), desmin (Bell et al., 2010; Jung et al., 2017), Zic-1 (Daneman et al., 2010), α-smooth muscle actin (αSMA) (Grant et al., 2017), and platelet-derived growth factor receptor-β (PDGFRβ) (Winkler et al., 2010), and less frequently with CD146 labelling (Patenaude et al., 2015; Chen et al., 2017a, b). There are also selective markers for pericytes that do not also identify vSMCs,

* Corresponding author at: Department of Pharmacology and Clinical Pharmacology, The University of Auckland, Private Bag 92019, 1142, Auckland, New Zealand.
E-mail address: m.dragunow@auckland.ac.nz (M. Dragunow).

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Table 1
Antibody specifications used for these studies.

Primary antibody							
Antibody	Species	Company	Catalogue	ICC	IHC	WB	Flow
PDGFRβ	Rabbit	Abcam	ab32570	1:500	1:250	1:500	–
PDGFRβ	Goat	R&D	AF385	–	1:500	–	–
PDGFRβ-PE	Mouse	BD Pharmingen	558820	–	–	–	1:5
Collagen IV	Rabbit	Abcam	ab6586	–	1:1000	–	–
Collagen IV	Mouse	Dako	m0785	1:500	1:1000	–	–
Fibronectin	Rabbit	Dako	A0245	1:100000			
αSMA	Mouse	Dako	iS611	1:10	1:10	1:10	1:0
αSMA	Rabbit	Abcam	ab5694	1:500	1:500	–	–
CD13	Mouse	Biolegend	301702	1:250	1:100	1:100	–
CD146	Mouse	Abcam	ab24577	1:500	1:250	1:250	–
CD146-APC	Mouse	Biolegend	134703	–	–	–	1:20
NG2	Mouse	Santa Cruz	sc53389	1:500	1:100	1:100	–
Desmin	Rabbit	Abcam	ab53765	1:500	1:250	1:250	–
CD45	Mouse	Abcam	ab65274	1:500	–	–	–
GFAP	Chicken	Abcam	ab4674	1:50,000	–	–	–
GAPDH	Mouse	Abcam	ab9484	–	–	1:2000	–
GAPDH	Rabbit	Abcam	ab9485	–	–	1:2000	–
Secondary antibody							
Anti-goat Alexa 594	Donkey	Life technologies	A11058	–	1:500	–	–
Anti-rabbit Alexa 488	Donkey	Life technologies	R37118	–	1:500	–	–
Anti-mouse Alexa 488	Donkey	Life technologies	R37114	–	1:500	–	–
Anti-mouse biotinylated	Goat	Sigma-Aldrich	B7264	–	1:500	–	–
Anti-rabbit biotinylated	Goat	Sigma-Aldrich	B7389	–	1:500	–	–
Anti-rabbit Alexa 488	Goat	Life technologies	A11008	–	1:500	–	1:500
Anti-rabbit Alexa 594	Goat	Life technologies	A11012	1:500	1:500	–	–
Anti-mouse Alexa 488	Goat	Life technologies	A11001	1:500	1:500	–	–
Anti-mouse Alexa 594	Goat	Life technologies	A11005	–	1:500	–	–
Anti-mouse Alexa 647	Goat	Life technologies	A21235	–	1:500	–	1:500
Anti-Chicken Alexa 647	Goat	Life technologies	A21449	1:500	–	–	–
Anti-mouse IRDye-680LT	Goat	LiCOR	926-68020	–	–	1:10,000	–
Anti-rabbit IRDye-800CW	Goat	LiCOR	926-32211	–	–	1:10,000	–

with transcriptomic studies having identified *Ifitm1* and *Vtn* as potential specific markers in mouse (He et al., 2016), though *Vtn* is a basement membrane component making it difficult to determine its cellular localisation, and has also been observed in large vessels (Yuan et al., 2018). Furthermore, a fluorescent tracer dye has been reported to be taken up specifically by pericytes in mouse brain *in vivo*, indicating that capillary pericytes are indeed a distinct mural cell type (Damisah et al., 2017). There have been recent attempts to classify the diversity of mural cell phenotypes. Distinct morphologies of pericytes have been observed and classified in mouse brain using αSMA and PDGFRβ, identifying intermediate forms between vSMCs and pericytes such as 'ensheathing pericytes' (Grant et al., 2017; Kisler et al., 2017a; Attwell et al., 2016; Sweeney et al., 2016). Indeed, a recent report has used single cell RNAseq to characterise the diversity of mural cell phenotypes, defining the diversity of mural cell phenotypes (Vanlandewijck et al., 2018). While no subtypes of pericytes were observed, principal component analysis showed that there were three different vSMC phenotypes that clustered apart from pericytes, along with a smear of transitional forms that were detected (Vanlandewijck et al., 2018). However, the majority of this work has been performed in animals, and the expression of mural cell markers and their morphologies has been less well characterised in the human brain.

Likewise, in the absence of anatomical context to reinforce immunohistochemical staining, identification of pericytes *in vitro* is difficult. It is therefore important to the field to develop markers that are specific to capillary pericytes and vSMCs as there has been considerable debate as to the definition of these cell types in recent years (Attwell et al., 2015). This has mostly been in relation to work on cerebral blood flow, where a number of groups have determined that pericytes play a role in blood flow regulation, in the brain (Hall et al., 2014; Kisler et al., 2017b; Peppiatt et al., 2006), the retina (Biesecker et al., 2016; Kawamura et al., 2004), cochlea (Mishra et al., 2016), and pancreatic islets (Almaça et al., 2018). However, this has been challenged by several reports that could not detect pericyte-induced constriction of capillaries, and inferred that the previous studies had been misattributing vSMCs as pericytes due to lack of definitive markers (Hill et al., 2015; Damisah et al., 2017; Mazzoni et al., 2015; Wei et al., 2016).

It is vital, particularly with primary cultures, to ensure that they recapitulate the *in vivo* phenotype *in vitro*. Additionally, the development of selective markers for mural cell phenotypes *in situ* will allow researchers to distinguish these cell types to dissect aspects of their biology *in vitro*. With this in mind, we validated protein markers of pericytes and vSMCs, αSMA and PDGFRβ, with lectin and collagen IV to ensure they were specific to mural cells and did not stain other vascular-associated cells in human brain tissue. Using PDGFRβ and αSMA we determined the presence of CD13, NG2, CD146, and desmin on pericytes and vSMCs, and quantified their distribution in small and large vessels *in situ*. Following from this, we tested the presence of these proteins and their transcripts in primary human brain pericyte cultures derived from neurologically normal post-mortem brain, to determine if these markers also define different populations of pericytes *in vitro*.

2. Materials and methods

2.1. Ethics and human tissue processing

Tissue used in this study was obtained from the Neurological Foundation of New Zealand Human Brain Bank at the University of Auckland. All tissue was donated with written informed consent from subjects and next of kin, and its use for study was approved by the University of Auckland Human Participants Ethics Committee. All methods were carried out in accordance with the approved guidelines. All brain tissue collection and processing protocols were approved by