Origins of fibrosis: pericytes take centre stage

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Abstract

Pericytes are ubiquitous perivascular cells that have recently attracted interest as potential myofibroblast precursors. In turn, myofibroblasts are the major source of extracellular matrix components that accumulate during tissue fibrosis. Given the worldwide burden of fibrotic disease and paucity of therapeutic options available to halt its progression, elucidating the origins of myofibroblasts is of prime importance. The advent of genetic strategies that permit fate-mapping of specific cell populations through permanent and heritable expression of reporter proteins has begun to shed light on the source of the fibrogenic myofibroblast. Here we discuss recent studies in multiple organs that highlight the central role of pericytes in the origins of fibrosis.

Introduction

Fibrosis, the accumulation of extracellular matrix molecules that constitute scar tissue, is a global health-care concern. As a basic wound-healing response that occurs in any organ undergoing iterative injury, tissue fibrosis is a major component of the pathophysiology of many common diseases, including cardiovascular disease, cancer and chronic diseases of the liver, lung and kidney. This highly conserved response to repetitive injury and inflammation ultimately results in disordered tissue architecture and organ failure, making fibrosis a major cause of mortality worldwide. Currently there are no European Medicines Agency (EMEA)- or Food and Drug Administration (FDA)-approved anti-fibrotic therapies, underscoring the urgent need for potent and novel treatments for tissue fibrosis.

The myofibroblast is generally acknowledged to be the key cell regulating tissue fibrosis through extracellular matrix deposition and exertion of tensile force [1]. Therefore, in order to accelerate the design of rational, targeted, anti-fibrotic therapies, recent research has focussed on defining the origin of scar-producing myofibroblasts in various organs, since gaining control of the myofibroblast progenitor cell population may allow us to arrest or even induce reversion of fibrosis in certain disease settings [2]. Multiple cell types have been proposed to fulfil this myofibroblast precursor role, including epithelial cells (via the process of epithelial-mesenchymal transition [EMT]) [3,4], bone marrow-derived cells including fibrocytes [5-10] and tissue-resident cells [11,12]. EMT was initially proposed as a major source of myofibroblasts in fibrotic disease, but recent cell fate-mapping studies in multiple organs in rodent models have shown that EMT does not directly contribute to the pool of collagen-producing myofibroblasts during fibrogenesis *in vivo* [13-18]. Furthermore, several recent studies utilising cutting-edge genetic cell-labelling techniques have drawn attention to one particular cell type, the pericyte, as a major myofibroblast progenitor in a diverse range of organ fibroses. In this review, we discuss recent advances demonstrating that this ubiquitous perivascular cell is a major source of myofibroblasts and how these studies have paved the way for a universal hypothesis linking pericytes to the origins of fibrosis (Figure 1).

What are pericytes?

The pericyte is most simply defined morphologically: a periendothelial cell with a rounded cell body and

Figure 1. Pericytes are a major source of myofibroblasts during fibrosis in multiple organs
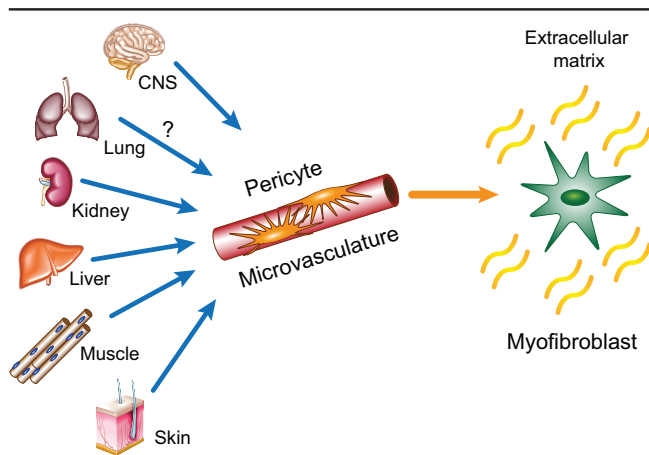
cytoplasmic processes encircling one or more endothelial cells [19]. Given the anatomic location of pericytes, it is perhaps unsurprising that their primary role in normal tissue homeostasis relates to vascular development, stabilisation and integrity [20-23]. Pericytes are a type of mural cell, principally located in the microvasculature (consisting of capillaries but also terminal arterioles and postcapillary venules) and as such are present in every organ and vascular bed [24]. A more exacting definition states that, in healthy tissue, pericytes are embedded within the vascular basement membrane [25]. However, as this requires electron microscopy, most studies do not confirm this feature, relying instead on a combination of light microscopy and multiple molecular markers to identify pericytes. Commonly used pericyte markers include platelet-derived growth factor receptor beta (PDGFRβ), chondroitin sulphate proteoglycan 4 (also known as nerve/glial antigen 2 or NG2), desmin and alpha-smooth muscle actin (αSMA) [19]. However, careful interpretation of these markers is required, as no single marker is specific for pericytes alone; some only label a subpopulation of pericytes and several are not constitutively present throughout the lifespan of every pericyte. Furthermore, pathological states, ongoing angiogenesis and increased vascular basement membrane turnover make the relationship between pericytes and the vascular basement membrane much less clear cut. Therefore, in the context of tissue fibrosis, discriminating between pericytes, other mesenchymal perivascular cells and resident tissue fibroblasts becomes even more challenging.

Tracing pericytes in organ fibrosis
In recent years, the rapid increase in powerful, sophisticated mouse genetic tools has greatly facilitated cellular

fate-mapping in multiple biological processes, including tissue fibrosis. The Cre/loxP system is widely used for this purpose [26]. It employs the gene for bacterial Cre recombinase (Cre), which is linked to a cell- or lineage-specific promoter prior to incorporation in the genome of a transgenic mouse. Furthermore, cell fate-mapping experiments commonly employ inducible Cre mouse strains, as Cre expression in these mice only occurs in the presence (or absence) of exogenous compounds (commonly tetracyclines or tamoxifen), thereby allowing both temporal and spatial control over Cre expression. When combined with a reporter mouse strain, Cre expression in a specific cell type in the embryo or adult causes a permanent alteration in the genome of these cells, resulting in reporter protein expression in the targeted cell type and its progeny.

Skin and muscle fibrosis
The origin of profibrotic cells in skin and muscle has most recently been explored in the elegant studies of Dulauroy *et al.* [27]. They examined the role of ADAM12 (a disintegrin and metalloprotease 12)-positive cells during skin and muscle injury. ADAM12 is a membrane-anchored metalloprotease, expressed in several human diseases with a fibrotic component, but is also, in a fashion, restricted during development. Initial studies by Dulauroy *et al.* demonstrated that transient expression of ADAM12 identifies a distinct pro-inflammatory subset of stromal cells that become activated following acute injury in the skin and skeletal muscle. The authors then fate-mapped these cells using an inducible, tetracycline transactivator-based system. This involved the generation of triple transgenic mice that expressed tetracycline transactivator under control of the *Adam12* locus, Cre under control of the tetracycline transactivator and the conditional reporter *Rosa26*$^{floxSTOP-YFP}$ locus. In these mice, yellow fluorescent protein (YFP) labelling of the progeny of ADAM12$^+$ cells was temporally controlled by the administration of doxycycline to prevent Cre expression. This allowed the separate fate-mapping of foetal and adult ADAM12$^+$ cells following skin or muscle injury, induced by injection of complete Freund's adjuvant into ear dermis or cardiotoxin into tibialis anterior muscle respectively. The genetic strategies employed by the authors, combined with a parabiosis experiment utilising a transgenic mouse sutured to a wildtype mouse, allowed them to demonstrate that the majority of collagen-producing, αSMA$^+$ myofibroblasts developing following acute dermal or muscle injury are generated from tissue-resident ADAM12$^+$ cells. Furthermore, ablation of ADAM12$^+$ cells in skeletal muscle (using mice that also expressed the human diphtheria toxin receptor under control of the *Adam12* locus) markedly reduced the generation of profibrotic cells and interstitial collagen accumulation.

The progeny of foetal ADAM12^{+} cells includes a sub-population of PDGFRβ[+] NG2^{+} perivascular cells, in addition to nerve and glial cells. Using mice that expressed Cre under control of the *Wnt1* promoter, crossed to a YFP reporter mouse, the authors found no contribution from the progeny of Wnt1^{+} nerve and Schwann cells to the stromal cell population following skeletal muscle injury. They therefore concluded that the ADAM12^{+} profibrotic progenitors developing in injured skeletal muscle do not originate from a neural crest cell lineage and ADAM12^{+} Schwann cells, but from ADAM12^{+} perivascular cells. In addition, ADAM12 itself was identified as a potential antifibrotic target, since its silencing with siRNA reduced collagen deposition and upregulated regenerative growth factors following muscle injury. Overall, the findings of Dulauroy *et al.* provide strong evidence that pericytes are a major contributor to the myofibroblast population that develops during skin and muscle scarring.

Kidney fibrosis
Pericytes in the kidney have also been intensively studied in order to analyse their contribution to renal myofibroblast formation and fibrosis. Almost a decade ago, Faulkner *et al.* reported that the myofibroblast population in a Habu venom and angiotensin II model of renal fibrosis appeared to expand from a perivascular location [28]. This observation was subsequently supported by Lin *et al.* using a Col-green fluorescent protein (GFP) mouse, which expresses the GFP reporter under control of the collagen-α1(I) promoter/enhancer, and subsequently co-staining for commonly used pericyte markers including NG2, PDGFRβ and αSMA [29]. This allowed them to identify pericytes and perivascular fibroblasts in the uninjured kidney and their expansion into an interstitial myofibroblast population following induction of renal fibrosis.

However, it is only lately that the appropriate genetic tools have permitted true fate-mapping of renal myofibroblast precursors during renal fibrogenesis. Humphreys *et al.* utilised a Cre reporter strategy to label renal epithelial cells or renal stromal cells in mice permanently and heritably [15]. Renal stromal cells, which include mesangial cells, vascular smooth muscle cells and pericytes, were labelled using a *FoxD1-Cre* driver bred into the ROSA26 reporter mouse, leading to LacZ expression in the stromal cell population. FoxD1 is a transcription factor expressed in metanephric mesenchyme during kidney development. Because FoxD1 can also be expressed in the adult kidney, a transgenic mouse in which Cre expression by FoxD1^{+} cells was inducible (through tamoxifen administration) and could therefore be limited to embryonic development was also utilised. Prior to injury, LacZ-expressing stromal cells in the adult

kidney interstitium co-expressed the pericyte / perivascular fibroblast markers PDGFRβ and CD73. Following unilateral ureteric obstruction to induce renal fibrosis, there was marked expansion of the LacZ-expressing cell population and these cells made up almost the entire population of αSMA[+] myofibroblasts. No epithelial cells contributed to the interstitial myofibroblast population when renal fibrosis was induced in the mice in which renal epithelial cells were heritably labelled. Therefore, the results obtained, using both the unilateral ureteric obstruction model and also an ischaemia-reperfusion model, provide strong evidence that renal myofibroblasts almost exclusively derive from stromal precursors and not through EMT of renal epithelial cells. Furthermore, co-localisation of the inherited marker with PDGFRβ and CD73 suggests that these precursor cells are pericytes or perivascular fibroblasts.

Fibrosis in the central nervous system
Many studies investigating scar formation in the central nervous system (CNS) have focussed on the role of astrocytes and indeed reference is often made to the 'glial scar' [30]. However, in a recent study, Göritz *et al.* switched focus to the stromal, non-glial core of the CNS scar [31]. Using tamoxifen-inducible Glast-CreER mice crossed with a YFP reporter, the authors labelled a subpopulation of pericytes lining blood vessels in the spinal cord parenchyma prior to spinal cord injury. These labelled cells, referred to as Type A pericytes, expressed PDGFRα and PDGFRβ and electron microscopy confirmed a perivascular location surrounded by basal lamina. Following spinal cord injury by dorsal funiculus incision or dorsal hemisection, the number of labelled cells increased and formed the core of the scar in the spinal cord. These cells also began to express fibronectin and (transiently) αSMA which, combined with ultrastructural examination, led the authors to conclude they were the main source of connective tissue in the scar.

Furthermore, the authors devised an elegant genetic strategy to prevent the Type A pericyte population expanding following spinal cord injury. They generated multi-transgenic mice that, in addition to carrying the Glast-CreER and YFP reporter alleles, were homozygous for H-ras and N-ras null alleles and for floxed K-ras alleles. As ras genes are necessary for cell cycle progression and mitosis, this allowed the investigators to inhibit mitosis in Type A pericytes in a temporal fashion through tamoxifen induction of Cre expression. Following spinal cord injury, these mice had a markedly reduced stromal cell core within the scar, with some of the mice even manifesting an open tissue defect, demonstrating that type A pericytes in the CNS are required for sealing spinal cord lesions.

Notwithstanding the detailed ultrastructural analysis undertaken by the authors, the use of Glast-CreER to target pericytes has been questioned, as it may also be expressed in other CNS cell types [32]. However, it is clear that these PDGFRβ[+] Glast^{+} perivascular cells are of prime importance in scar formation following spinal cord injury.

Liver fibrosis

Hepatic stellate cells (HSCs) are liver-specific pericytes that reside in the space of Disse in close contact with sinusoidal endothelial cells [33,34]. Over one hundred years after their first description, a major advance in the understanding of HSC function occurred with the discovery that HSCs are the principal collagen-producing cells in the liver [35,36]. These seminal studies, which involved *ex vivo* culture of hepatic cell populations, stimulated the genesis of the now classical paradigm of HSCs undergoing activation to a proliferative, contractile, matrix-secreting, myofibroblast phenotype following liver injury.

Recent work by Kisseleva *et al.* in a study primarily designed to explore the fate of hepatic myofibroblasts during liver fibrosis regression, reported that HSCs are the major source of myofibroblasts in a murine model of liver fibrosis [37]. Following carbon tetrachloride (CCl_4)-induced liver injury in Col-GFP mice, flow cytometry was performed on isolated non-parenchymal liver cells and GFP^{+} cells were considered to be myofibroblasts. In addition, autofluorescence was used to detect vitamin A expression, since HSCs produce and store vitamin A. Ninety-two percent of GFP^{+} cells co-expressed vitamin A and the authors concluded that HSCs are the major source of myofibroblasts in CCl_4-induced liver injury. The observed expression of GFP (driven by the collagen promoter) concurrent with vitamin A autofluoresence is somewhat surprising, as it has previously been reported that quiescent, non-myofibroblastic HSCs are vitamin A rich (and hence autofluorescent), but subsequently lose vitamin A upon activation to a myofibroblast phenotype [38]. Furthermore, this was not a fate-mapping experiment in the truest sense, since a defined cell population was not heritably labelled. Therefore, although this study is strongly supportive of liver pericytes (HSCs) constituting a major source of collagen-producing myofibroblasts following CCl_4-induced liver injury, definitive fate-mapping of HSCs demonstrating their contribution to the hepatic myofibroblast pool *in vivo* has yet to be undertaken.

Lung fibrosis

A continuing challenge in the field of pericyte biology is the accurate identification and use of appropriate nomenclature relating to pericytes / pericyte-like cells / perivascular cells and their distinction from tissue fibroblasts, particularly in highly vascular tissues. This issue is exemplified in the recent work by Rock *et al.* examining the contribution of various cell types to pulmonary fibrosis [18]. The authors observed abundant NG2^{+} PDGFRβ[+] desmin^{+} interstitial cells intimately associated with pulmonary capillaries, but classified these cells as 'pericyte-like' as they did not have supplementary electron microscopy data. The number of cells expressing these markers increased in bleomycin-induced pulmonary fibrosis.

The authors then utilised an inducible NG2-CreER transgenic mouse crossed to a fluorescent reporter in a fate-mapping study. Tamoxifen administration permitted Cre expression in NG2^{+} cells, resulting in expression of GFP. However, as the authors note, this strategy was unfortunately inefficient at driving recombination, since it labelled only around 14% of NG2^{+} cells. They found that these labelled cells do proliferate during bleomycin-induced pulmonary fibrosis and continue to express NG2 and PDGFRβ. However, most of these cells do not express what the authors refer to as 'high levels' of αSMA, suggesting that they may not be myofibroblasts. As the authors highlight, αSMA expression is dynamic during the course of pulmonary fibrosis, so assessing expression at a single time point has inherent limitations. In addition, assessment of type I collagen expression as an adjunctive myofibroblast marker may have been useful to interrogate the NG2^{+} cell population further. The authors suggest their observation that NG2^{+} PDGFRβ[+] pericyte-like cells proliferate during a murine model of pulmonary fibrosis but do not become αSMA[+] myofibroblasts may be due to organ-specific differences in fibrotic responses or because another pericyte (-like) subpopulation, distinct from the NG2^{+} subpopulation labelled in this study, may be the source of αSMA[+] myofibroblasts in the lung. Indeed, replicating cells were detected by bromodeoxyuridine (BrdU) staining that were not lineage-labelled and probably represented additional stromal subpopulations proliferating in response to fibrogenic stimuli. This possibility could be explored further using alternative inducible transgenic systems to label a larger percentage of the lung pericyte population, including that which is NG2^{−}, and thus further define the role of pericytes in pulmonary fibrosis.

Future directions

The wealth of data generated in recent years implicating pericytes as a major source of myofibroblasts in multiple models of organ fibrosis has drawn attention to the pericyte as a prime target for new anti-fibrotic therapies. In addition, this rapidly evolving field has generated

a number of important questions. Are there common, basic pathways operating in pericytes across different tissues during their transition to myofibroblasts following injury that will allow a "one size fits all" drug targeting approach? Or will we find that pericyte populations in different fibrosing organs exhibit markedly different biological behaviour, requiring a diverse approach to the development of potent pharmacologic inhibitors? Clearly, the more information we can accrue regarding pericyte biology across the full range of organ fibroses, the more able we shall be to inform and accelerate the acquisition of potent, new treatments for a wide range of fibrotic diseases. An area that warrants a great deal more attention is the careful investigation and phenotyping of pericyte subpopulations within a fibrosing organ, with the aim of identifying the most pro-fibrogenic subpopulations and the mechanisms underlying the acquisition of this phenotype, both of which will allow further refinement of drug targeting.

There is a more fundamental question that has gained credence as pericytes have taken centre stage in the field of fibrosis: what differentiates a pericyte from a resident tissue fibroblast? This is particularly salient when the pathological changes that occur during fibrosis lead to turnover of the vascular basement membrane and migration of the pericyte away from its perivascular location. The margins separating these two cell populations are increasingly unclear and, in the absence of unique markers for each cell type, this is an area that requires further clarification. The generation of panels of markers that could definitively identify the various pericyte subpopulations and tissue fibroblasts within different organs would be of great benefit. Furthermore, although it appears attractive to target pericytes to treat fibrosis, we cannot neglect their vital roles in normal tissue homeostasis which, if significantly perturbed, may exacerbate tissue dysfunction in a fibrotic organ with already limited reserve. To this end, and in an era where we strive towards translational medicine, further efforts to phenotype pericytes carefully, both in healthy human tissue and fibrotic organs, are a pre-requisite for the successful translation of this burgeoning field into potent new anti-fibrotic therapies.

Abbreviations

αSMA, alpha smooth muscle actin; ADAM12, a disintegrin and metalloprotease 12; BrdU, bromodeoxyuridine; CCl_4, carbon tetrachloride; CNS, central nervous system; Cre, Cre recombinase; CreER, tamoxifen-inducible Cre recombinase; EMEA, European Medicines Agency; EMT, epithelial-mesenchymal transition; FDA, Food and Drug Administration; GFP, green fluorescent protein; HSC, hepatic stellate cell; NG2, nerve/glial

antigen 2 (also known as chondroitin sulphate proteoglycan 4); PDGFRα, platelet-derived growth factor alpha; PDGFRβ, platelet-derived growth factor beta; YFP, yellow fluorescent protein.

Disclosure

The authors declare that they have no disclosures.

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