Chapter 7
Fibroblasts, Fibrosis and Autophagy

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Abstract The major distinguishing feature of fibrosis is significant deposition of collagen and other extracellular matrix (ECM) proteins, which can result in scarring if sufficiently excessive. Fibrosis affects many tissue types, and thus contributes to a broad group of diseases which, with few exceptions, continues to lack specific therapy. It has been estimated that nearly 45% of deaths in the developed world are caused by fibroproliferative diseases, which contribute to cardiovascular disease, pulmonary, renal, gut and liver fibrosis, and scleroderma (Bitterman and Henke in Chest 99:81S–84S, [1]). Fibroblasts are the most common stromal cell type of the connective tissues found in the body, and are the primary source of ECM in physiological conditions, i.e. in the absence of disease. The conversion of fibroblasts or similar stromal cells to myofibroblasts is a principal mediator of pathological fibrosis in many tissue types, and frequently occurs in response to ongoing tissue injury and chronic inflammation. While the fibrotic response can occur in response to existing disease, the phenotype conversion of fibroblasts to myofibroblasts due to transient stress or damage may lead to the initiation of long-term fibrotic disease (Bagchi et al. in BMC Biol 14:21, [2]). Inflammation has been found to be a critical inducer of fibrosis, with immune cells generating a variety of growth factors and cytokines that play critical roles in fibroblast activation and subsequent tissue remodelling and fibrosis. A common cellular response to stress stimuli such as inflammation is autophagy, and recent studies have tightly linked the activation or inhibition of autophagy with fibrotic diseases in myriad tissues. Here, we discuss the inter-relationship of these pathways to provide insight into their potential as therapeutic targets in fibrotic disease.

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Introduction

Fibrosis occurs due to the abnormal regulation of the synthesis and/or degradation of ECM, resulting in excessive extracellular deposition of fibrillar collagens (particularly type I and III) and other proteins and proteoglycans, and can alter the function of virtually every organ system in the body. While the precise impact of altered ECM production varies according to tissue type, fibrosis typically results in significant organ dysfunction, and frequently organ failure, contributing to patient morbidity and mortality. For example, idiopathic pulmonary fibrosis (IPF) progressively and severely compromises lung function, and shortens lifespan in affected individuals by many years [3]. Fibrosis frequently occurs secondary to other comorbidities—in the heart, hypertension and diabetes can both be significant drivers of fibrosis, as can smoking in the lung or acute or chronic toxic agent exposure in the liver [4–8]. Cardiac fibrosis can present in multiple patterns, but mid-wall fibrosis appears to be particularly dangerous, increasing the risk of death or hospitalization by up to 18-fold in dilated cardiomyopathy patients exhibiting this pattern compared to those without [9]. Thus, fibrosis is not only an outcome of pre-existing disease, it can also be a risk factor for further organ dysfunction, exacerbating adverse outcomes for patients. Clinical treatments for fibrosis in any organ remain scant, thus a better understanding of fibrosis pathogenesis is critically needed to enable the identification and development of novel therapeutics.

Fibroblasts are a polymorphic cell type which may arise from a variety of precursors such as endothelial or epithelial cells, depending on tissue type. Stromal fibroblasts play a variety of roles, including facilitating organ development, generating the supportive ECM of a tissue, and communicating with nearby parenchymal cells to maintain tissue homeostasis [10]. The ECM generated by fibroblasts consists of fibrous proteins such as collagen and elastin, gelatinous ground substance rich in glycosaminoglycans, and adhesive proteins such as laminin. This complex ECM provides overall tissue, organ and body integrity, and fibroblasts not only synthesize this material, they also play central roles in ECM maintenance and reabsorption. The specific composition of ECM can vary widely across tissues, depending on the specific mechanical stresses to which the tissue is subjected [11]. The critical importance of ECM, and thus of the fibroblasts that produce it, is reflected in the population of virtually all tissue types by fibroblasts or cells that fulfil a fibroblast function, such as hepatic stellate cells. Fibroblasts themselves are heterogeneous, even within tissues, and this is likely to help tune tissue integrity to local mechanical stresses [11, 12].

A general hypothesis that has gained significant consensus is that fibrosis represents a wound healing process that has somehow gone awry. In brief, the normal reparative process following tissue injury involves an initial inflammatory response,

increased stromal tissue coupled with elevated ECM synthesis to support the healing injury, replacement of the dead or injured tissue with new parenchyma and a resolution phase in which the temporary ECM and stromal cells such as myofibroblasts are removed [13]. However, if the transition from the inflammatory phase to subsequent proliferation (of stroma and parenchyma) and wound remodelling (including ECM synthesis) fails to execute properly, fibrosis may occur.

A critical event during wound healing and the development of fibrosis is the activation of fibroblasts to a proliferative, migratory state, followed by a further phenotype transition to that of the myofibroblast [11, 14]. While fibroblasts synthesize and maintain ECM levels in homeostatic balance, myofibroblasts are the arbiters of excessive ECM production that occurs during tissue fibrosis. Not all myofibroblasts arise from fibroblasts, however they are largely responsible for disease progression and dysfunction in organs as varied as the heart, lungs, skin, kidneys, liver and gastrointestinal tract [12]. Myofibroblasts secrete high levels of ECM, and both secrete and are hypersensitive to a variety of cytokines, growth factors, and chemokines that promote and maintain the pro-fibrotic myofibroblast phenotype [15]. Their hallmark functional change is the acquisition of a contractile apparatus due to the induction of expression of α-smooth muscle actin which is incorporated into stress fibers, which may permit these cells to exert physical traction to help close or reduce the margins of wounds [12, 16, 17]. Cardiac myofibroblasts have been shown to also express the matricellular protein periostin, which itself has been implicated as a driver of fibrosis, and which is not expressed in non-activated fibroblasts [18, 19]. Fibroblast activation is unquestionably important in the wound healing process, but long-term maintenance of a myofibroblast-like phenotype contributes to pathological fibrosis [20, 21].

A number of cytokines and growth factors have been demonstrated to induce or facilitate the conversion of fibroblasts to myofibroblasts. While the most potent of these is TGFβ, a variety of other factors have also been implicated in fibroblast activation and fibrosis in multiple tissue types, including endothelin-1, Platelet-Derived Growth Factor (PDGF), angiotensin II, and Connective Tissue Growth Factor (CTGF/CCN2) [22–27]. Elevated TGFβ expression has long been associated with a host of fibrotic diseases including cystic fibrosis, scleroderma, and fibrosis of the lungs, heart, kidneys and liver [28–34]. TGFβ is produced and secreted to the extracellular matrix in a protein-bound, latent form by various cell types, including fibroblasts themselves as well as inflammatory cells [35, 36]. Various processes, including protease action or physical disruption, results in the release of active TGFβ, which acts via cell surface receptors to activate intracellular signalling cascades that can be Smad-dependent (canonical) or Smad-independent (non-canonical) [35, 37–39]. TGFβ-mediated expression of CTGF/CCN2, a matricellular protein, can further amplify pro-fibrotic processes, while upstream agents such as angiotensin II can up-regulate expression of TGFβ itself [40–43]. Antagonism of TGFβ signaling is effective in reducing evidence of fibrosis, and drugs that interfere with the renin–angiotensin–aldosterone system have been shown to exert anti-fibrotic effects [44–46]. It is unclear if these various factors act solely as a trigger to activate fibroblasts, or if their presence is required for ongoing maintenance of the

myofibroblast phenotype. In either scenario, the transition of fibroblasts to myofibroblasts appears to be a critical step in the development and progression of fibrosis across tissue and organ types.

Immune Cells as Mediators of Fibrosis

Inflammation is a potent inducer of fibrosis across tissue types. While inflammation is an important initial step of the normal wound healing process, it is critical that inflammation resolves in a timely manner as a chronic inflammatory state leads to tissue remodelling and fibrosis [47]. Inflammation is mediated by a variety of immune cells including macrophages, mast cells, eosinophils, neutrophils, and CD4+ and CD8+ lymphocytes, and fibroblasts themselves can generate pro-inflammatory products such as growth factors and cytokines. Two primary types of immune responses can contribute to fibrosis. In type 1 immunity, Type 1 T helper (Th1) cells release factors such as interferon gamma (IFNγ) and interleukin-2 (IL-2). In turn, these factors stimulate phagocytic cells including mast cells and macrophages. In type 2 immunity, characterized by high antibody titers, Th2 cells secrete a variety of cytokines including IL-4, IL-5, IL-9, IL-10, and IL-13, resulting in eosinophil activation [48]. Fibrosis is initially characterized by the production of Th1 cytokines followed by the Th2 response, producing TGFβ and IL-13 and leading to activation of fibroblasts and their conversion to myofibroblasts to promote fibrosis [14, 49, 50]. Th17 cells have also been implicated in fibrosis of the skin and lung [51].

Mast Cells

Mast cells are involved in both innate and adaptive immunity, and play a pivotal role in inflammation as well as in tissue remodelling leading to fibrosis [52]. Mast cells produce, store and release various growth factors, inflammatory factors and cytokines which contribute to fibrosis, including TGFβ [53]. In addition, mast cells can produce proteoglycans such as hyaluronic acid which contribute to matrix composition directly, but which can also stimulate fibroblast activation [54]. Mast cells release a variety of proteases, including chymase, leukocyte elastase, and plasmin, that in turn release latent TGFβ from ECM niches to induce fibroblast activation [39]. Mice that were mast cell deficient were protected from bleomycin-induced pulmonary fibrosis, and this same study found that mast cell release of histamine and renin could activate fibroblasts either directly, or via the eventual generation of angiotensin II, respectively [55]. Thus, mast cells can trigger the initial activation of fibroblasts leading to their proliferation and ECM production via several complementary mechanisms, demonstrating their key role in the development of fibrosis.

Macrophages

Macrophages can be broadly classified into two phenotypes: the classical M1 phenotype that is pro-inflammatory and activated by cytokines such as IFNγ, and the alternative M2 phenotype that is involved in the resolution of inflammation and is activated by interleukins such as IL-4 and IL-13 [56, 57]. While M2 macrophages have been shown to specifically promote tissue remodelling and fibrosis, M1 macrophages can also drive fibrosis via the sustained maintenance of tissue inflammation, although the specific interplay of factors that determine whether M1 macrophages act in a pro- or anti-fibrosis fashion remain to be determined.

The pro-fibrotic role of M2 macrophages has been demonstrated in multiple tissue types [58]. When human monocyte THP-1 cells were polarized to M1 or M2 macrophages and exposed to human dermal fibroblasts, M2 macrophages, specifically, induced fibrotic responses including up-regulation of αSMA and expression of collagen [59]. However, the source of these macrophages appears to be important for their role in promoting fibrosis. Alveolar macrophages derived from monocytes were required for lung fibrosis in mice, whereas tissue-resident alveolar macrophages were not [60]. While M2 macrophages can serve as a source of TGFβ that subsequently acts directly on fibroblasts to induce their activation and promote fibrosis, TGFβ can instead act on bone marrow-derived macrophages to induce their conversion to myofibroblasts [61]. Macrophages thus exert a variety of effects on the induction of fibrosis across tissue types.

Interleukins

Interleukins are cytokines produced by white blood cells. They play a central role in the body's immune and inflammatory responses, and their action is modulated by the inflammasome—a cytosolic multiprotein complex produced by myeloid cells which can contribute to tissue fibrosis [62]. In response to stress or injury, inflammasome assembly results in the activation of inflammatory caspases that in turn activate inflammatory cytokines via cleavage of inactive precursors [63]. The inflammasome acts as a binding site for a variety of caspases responsible for activating different interleukins, including IL-1 family members and related cytokines that stimulate fibrosis via the inflammatory response [63, 64].

Inflammatory cytokines including IL-1, Tissue Necrosis Factor and IL-33, can promote fibroblast activation, proliferation and collagen synthesis, potentially by increasing TGFβ expression [65–67]. Activated cytokines IL-18 and IL-33, along with IL-1, enhance inflammation and through the involvement of TGFβ can lead to the production of other cytokines such as IL-4 and IL-13, which in the setting of the lung can activate fibroblasts to behave as inflammatory cells, releasing pro-inflammatory cytokines and chemokines [68]. IL-5 is secreted by several inflammatory cell types, including mast cells and eosinophils, and eosinophils in turn are activated by IL-5

and Granulocyte–Macrophage Colony Stimulating Factor to contribute to activation of fibroblasts or other stromal cells to promote lung and intestinal fibrosis [69, 70].

In contrast to these pro-fibrotic cytokines, some demonstrate anti-fibrotic activity. IL-37 is an IL-1 family member, but has been found to attenuate the production of pro-inflammatory cytokines and chemokines by mast cells, macrophages or other immune cells that contribute to inflammation, and has shown promise as an anti-fibrotic in the lung and liver [71, 72]. Kim et al. reported that IL-37 reduced extracellular matrix protein expression in primary human lung fibroblasts, attenuated their proliferation in response to TGFβ, and was more highly expressed in alveolar epithelial cells and macrophages compared to cells from IPF patients [73]. In this study, the beneficial effect of IL-37 was dependent upon its ability to induce autophagy by inhibition of mTOR. IL-37 may also induce the expression of IL-10, and over-expression of IL-10 in the lung was able to reduce pulmonary fibrosis induced by bleomycin [74]. IL-10 has been reported to promote wound healing via activation of fibroblast-specific STAT3 and down-stream hyaluronan synthesis without driving fibrosis [75]. IL-22 is an IL-10 family member that has been found to act as an anti-inflammatory and anti-fibrotic cytokine in liver injury [76].

Autophagy

Autophagy is a critical process for the removal of excessive or defective cellular components including proteins and organelles, which can be recycled both to remove their detrimental effects on cell function and to provide energy and resources back to the cell. While autophagy can help the body to cope with stress, damage, injury, or pathogen infection, excessive levels of autophagy can be detrimental, potentially leading to cell apoptosis and tissue dysfunction.

Recent studies have linked autophagy, apoptosis and fibrosis in a variety of tissue types (Fig. 7.1). Stimulation of human atrial myofibroblasts with TGFβ induced both collagen synthesis and autophagy, while blockade of autophagy attenuated the fibrotic effect of TGFβ and in a separate study, prevented the conversion of rat cardiac fibroblasts to myofibroblasts [77, 78]. Over-expression of the TGFβ/Smad repressor Ski induced apoptosis in rat cardiac myofibroblasts, and this effect was increased if autophagy was simultaneously inhibited, suggesting that autophagy provides energy required for cell survival [79]. In normal human lung parenchymal and airway fibroblasts, TGFβ induced collagen synthesis and autophagy in parallel, however while the effect of TGFβ on collagen synthesis was increased in cells derived from IPF patients, autophagy induction was reduced demonstrating that the relationship between autophagy and fibrosis is variable [80]. Instead, the unfolded protein response was induced in IPF cells. These pathways may thus represent mechanisms to meet the high energy demands of ECM synthesis when fibroblasts become myofibroblasts, including in the setting of fibrosis. Conversely, apoptosis may be a means by which myofibroblasts are removed when energy levels are insufficient to support their ECM synthesizing function. These mechanisms may be highly

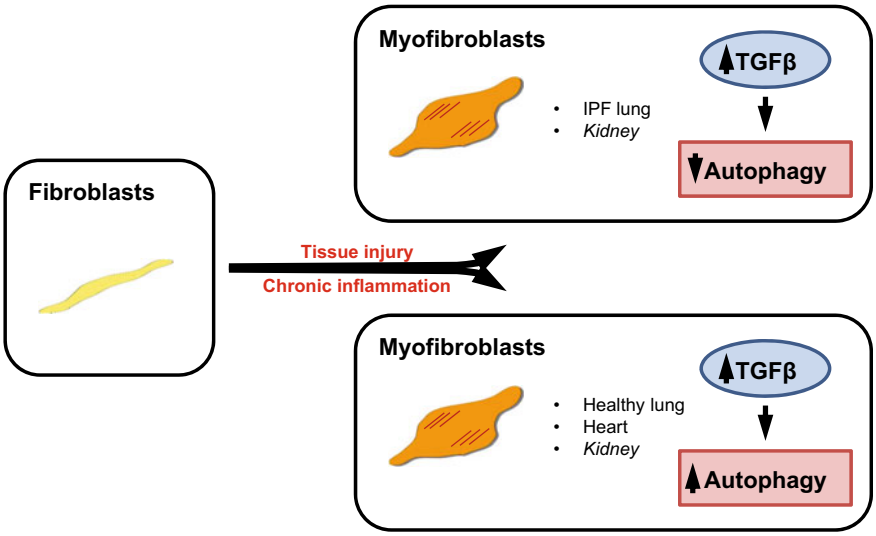

Fig. 7.1 The association of autophagy with TGFβ treatment is context-dependent. In response to tissue injury, chronic inflammation or impaired healing, fibroblasts undergo a phenotype conversion to myofibroblasts. The cytokine TGFβ can also independently induce this cellular change, however the induction of autophagy concomitantly with fibrosis is context-dependent. In a variety of cells including healthy lung fibroblasts, and atrial and ventricular cardiac fibroblasts, TGFβ induces both fibrotic gene expression and autophagy during conversion to myofibroblasts, and inhibition of autophagy typically attenuates fibrosis (lower panel). Conversely, in lung fibroblasts isolated from idiopathic pulmonary fibrosis (IPF) patients, autophagy decreases in response to TGFβ despite an induction in fibrotic gene expression during myofibroblast conversion. In kidney mesangial cells, TGFβ induced autophagy and collagen synthesis together, however reducing autophagy by decreasing Beclin-1 expression also stimulated collagen synthesis, further demonstrating that these pathways can be separated. Autophagy is positively correlated with fibrosis in other collagen-producing non-fibroblast cell types such as hepatocellular carcinoma cells, while in gut epithelium, attenuation or stimulation of autophagy increased or decreased fibrosis, respectively. The link between autophagy and fibrosis thus likely depends not only on cell type, but also on the specific environment and/or health of the cells

context- and signalling pathway-dependent, however: in kidney mesangial cells, TGFβ induced both collagen synthesis and autophagy, while knockout or knockdown of the autophagy protein Beclin 1 also increased collagen synthesis [81].

While autophagy may be required for fibroblast activation to myofibroblasts, the response of fibroblasts to the induction of autophagy may be distinct depending on the relative health status of the tissue, further supporting the idea that the specific cellular context is important in the relationship between autophagy and fibrosis. In normal lung fibroblasts, culturing on polymerized collagen can induce cell stress that results in apoptosis, but in contrast, IPF fibroblasts resist this stress and instead become proliferative and pro-fibrotic [82]. This was found to be due to alterations in PTEN/Akt/mTOR signalling in IPF fibroblasts compared to healthy

lung fibroblasts, such that healthy cells undergo autophagy and subsequent apoptosis. Conversely, autophagy is down-regulated in IPF fibroblasts following culture on collagen resulting in increased cell survival [82]. A recent study suggests that IPF fibroblasts exhibit a senescent phenotype with reduced apoptosis and proliferation, rather than an activated fibroblast phenotype, with TGFβ inducing endoplasmic reticulum stress [83]. The disparity in these studies may represent differences in cell collection, nature or length of culture, biological variability across patients from which the cells are derived, or may simply be reflective of high levels of fibroblast heterogeneity. It is noteworthy, however, that both studies identified reduced apoptosis as a feature of IPF fibroblasts. It remains to be seen whether similar mechanisms linking fibrosis, autophagy and apoptosis occur in fibroblasts derived from other healthy or diseased tissues.

Regulation of Autophagy by TGFβ

TGFβ has been shown to regulate autophagy via both Smad-dependent and Smad-independent pathways, in a variety of disease contexts including cancer and fibrosis. In human hepatocellular carcinoma cells, for example, knockdown of Smad2/3 or Smad4 resulted in the inhibition of TGFβ-induced autophagy, although knockdown of c-Jun NH_2-terminal kinase had a similar effect, implicating both canonical and non-canonical TGFβ pathways [84]. In several cancer cell lines, TGFβ induced pRb/E2F1-mediated up-regulation of autophagy genes and induction of autophagosome formation [85]. Conversely, berberine administration in a rat bleomycin model of IPF attenuated Smad and PI3K/Akt signalling, but also inhibited mTOR to increase autophagy [86]. As noted above, TGFβ both induced or inhibited autophagy in lung fibroblasts depending on whether they were derived from healthy or IPF donors, respectively, despite inducing collagen expression in both cell types [80]. Thus, the variable induction of canonical and non-canonical signalling pathways downstream of TGFβ may account, in part, for the differential effects noted on fibrotic gene expression and the induction of autophagy. In turn, alterations in the level of autophagy may be beneficial or detrimental with respect to fibrosis in different contexts. Clearly, additional investigation in this area is required.

Potential Therapeutic Targets for Fibrosis

Given the central roles of inflammation and autophagy in the induction and/or progression of fibrosis in various tissue types, it is tempting to consider these areas for exploitation in the quest for novel anti-fibrotic treatments, particularly given the current and conspicuous lack of such medications. Given its central role as a product of inflammatory cells and inducer of fibrosis and autophagy, TGFβ presents a tempting target. However, with its myriad roles within individual cells and across

cell and tissue types, some of which may oppose one another depending on context or disease state, direct therapeutic interference with TGFβ itself is complicated at best [87].

Other targets may be more tractable for development [11]. As noted, IL-37 is an anti-inflammatory cytokine which can act directly by downregulating pro-inflammatory mediators and interfering with TGFβ signalling, and indirectly by promoting the production of other anti-inflammatory cytokines such as IL-10 [73, 74]. IL-37 may be of particular use in the setting of IPF. IL-10 and its family member IL-22 may similarly be useful therapeutically via their anti-inflammatory and anti-fibrotic properties [74, 76].

The regulation of autophagy provides another opportunity to reduce fibrosis. Autophagy may be required for the activation of fibroblasts to myofibroblasts, and autophagy inhibition leads to cardiac myofibroblast apoptosis [79]. However, the link between autophagy and fibrosis appears to be variable across tissues, and may further be influenced by not only the presence of disease, but also by the specific nature of the disease. For example, autophagy inhibition may actually increase activation of lung fibroblasts, with consequences for IPF [88]. Stimulation of autophagy by rapamycin in a gut fibrosis model reduced fibrosis, while autophagy inhibition with 3-methyladenine exacerbated fibrosis [89]. Thus, the positive or negative manipulation of autophagy to treat fibrosis will be critically dependent on the specific pathology involved.

Conclusion

Fibrosis is an aberrant wound healing process in which fibroblasts, in response to various stimuli including growth factors like TGFβ, which in turn is secreted and activated through inflammation-activated immune cells, increase collagen and extracellular matrix protein deposition. The end result is tissue remodeling leading to organ failure, with increased risk of patient morbidity and mortality. The interplay of fibrosis development and autophagy is complex, variable across cell and tissue types, and dependent on a variety of intracellular signaling pathways including mTOR/AKT/PTEN, Smads and others, despite the superficial similarity of fibrosis in different tissues.

The identification of novel anti-fibrotic medications is arguably one of the most urgent clinical challenges at present, given the widespread occurrence of fibrosis and dearth of treatments. Targeting the relationships between inflammation, autophagy and fibrosis provides an exciting new frontier for therapeutic development. However, caution is required given the heterogeneity of fibrotic disease mechanisms, and further mechanism-focused research in this area is critically needed.

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