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Portal Fibroblasts in Biliary Fibrosis

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Abstract

Portal fibroblasts are a minor population in the normal liver, found in the periportal mesenchyme surrounding the bile ducts. While many researchers have hypothesized that they are an important myofibroblast precursor population in biliary fibrosis, responsible for matrix deposition in early fibrosis and for recruiting hepatic stellate cells, the role of portal fibroblasts relative to hepatic stellate cells is controversial. Several papers published in the past year have addressed this point and have identified other potential roles for portal fibroblasts in biliary fibrosis. The goal of this review is to critically assess these recent studies, to highlight gaps in our knowledge of portal fibroblasts, and to suggest directions for future research.

Keywords

portal fibroblast; hepatic stellate cell; myofibroblast; desmin; biliary fibrosis; elastin; mesothelin; collagen XV; lecithin-retinol acyltransferase; PDGF receptor; angiogenesis; liver fibrosis; bile duct ligation; bile duct

Introduction

Fibrosis is often referred to as the “final common pathway” occurring after liver injury. The location and etiology of liver injury, however, have pronounced effects on the nature and mechanism of fibrosis. This is particularly true in biliary fibrosis, also known as cholestatic fibrosis, such as occurs in primary sclerosing cholangitis, primary biliary cirrhosis, and biliary atresia. Biliary fibrosis differs from other forms of liver fibrosis in significant ways: it is often more rapid, involves dysregulated proliferation of bile ducts and is associated with cholestasis, and is localized, at least initially, to the periductal region. Most important, biliary fibrosis clearly results from injury to cholangiocytes, rather than to hepatocytes, and these cholangiocytes are active participants in the process [1, 2]. What is still unclear, however, is whether biliary fibrosis requires specialized fibrogenic myofibroblasts derived not from hepatic stellate cells but from portal fibroblasts. This controversy, and the potential

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Compliance with Ethics Guidelines

Conflict of Interest

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Human and Animal Rights and Informed Consent

This article does not report any studies with human or animal subjects.

roles – fibrogenic and otherwise – of portal fibroblasts in biliary fibrosis are the subject of this review.

Over the last decade four major categories of cells have been identified as potential precursors of the fibrogenic myofibroblasts that drive liver fibrosis: cholangiocytes and hepatocytes which undergo an epithelial to mesenchymal transition (EMT), fibrocytes from the bone marrow, portal and other fibroblasts, and hepatic stellate cells [3–5]. Recent research suggests that, at least in rodent models, fibrocytes contribute only a small number of cells to the myofibroblast population [6, 7], and epithelial cells undergoing EMT virtually none [8–10]; most authors agree that hepatic stellate cells are the predominant myofibroblast population in fibrosis resulting from hepatocyte injury. In spite of elegant work from several groups, however, the relative contributions of hepatic stellate cells and portal fibroblasts in biliary fibrosis remain uncertain. Specifically, it is unclear whether portal fibroblasts yield a major myofibroblast population and, if yes, whether that contribution is primarily at early time points. Additionally, research has not yet established whether portal fibroblasts *in vivo* deposit the fibrillar collagens typical of fibrosis or whether they have other roles in fibrosis.

Portal fibroblasts are a heterogeneous group of fibroblasts found in the mesenchyme of the portal tract, surrounding the intrahepatic bile ducts. (Note that the cells responsible for fibrosis of the *extrahepatic* bile ducts are not known. This discussion concerns only fibrosis occurring in the liver proper, although it includes intrahepatic biliary fibrosis resulting from extrahepatic ductal fibrosis and obstruction.) In comparison to hepatic stellate cells, which were first visualized (and beautifully sketched) a century and a half ago by Carl Wilhelm von Kupffer [11], fibroblasts in the portal tract were, to our knowledge, first illustrated in the literature in 1961 [12], and received significant attention as potential mediators of biliary fibrosis relatively recently [13–16]. While lineage tracing studies show that portal fibroblasts and hepatic stellate cells (as well as smooth muscle cells and fibroblasts located around the central veins) are derived from a common Wilms tumor 1-expressing precursor lineage in the mesenchyme of the septum transversum [17], they are distinct cell populations in the adult animal, with different localizations (portal mesenchyme *vs.* space of Disse), different functions [18], and different marker expression. Hepatic stellate cells are commonly identified by the presence of vitamin A-containing lipid droplets or by the expression of a variety of markers including desmin, cytoglobin, glial fibrillary acidic protein (GFAP), cellular retinol binding protein 1, and H and 2, although the usefulness of these markers varies with the species and the degree of myofibroblastic activation [19, 20]. Two recent studies, both carefully validated, identified the PDGF receptor subunit and lecithin-retinol acyltransferase (LRAT) as new markers that specifically label mouse hepatic stellate cells, regardless of activation state [21, 22]; these will likely see widespread use in the future. Portal fibroblasts have been identified by a variety of markers including Thy1, fibulin-2, elastin, IL-6, cofilin-1, and the ectonucleotidase NTPDase 2 [19, 23]. Two groups recently compared the transcriptomes of portal fibroblasts and hepatic stellate cells in order to identify new portal fibroblast markers. One group identified COL15A1 [24], while the other found calcitonin and mesothelin (along with a long list of other genes) to be specific portal fibroblast markers [25]. All of these new portal fibroblast markers require validation.

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The relative contributions of hepatic stellate cells and portal fibroblasts in matrix deposition in fibrosis

Studies showing that portal fibroblasts are a major myofibroblast population in fibrosis

There are conflicting data in the literature about the role of portal fibroblasts in matrix deposition in biliary fibrosis. While the ability of portal fibroblasts to differentiate into fibrogenic,-smooth muscle actin (SMA)-expressing myofibroblasts *in vitro* has been well documented, their role *in vivo* is less certain. In favor of portal fibroblasts being major players in biliary fibrosis, many groups have used marker analyses to argue that there is clear *in vivo* evidence that there is a significant population of fibrogenic myofibroblasts in biliary fibrosis that is *not* derived from hepatic stellate cells. Given the controversy in this area, it is important to examine carefully the methods used in these studies.

One of the first papers implicating portal fibroblasts in biliary fibrosis was published in 1996 by Tuchweber *et al.* [16]. This group carried out bile duct ligations in rats, examining time points from 1 to 7 days post procedure. Using double immunostaining techniques and defining portal fibroblast-derived myofibroblasts as-SMA-positive, desmin-negative cells, and hepatic stellate cell-derived myofibroblasts as -SMA-positive, desmin-positive cells, these investigators concluded that myofibroblasts were mostly derived from portal fibroblasts in the first 72 hours after injury, and from hepatic stellate cells thereafter.

A decade later, Beaussier *et al.* induced biliary injury in rats by two methods, bile duct ligation and arterial ischemia [26]. They found that prominent fibrosis developed, with the appearance of many -SMA-positive myofibroblasts; few of these, however, were in the same region as desmin-expressing cells, leading the authors to conclude that most myofibroblasts in the portal region were derived from portal mesenchymal cells such as portal fibroblasts, and not from hepatic stellate cells. Note, however, that double staining was not done. Like the work by Tuchweber *et al.*, this study relied on desmin staining to identify hepatic stellate cell-derived myofibroblasts, and defined portal fibroblasts by the absence rather than the presence of a specific marker. Whether immunostaining for desmin was sufficient in either study to identify all hepatic stellate cells is unclear; some desmin-negative cells express the PDGF receptor subunit [14], which has since been found to be specific for hepatic stellate cells [21]. Thus, the strength of the conclusions in these two influential studies is dependent on the efficiency of desmin immunostaining and on the validity of desmin as a sensitive and specific stellate cell marker *in vivo*, and is therefore difficult to assess.

In a recently published study, Iwaisako *et al.* used transgenic mice expressing green fluorescent protein (GFP) under the control of the collagen 1(I) promoter (such that all collagen-expressing cells are green) to support the contention that portal fibroblasts play a prominent role in biliary fibrosis [25]. The authors identified hepatic stellate cells by the presence of vitamin A, arguing in this and a previous paper that all stellate cells, even after myofibroblastic differentiation, contain at least small amounts of vitamin A [25, 27]. Portal fibroblasts and myofibroblasts were defined as vitamin A-negative cells in the non-parenchymal cell fraction after liver digestion. Control experiments making use of other known hepatic stellate cell and portal fibroblast markers (desmin and GFAP *vs.* Thy1 and

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elastin, respectively) suggested that this approach effectively segregated the two cell types. The authors found a significant percentage of vitamin A-negative, GFP-positive cells in all forms of fibrosis, including after hepatocyte injury from chronic carbon tetrachloride (CCl_4) intoxication. Remarkably, they observed that an average of 73% of myofibroblasts in livers 5 days after bile duct ligation were vitamin A negative and therefore not stellate cell derived; this decreased with time but still remained significant at 46% on day 20 after bile duct ligation. Only a small percentage of these cells (<4%) expressed fibrocyte markers, suggesting that portal fibroblasts give rise to the dominant myofibroblast population in biliary fibrosis, particularly at early time points.

Iwaisako *et al.* also carried out gene expression analyses comparing stellate cells and portal fibroblasts after different forms of injury. Surprisingly, the gene profiles of stellate cells and portal fibroblasts from bile duct ligated animals had more in common than did the gene profiles of stellate cells from bile duct ligated *vs.* CCl_4-treated animals [25]. This raises the possibility that the mechanism of injury has as much to do with myofibroblast function as does the cell of origin. These authors also found that the level of *Col1a1* mRNA expression in a given myofibroblast population was directly related to the prominence of that population at a given time [25]; in other words, portal myofibroblasts are particularly fibrogenic at early time points after biliary injury when they comprise the bulk of the myofibroblast population.

These findings are consistent with portal fibroblasts serving as “first responders” after biliary injury, as proposed in 2002 by Kinnman and Housset [28]. Iwaisako *et al.* suggest a potential mechanism whereby portal myofibroblasts could mediate the first wave of collagen deposition but recruit and ultimately be supplanted by hepatic stellate cells. They show that portal myofibroblasts express IL-25R, enabling them to respond to IL-25, which is upregulated in the serum after bile duct ligation [25]; IL-25-treated portal myofibroblasts secrete IL-13, which increases hepatic stellate cell activation and fibrogenesis.

Studies showing that portal fibroblasts are a minor myofibroblast population in fibrosis

At odds with the data reviewed above is a recently-published, methodologically-sophisticated lineage tracing paper showing that portal fibroblasts are at best minor contributors to biliary fibrosis [22]. Mederacke *et al.* generated transgenic mice with a bacterial artificial chromosome expressing Cre recombinase under the control of the lecithin retinol acyl transferase (*Lrat*) gene; this resulted in highly efficient and specific labeling of hepatic stellate cells, as defined by expression of desmin and the PDGF receptor subunit (*Pdgfrb*) and by the presence of vitamin A-containing lipid droplets. There was no cross-labeling of portal fibroblasts or other cells of the liver (excepting a small number of vascular smooth muscle cells) after crossing to a reporter mouse. LRAT drives the formation of lipid droplets containing retinyl esters, and it is thus reasonable that its expression is specific to hepatic stellate cells.

Mederacke *et al.* tested a total of 7 fibrosis models (hepatotoxic, biliary, and metabolic) using these transgenic mice, demonstrating that 82–96% of myofibroblasts are derived from hepatic stellate cells, even in the early stages of biliary fibrosis. They also isolated a small number of *Lrat*-negative portal myofibroblast-like cells, but found that they secreted less

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collagen than hepatic stellate cell-derived myofibroblasts. Thus, these authors concluded that the majority of myofibroblasts, and the dominant population of collagen-producing cells in both biliary and non-biliary fibrosis, consisted of hepatic stellate cells, with portal fibroblast-like cells playing a minor role in matrix deposition.

Reconciling conflicting data on the importance of portal fibroblasts in fibrosis

Reconciling these disparate pieces of data, particularly the work of Iwaisako *et al.* and Mederacke *et al*., is difficult. While Mederacke *et al.* did observe a small number of portal myofibroblasts, the number of these cells was low compared to hepatic stellate cells, they were minimally fibrogenic, and there was no significant shift from portal fibroblast-derived to hepatic stellate cell-derived myofibroblasts as biliary fibrosis progressed – all findings in contrast to those of Iwaisako *et al.* One of the most significant problems in interpreting these studies is that portal fibroblasts are typically defined by the absence, rather than the presence, of specific markers. It will be key to validate newly identified markers for portal fibroblasts and to carry out portal fibroblast lineage tracing studies, following the model of Mederacke *et al.* Similarly, new stellate cell markers, including *Lrat* and *Pdgfrb*, need to be incorporated into studies similar to those of Iwaisako *et al.* An ideal, although technically daunting, approach would be to differentially label portal fibroblasts and hepatic stellate cells in the same mouse model, and thereby to directly compare their contributions in biliary and non-biliary models.

Henderson *et al.* developed a *Pdgfrb*-Cre mouse [21] and demonstrated that, in the liver, they were able to label hepatic stellate cells (at all stages of differentiation) efficiently and specifically. This mouse, which was used to show that almost all myofibroblasts that appear after CCl_4 treatment are derived from hepatic stellate cells, would be useful for validating the experiments of Mederacke *et al.* [22], especially in biliary fibrosis. Additionally, the approach of Puche *et al*. would be valuable if applied specifically to portal fibroblasts and hepatic stellate cells in biliary fibrosis models [29]. Puche *et al.* expressed the herpes simplex virus thymidine kinase under the control of the GFAP promoter; expression of the viral gene led to death of cells in which the gene was expressed. These authors considered GFAP to be specific for hepatic stellate cells, although others have shown it to be expressed in other cells including portal fibroblasts [22, 23]; use of this method in combination with new and highly specific stellate cell and portal fibroblast promoters would enable a direct determination of the functional relevance of these two cell types in various models of murine liver fibrosis.

A related issue (but one that could be studied with similar techniques) is the fate of portal fibroblasts and myofibroblasts after fibrosis regression. Two groups recently demonstrated independently and using different methods that, while about half of myofibroblastic hepatic stellate cells undergo apoptosis, the remaining cells revert to an intermediate, “primed” state [27, 30]. Whether the same is true for portal fibroblasts during regression of biliary fibrosis (or, potentially, after the initial phases of biliary fibrosis) requires study, as it has significant implications for hepatic stellate cell function, cholangiocyte proliferation and, potentially, bile duct integrity (see below).

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Potential roles for portal fibroblasts in biliary fibrosis beyond fibrogenesis

Portal fibroblasts regulate cholangiocyte behavior

Portal fibroblasts have potential roles in biliary fibrosis independent of matrix deposition, including crosstalk with cholangiocytes. Jhandier *et al.* proposed a model whereby portal myofibroblasts regulate bile duct proliferation [31]. These authors showed that portal fibroblasts in the normal liver express the ectonucleotidase NTPDase 2 (which is used as a marker of these cells). This enzyme hydrolyzes extracellular nucleotides which would otherwise bind to P2Y family receptors on cholangiocytes and stimulate cholangiocyte proliferation. Thus, portal fibroblasts in the normal liver prevent, or at least fail to stimulate, ductular expansion. Portal myofibroblasts, however, do not express NTPDase 2, such that in the diseased state extracellular nucleotides are theoretically available to enhance bile duct proliferation [3, 31].

Portal fibroblasts may also regulate cholangiocyte function via a peribiliary stem cell niche, or by deposition of hyaluronic acid or other matrix proteins during development or after injury [32–36]. Additional studies examining reciprocal expression of growth factors and their receptors by cholangiocytes and portal fibroblasts (and the impact of these interactions on hepatic stellate cells), similar to those carried out by Iwaisako *et al.* (above) will be important to understanding the full role of portal fibroblasts in biliary injury [1, 37].

Portal fibroblasts may regulate angiogenesis and maintain bile duct integrity in fibrosis

It is possible that the major function of portal fibroblasts in cholestatic fibrosis is unrelated to collagen deposition or to myofibroblasts. We have previously suggested that portal fibroblasts should be studied in more detail with regard to classical fibroblast functions, including site-specific matrix protein deposition, interactions with the epithelium, and expression of cell migration guidance signals [38]. There is evidence supporting important roles for portal fibroblasts in at least the first two categories [38].

Additional functions are worth considering. A recent paper from Lemoinne *et al.* puts forth the novel hypothesis that portal myofibroblasts are a minor population of myofibroblasts that nonetheless play a key role in angiogenesis associated with fibrosis and cirrhosis [24]. This group compared the gene expression profiles of activated hepatic stellate cells and portal fibroblasts in culture, determining that expression of the basement membrane collagen component *Col15a1* was specific to portal myofibroblasts in the liver. Cells expressing this marker increased dramatically in advanced fibrosis and cirrhosis, and were closely apposed to vascular structures in fibrotic septae and around reactive ductules. *In vitro*, these cells were stimulated by cholangiocytes to produce VEGFA-containing microparticles, which markedly upregulated tubulogenesis and angiogenesis in several assays [24]. Validation of *Col15a1* as a portal fibroblast marker is required, as are additional studies to confirm that these cells are the same population of portal myofibroblasts studied by other groups. Nonetheless, this work suggests new roles for portal fibroblasts in fibrosis and new modes of interacting with cholangiocytes, particularly in established cirrhosis. Note that most of the other studies discussed in this review have viewed portal fibroblasts as potential early responders after injury and none, including those from Mederacke *et al.* and Iwaisako *et al.,*

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used animal models of established cirrhosis; these models will be critical to developing a full understanding of portal fibroblast function.

Portal fibroblasts possibly maintain ductal structure and integrity. Unlike hepatic stellate cells, portal fibroblasts deposit elastin in addition to fibrillin (both components of elastic fibers), particularly after bile duct ligation [39]; elastic fibers may enhance the resilience of the duct in the setting of obstruction, where there is distension and increased ductal pressure. It may be relevant that portal myofibroblasts secrete collagen in addition to elastin, since this combination could enhance the structural stability of the duct. Collagen XV, which was recently identified as a portal fibroblast marker, is known primarily as a structural collagen that underlies blood vessels and maintains basement membrane integrity [24, 40, 41]. It has yet not been well studied in liver, but could also conceivably provide mechanical benefits to the bile ducts.

Conclusions

Portal fibroblasts were first described more than 50 years ago, yet in spite of rigorous studies published in the last year, their function in the normal and fibrotic liver remains controversial. Confusion stems in part from the heterogeneous and, to some extent, poorly defined nature of this population. The recent identification of new, highly specific markers for both stellate cells and portal fibroblasts should lead to the development of new tools to study the behavior of both cell types in more depth. Portal fibroblasts may have unexpected functions, including roles in angiogenesis and bile duct structure, and thus future research on portal fibroblasts in biliary fibrosis should include not only early fibrosis models but also those with established fibrosis and significant angiogenesis. The fate of portal myofibroblasts as fibrosis progresses and regresses should also be part of these studies. Finally, while initial studies must include lineage tracing analyses in mice, it will ultimately be important to extend this work to human materials.

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