Hypoxia-sensitive pathways in inflammation-driven fibrosis.

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Abstract:

Tissue injury can occur for a variety of reasons including physical damage, infection and ischemia. The ability of tissues to effectively recover from injury is a cornerstone of human health. The healing response in tissues is conserved across organs and typically involves distinct but overlapping inflammatory, proliferative and maturation/resolution phases. If the inflammatory phase is not successfully controlled and appropriately resolved, an excessive healing response characterized by scar formation can lead to tissue fibrosis, a major clinical complication in disorders such as Crohn’s disease (CD). As a result of enhanced metabolic and inflammatory processes during chronic inflammation, profound changes in tissue Oxygen levels occur leading to localized tissue hypoxia. Therefore, inflammation, fibrosis and hypoxia are co-incidental events during inflammation-driven fibrosis. Our current understanding of the mechanism(s) underpinning fibrosis is limited as are the therapeutic options available. In this review, we discuss what is known about the cellular and molecular mechanisms underpinning inflammation-driven fibrosis and how hypoxia may play a role in shaping this process.
Introduction

The capacity to heal injured, wounded or damaged tissue is of critical importance for organism survival. The process of successful tissue recovery involves a significant but transient disruption of homeostasis. The mechanisms underpinning tissue healing are generally conserved between tissues and involve a series of discreet yet overlapping inflammatory, proliferative and maturation/resolution phases with each phase requiring the participation of specific cell types and mediators(5, 46, 47, 89, 93).

Inflammation is initiated shortly after tissue damage occurs. The main purpose of the inflammatory response is to ensure the isolation and sterilization of the wounded area, thus controlling and eliminating the risk of infection. Physiologic inflammation is thus the first phase of wound healing(5, 46, 47, 89, 93). The infiltrating inflammatory cells and the substances they produce during this phase are also responsible for promoting the development of subsequent proliferative and maturation stages of the healing response which promote the regeneration and recovery of the tissue respectively(85-87, 125, 132, 133). Therefore, a controlled and resolving initial inflammatory stage is essential for the successful regeneration and recovery of wounded tissue.

Subsequent to the inflammatory phase, wound healing enters a proliferative phase which is directed towards regenerating the original structure of the damaged tissue. To accomplish this, a series of mediators released during the inflammatory stage direct the recruitment and activation of fibroblasts which initiate a fibrogenic response(5, 46, 47, 89, 93). Tissue fibrogenesis is characterized by the production of extracellular cell matrix components in order to replace / renew damaged tissue. The
newly formed extracellular matrix (ECM) provides the physical scaffold to support subsequent wound closure, remodeling and repair.

Despite its beneficial role in tissue repair, if uncontrolled or unresolved due to adverse genetic or environmental factors, inflammation can pose a significant risk to long-term tissue homeostasis resulting in chronic inflammatory diseases such as Inflammatory Bowel Disease (IBD). Inflammatory lesions in such conditions resemble non-healing wounds with physiologic inflammation and associated fibrogenesis developing into chronic inflammation and fibrosis respectively (46, 133).

Perturbations in the mechanisms controlling the duration and intensity of the inflammatory response underpin the transformation from acute inflammation into a chronic, non-resolving process.

In the context of tissue repair following injury, the degree and duration of the inflammatory step is critical for its ultimate success. An example of the importance of this comes from the observation that adult skin wound healing usually results in the formation of scar tissue, whereas in foetal skin, wound healing occurs in a scarless manner. This is in part due to the different degrees of inflammation associated with adult or foetal skin wound healing, which is milder in foetal skin (33, 87). While an appropriately driven and resolving inflammatory process results in successful wound healing, an inappropriately sustained inflammatory reaction is often related to an overactive wound healing response leading to tissue fibrosis which can represent a threat to tissue structure and function.

Therefore normal physiologic wound healing involves controlled self-resolving inflammation and fibrogenesis which provides the mechanism for a successful return to homeostasis. In contrast, chronically inflamed tissues are characterized by
sustained, non-resolving inflammation and fibrosis leading to tissue dysfunction. Of 
note, the process of inflammation has significant effects on the tissue 
microenvironment including the development of relative tissue hypoxia as a result of 
increased Oxygen consumption by highly metabolically active resident cells and 
recruited inflammatory cells(118). This tissue hypoxia regulates inflammatory and 
fibrotic pathways through Oxygen-sensitive signaling pathways such as the Hypoxia 
Inducible Factor and may represent a new therapeutic target for the treatment of 
fibrosis(24). In this review we will discuss the dynamic mechanisms underpinning 
physiologic wound healing and how disruption of these processes can lead to 
chronic inflammation and fibrosis. More specifically, we will interrogate the interplay 
between inflammatory, hypoxic and fibrotic pathways and discuss potential new 
therapeutic approaches in fibrotic disease based on our developing understanding of 
these inter-related processes.

The physiologic Wound healing process.

Internal or external tissue injury is a common occurrence which can be the result of a 
broad range of adverse events including (but not limited to) physical damage, 
infection and ischemia/reperfusion injury. The ability to elicit and execute an effective 
healing response which restores normal physiologic function is therefore central to 
directing a successful return to homeostasis in a wounded tissue. This healing 
response is largely conserved between tissue types and can be divided into three 
temporally distinct yet overlapping phases termed the inflammatory phase, the 
proliferative phase and the resolving phase respectively(5, 46, 47, 89, 93). These 
phases require the interdependent activity of a range of cell types (including immune, 
mesenchymal and epithelial cells) and signaling pathways, which act in concert to 
promote an effective healing response:
Inflammatory phase: the first phase of physiologic healing is characterized by a hemostatic response directed towards vascular isolation of the wounded area in order to prevent blood loss. This involves rapid vasoconstriction and localized thrombosis. Platelet activation in response to factors released by injured endothelial cells in a wounded tissue is an essential early event in this process (5, 85, 86). Circulating fibrinogen is cleaved to produce fibrin which facilitates platelet aggregation. Activated platelets release a range of factors including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) which in turn promote an inflammatory response, the function of which is to kill and remove any bacteria present and to initiate the process of tissue repair. For the inflammatory phase to progress, the vascular tone must be reduced through a decrease in vascular smooth muscle contractility (initiated by histamine and leukotrienes released from resident mast cells) and an increase in vascular permeability promoted by factors such as VEGF. Chemoattractants such as interleukin-8 (IL-8) are released and promote the recruitment of immune cells from the circulation into the wounded tissue. The first immune cells to arrive at the wounded site due to the established gradients of chemoattractants are typically polymorphonuclear cells (neutrophils) which kill exogenous infectious agents and release factors which promote the infiltration of monocytes which, upon differentiation into macrophages are key players in driving (and resolving) an effective immune response through phagocytosis of pathogens and apoptotic neutrophils (5, 46, 89, 125). Macrophages are also responsible for the propagation of the inflammatory response through the release of cytokines which drive inflammation in the wounded tissue (5, 46, 125, 132, 133). As noted above, during the inflammatory phase of normal wound healing, the consumption of Oxygen by infiltrating immune cells such as neutrophils along with the increased
consumption of Oxygen by highly metabolically active inflamed resident cells renders the normally healing tissue transiently hypoxic (Figure 1) (24, 117, 118).

- Proliferative phase: following an effective inflammatory phase where injured tissue is sterilized by an effective immune response, the wound healing process enters the proliferative phase where the laying down of a new extracellular matrix (ECM) formed from collagens and laminins takes place (5, 8, 125, 133). A key cell type in this process is the fibroblast which as well as producing the major components of the ECM, is important in defining the physical parameters of the wound. In order for this to occur, fibroblasts must migrate into the wounded area, an event stimulated by factors including TGFβ, PDGF and connective tissue growth factor (CTGF)(5, 46, 69, 125). Angiogenesis occurs during this phase of the healing response as a result of tissue hypoxia driving the release of factors such as VEGF, a potent endothelial growth factor. During this phase of the healing response, the removal of neutrophils by phagocytosis and the restoration of blood flow likely results in a return to tissue normoxia (Figure 1). Therefore, the normal healing response likely involves a transient period of tissue hypoxia. As will be discussed below, more than simply being a bystander effect, hypoxia activates pathways which actively regulate the nature, degree and duration of inflammation occurring.

- Maturation phase: this last step of physiological healing involves tissue remodeling and contraction of the wound area with the aim of reconstituting normal tissue structure as effectively as possible. Fibroblasts also play a key role here by differentiating into myofibroblasts which are capable of driving contraction of the wound area. Inflammatory mediators reduce collagen formation rates in fibroblasts and matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) are synthesized and released and promote the breakdown of the pre-formed ECM scaffold(27, 46, 70). The resolution of the wound healing response is characterized by a loss of capillaries as the mature scar becomes less vascular.
Antiangiogenic factors such as thrombospondin 1 contribute also to vascular regression and fibroblast numbers decrease through the induction of fibroblast apoptosis. Thus, under physiologic conditions, tissues have the ability to respond to injury in a manner which promotes recovery of tissue structure and function and a return to physiologic homeostasis (Figure 1).

**Chronic inflammation: a non-healing wound.**

The transition from an acute, physiologic immune response (evolved to promote the healing of injured tissue) to a state of chronic inflammation reflects the transformation from a physiologic to a pathologic state. Chronic inflammation leads to a condition resembling a non-healing wound and is associated with a diverse range of pathologies including the development of cancer(6). The success of the physiologic inflammatory process is determined by its ability to undergo a phase of adequate resolution. This requires the elimination of inflammatory cells by apoptosis, phagocytosis or migration, the production of anti-inflammatory agents and the cessation of pro-inflammatory mediator production (5, 44).

The transformation from physiologic, healing-associated inflammation to chronic pathologic inflammation occurs when the positive regulators of the inflammatory response outweigh the pro-resolving factors over a prolonged period. A number of underlying pathologies can cause this including persistent infection with aggressive pathogens, dysfunctional innate immunity, repetitive inflammatory insults, the presence of environmental antigens, genetic predisposition of the host and autoimmune reactions. These underlying factors have been related to some of the most common and severe chronic inflammatory diseases(24, 30, 43, 75, 121, 134, 136). Indeed, the combination of risk factors is often responsible for the development of a non resolving chronic condition. For example, in IBD, specific variants in genes
such as NOD2 or IL23R predispose patients to disease, but certain infections such as Salmonellosis serve as a trigger for disease development(24, 43, 121, 134). In the lung, the development of interstitial pulmonary fibrosis (IPF) has also been related to genetic predisposition combined with the alveolar injury caused by bacterial and/or viral infections(75) as well as to a strong environmental component represented by the exposure to inhaled agents such as cigarette smoke or metal dust(136). In these chronically inflamed environments the overactive mechanisms of the immune and wound healing response can be harmful to tissues. Macrophages are key immune cells in chronic inflammation which stimulate differentiation of T cells, whereas T cells secrete IFN-gamma which further activates proinflammatory macrophages and signals those macrophages to survive(44, 77), thus prolonging the inflammatory process.

While excessive pro-inflammatory activity can underpin chronic inflammation, it is also common that this condition arises as a result of a failure to resolve inflammatory processes due to diminished endogenous anti-inflammatory events. In this context, a key resolving mechanism is mediated by the expression of anti-inflammatory and pro-resolving mediators expressed in different cell types(60, 64). Amongst these specialised lipid mediators such as lipoxins, resolvins and maresins may play an important role. The beneficial effects of lipoxins are due to three temporally distinct activities(106): First, they inhibit neutrophil recruitment and activation(34, 60, 64). Second, they promote monocyte recruitment and stimulate the phagocytosis of apoptotic neutrophil residues, thus promoting in the clearance of the wound(60, 64). Finally, in experimental models lipoxins prevent fibrosis(11, 12), a final pathological outcome of chronic inflammation that will be discussed below. In this context, current evidence points to lipoxin A4 (LXA4) as a negative regulator of TGF-β1, key
mediator in wound healing and fibrosis(11) by mechanisms that include induction of
the miRNA let-7c which specifically targets a subunit of the TGF-β receptor (12).

**Impaired healing leads to tissue fibrosis**

As discussed above, successful wound healing is a tightly regulated process
involving a high degree of complex crosstalk between multiple cell types. Impaired
wound healing, either when defective or excessive, is often related to chronic
disease and poor prognosis. When the healing process is overactive or unresolved,
wounds can become fibrotic due to excessive deposition of ECM leading to tissue
dysfunction.

Fibrosis is associated with many disease states and because the wound healing
process is conserved across tissues, it can affect any tissue type(35, 95, 120, 133).
Fibrosis is a complication of chronic inflammatory conditions such as interstitial lung
disease (ILD)(2), chronic kidney disease (CKD)(143), chronic liver disease (CLD)(91)
or IBD(114). Fibrosis is associated with poor patient prognosis, as it often leads to
irreversible tissue dysfunction. For example, CKD-associated fibrosis causes
scarring, loss of nephron function and kidney failure(59). IBD-associated fibrosis,
particularly in transmural Crohn’s disease often leads to the formation of areas of
stenosis and intestinal obstruction. Indeed, in early stages of Crohn’s disease,
inflammation is more predominant while later stages become more penetrating and
associated with the formation of fibrotic strictures(21). Although fibrosis is a common
and important clinical complication, its mechanisms remain incompletely understood
and currently, it cannot be effectively pharmacologically reversed and surgery is
often ultimately required to remove dysfunctional fibrotic tissue(35, 95, 114, 133). An
example of the clinical importance of fibrosis is that the mortality rate in idiopathic
pulmonary fibrosis exceeds that of many cancers(146). Furthermore, 75-80% of patients with Crohn’s disease ultimately require surgical resection of fibrotic colon segments(95, 114). Thus, a better understanding of the mechanisms underlying fibrosis as well as further insights into possible therapeutic approaches are crucial to address this major health burden.

**Mechanisms of fibrosis**

Although the causes remain to be fully elucidated, altered signaling in the processes of physiological wound healing lead to fibrosis. At a cellular level, fibrosis is the consequence of excessive production and deposition of ECM components(2, 27, 35, 59, 95, 132, 143). Figure 1 shows a schematic representation of the comparison of the process of normal physiological wound healing with excessive wound healing leading to fibrosis.

As stated above, fibrosis is related to continuous or unresolved chronic inflammation. Under these conditions, excessive macrophage activity is related to increased and sustained ECM production through macrophage-dependent activation of ECM producing fibroblasts and (potentially) other cell types (132, 133). ECM production takes place mainly during the proliferative stage, when granular tissue is formed (5, 46, 93). Thus, a wound is likely to become fibrotic when the healing process is locked in the proliferative stage due to non-resolving chronic inflammation. However, it should be noted that fibrosis can also occur as a consequence of defective matrix breakdown during the remodelling stage of wound healing (27, 95). This aspect of fibrosis depends upon a dysregulated expression and activity of MMPs and/or TIMPs(27, 95, 143).

ECM production is primarily a fibroblast-driven process. Therefore, excessive activation of these cells is the critical event for the development of fibrosis(2, 8, 27,
95, 133). A prolonged inflammatory response causes increased fibroblast migration into the wounded area and also prolongs the duration of their activation, leading to an excessive accumulation of differentiated fibroblasts. This primary event has the direct consequence of increasing the amount of collagens and other ECM components. Alternatively, the resolution of fibroblast activity may be dysfunctional also leading to an excessive deposition of ECM. Therefore, fibrosis can occur as a result of non-resolving inflammation resulting in the overactivation of fibroblasts and the resultant excessive production and deposition of ECM. Understanding the pathways underpinning these events in immune cells and fibroblasts is key to developing new therapeutic opportunities in these conditions.

**Regulatory pathways in inflammation-driven fibrosis.**

While multiple molecular mechanisms govern the transcriptional regulation of inflammation-driven fibrosis, the NF-κB and Smad pathways are master regulators of inflammation and fibrosis respectively:

- **NF-κB pathway:** NF-κB is a family of transcription factors comprised of 5 subunits which form distinct homo- or hetero-dimers. While three of these isoforms (p65 / RelA, RelB and c-Rel) are synthesized as fully active forms, two isoforms (NF-κB1 / p105 and NF-κB2/p100) are synthesized as precursors which are cleaved to form fully active p50 and p52 isoforms(14, 53). In the steady state, NF-κB is repressed by IκB inhibitor proteins, which retain it in the cytoplasm. Upon exposure to an inflammatory stimulus, IKK-kinases (IKKs) phosphorylate IκB leading to its ubiquitination and proteosomal degradation, which in turn liberates NF-κB which is then able to translocate to the nucleus and bind to DNA(14, 53).

In inflammation and infection, the functions of NF-κB are mainly mediated through its canonical signalling pathway, the activation of which can be driven by the
engagement of multiple receptor sub-types(14, 53). Receptors linked to canonical NF-kappaB signaling include toll-like receptors (TLR), Interleukin receptor 1 (IL-R1), tumor necrosis factor receptor (TNFR) and antigen receptors. Typical stimuli for these receptors include lipopolyssacharide (LPS) and other bacterial components and major inflammatory cytokssacharide such as tumor necrosis factor-α (TNF-α) and interleukin 1-β (IL-1β). TLRs are expressed in a broad range of immune cells including macrophages, dendritic cells, B cells and T cells, supporting the global importance of the TLR/NF-κB pathway in inflammation and immunity. Stimulation of NF-κB pathway in immune cells leads to the production of chemokines such as IL-8, adhesion molecules, and cytokines such as IL-1β, TNF-α or IL-12 all of which promote immune/inflammatory reactions(14, 19, 53, 72, 80).

- The TGF-β pathway: The TGF-β family plays a pivotal role in regulating fibrosis. This family of secreted ligands comprises the TGFβs 1-3, activins and the bone morphogenetic proteins (BMPs). TGF-β1 is the prototypic cytokine driving fibrosis in numerous organs(5, 8, 27, 70, 95, 99, 110, 124). The bioactions of TGFβ1 are modulated by additional members of the family including the BMP agonists and antagonists whose activities may exacerbate or attenuate TGF-β1-elicited responses(126). All members of the TGF-β superfamily are ligands at serine threonine kinase receptors. TGF-β1 was first shown to activate fibroblasts in the early 1980’s, and its role in collagen production was first reported in 1986(54). The three TGF-β isoforms are designated TGF-β1, TGF-β 2 and TGF-β 3. Amongst these, TGF-β1 is the most prevalent(4, 8, 65) and is the main Isoform responsible for the development of tissue fibrosis(4, 8, 27). The ligands signal through a heterodimeric receptor complex composed of TGF-β RI and RII. The TGF-βRII is constitutively active and transphosphorylates the ligand-bound TGF-βRI (8, 65, 67). The classical pathway of TGF-β signaling involves the participation of a family of proteins known as Smads which are the vertebrate homologs of the drosophila protein mothers against
decapentaplegic homologue and the related C. elegans Sma. Smad proteins are
divided into subtypes depending on their functions: receptor activated Smads
(Smad1, 2, 3, 5 and 8), common mediator Smads (Smad4) and inhibitory Smads
(Smad6 and 7)(8, 58, 67). On receptor activation serine phosphorylation of the
receptor associated smads 2 and 3 results in the formation of a heterodimer with
cytosolic smad 4 and the smad 2,4 and smad 3,4 complexes translocate to the
nucleus and interact with smad binding elements recruiting coactivators, repressors
and or transcription factors to modulate expression of genes including those involved
in matrix deposition and inhibition of breakdown e.g. fibronectin, collagen and
plasminogen activator inhibitor. Smad7 inhibits TGF-β1 signalling by binding to the
TGFβRI and blocking smad 2, 3 activation, or by promoting Smad degradation(8, 59,
65, 67, 78).

TGF-β1 signals through both canonical (i.e. smad) and non-canonical pathways. The
non-canonical pathway depends on the ability of phosphorylated TGF-βRI and RII to
activate various mitogen activated protein kinase (MAPK) pathways such as ERK,
p38 and JNK(8, 65, 78) and PI3 kinase(137).

Many different studies highlight the importance of the canonical TGF-β pathway in
fibrosis. Smad2 and Smad3 have been related to fibrosis in different organs and
diseases. Both Smad2 and Smad3 are strongly activated in fibrotic conditions as
occurs in chronic kidney disease(59) or post-myocardial infarction scarring(49). In
this regard, Smad3 is critical for the expression of fibrotic molecules such as α-
smooth muscle actin (α-SMA), Collagen-1, CTGF, plasminogen activator inhibitor-1
(PAI-1) or TIMP-1. Evidence supporting a role for Smads in driving fibrosis includes
the observation that Smad3-deficient mice are protected against diverse models of
fibrosis such as bleomycin--induced pulmonary fibrosis(144), Skin irradiation induced
injury model(37) or ischemia reperfusion myocardial infarction model(28). In vitro,
Smad3 overexpression in hepatic stellate cells caused increase in α-SMA and
collagen-1(123) and enhanced α-SMA in human foetal lung fibroblasts(45).
Moreover, siRNA against both Smad2 and Smad3 attenuates the expression of TIMP-1 and Collagen-1 in rat intestinal myofibroblasts(71). On the other hand inhibitory Smads such as Smad7 appear to be key in restricting excessive wound healing through the inhibition of fibrosis. However, as TGF-β1 signalling also has an important role in limiting the inflammatory response(138), excessive Smad7 activity may also contribute to exacerbate inflammatory processes(42).
Integrins are a family of transmembrane adhesive proteins which participate in inflammatory processes and tissue organization. Their extracellular domains are important for cell-ECM interactions with some subtypes taking part in laminin and collagen binding(1). Integrins play an important role in TGF signaling and are thus relevant in fibrosis. In this regard, integrins regulate the activation of TGF-β1(1).
Furthermore, integrin-α1β1 is required for the dephosphorilation of TGF-β receptors and mice lacking this integrin, suffered of severe fibrosis in an unilateral uretral obstruction model of kidney fibrosis(20).
The non-canonical pathways have also been reported to be involved in the development of fibrosis(8, 99, 122, 141). For example, Smad1-dependent signaling plays a role in hepatic fibrogenesis(129) and in a model of dermal fibrosis, where TGF-dependent up-regulation of collagen and CTGF were dependent on Smad1 and ERK 1/2(90). ERK signaling has also been linked to fibrotic responses in other models of dermal fibrosis, where the pharmacological anti-fibrotic effects were shown to be ERK- rather than Smad-dependent(7). Transforming growth factor beta-activated kinase 1 (TAK1) is involved in pro-fibrogenic responses(108) and p38 kinase was proposed to play a role in the development of kidney fibrosis(8), whereas both canonical and non-canonical TGF-β pathways have been shown to be required for PAI-1 expression(99). Other studies have shown the participation of members of the Wnt pathway in TGF signaling(141). Therefore TGFβ is the key regulator of
fibrosis which mainly acts through regulation of Smad-dependent gene transcription, although other Smad-independent mechanisms have also emerged to be involved in TGF-dependent fibrotic responses.

**Oxygen in wound healing and fibrosis**

Molecular Oxygen (O$_2$) is the final electron acceptor in the electron transport chain and is thus the key substrate for oxidative metabolism. A sufficient level of Oxygen delivery is essential to provide the metabolic energy necessary for effective tissue repair. The importance of Oxygen is related to its requirement in almost every step of this metabolically demanding process(15, 103, 116). Examples of reparative processes that require significant amounts of Oxygen are outlined below.

Firstly, during the early inflammatory phase of wound healing, neutrophils are recruited to accomplish the task of cleaning the wound from pathogens and other injurious agents. For this, neutrophils generate significant amounts of reactive Oxygen species (ROS) from Oxygen, which act as bactericidal agents. ROS generation is a significant source of Oxygen consumption during the inflammatory phase(103, 116). Secondly, during wound healing, fibroblasts are required to produce large amounts of collagen to support the healing process. Collagens are unstable at 37°C. The stabilization of collagen polypeptides at body temperature therefore requires Oxygen-dependent hydroxylation on proline residues present in collagen subunits(81). This process is driven by collagen prolyl hydroxylases (C-PHDs) and likely requires the consumption of significant amounts of Oxygen. Thirdly, in injured tissues, cells need to be repaired and or replaced. The increased proliferation associated with the proliferative phase of the healing response requires
an increase in metabolic and proliferative activity for cells. Thus, Oxygen availability is critical for successful tissue replacement.

All the above-mentioned processes require an increase in Oxygen consumption in tissues undergoing a healing response. Furthermore, the Oxygen supply in chronically inflamed tissues is often compromised by inflammation-associated vasculopathy and vascular dysfunction(24, 117). Therefore it is not surprising that in chronic inflammatory disorders such as IBD, significant degrees of tissue hypoxia occur in inflamed regions(79, 82, 117, 118). It is now clear that a chronically inflamed / fibrotic environment is often co-incidentally hypoxic(15, 103). Further evidence of the importance of hypoxia in fibrotic environments comes from the observation that other hypoxia-induced proteins are often present in fibrotic disease. For example, the hypoxia induced mitogenic factor found in inflammatory zone 1 (FIZZ1), has also been associated with fibrosis. This protein, which has mitogenic, angiogenic and vascular remodeling roles, is up-regulated by chronic hypoxia in vivo. In addition, FIZZ1 is highly expressed in bleomycin induced lung fibrosis models and is been shown to induce the expression of fibrotic markers(62). Studies using FIZZ1 knockout mice showed attenuated susceptibility to bleomycin-induced lung fibrosis, thus providing evidence of the interplay between hypoxia and fibrosis(62).

Because of the central importance of Oxygen for continued metabolic activity, survival and function, it is not surprising that over the course of evolution, eukaryotic cells have evolved an adaptive mechanism to help deal with hypoxic stress. When cells are in an Oxygen rich environment, the majority of the Oxygen is consumed by mitochondria in the generation of ATP(117, 140). In this state, the remainder of non-mitochondrial Oxygen is available for other cellular processes such as reparative and immune responses(116).
Given the importance of Oxygen for cell survival, the existence of a mechanism of adaptation is required that allows cells to sense and respond to hypoxia(24, 117, 118). This response is mediated by a family of transcription factors known as hypoxia inducible factors (HIFs) which have been recently reviewed elsewhere (24, 105, 117, 118). The activation of HIF results in a transcriptional response which directs an adaptive response to hypoxia through the promotion of processes including angiogenesis, erythropoiesis, vasodilation and metabolic reprogramming.

HIFs are heterodimeric proteins formed by binding of HIF-α and HIF-β subunits. While only one HIF-β isoform is known, three different HIF-α isoforms (HIF-1α, HIF-2α and HIF-3α) have been described. HIF-1 and HIF-2 are known to form active transcription complexes which regulate discreet yet overlapping gene cohorts(38, 61). HIF-3 has been reported to act as a repressor through reducing the availability of HIF-β subunits(61).

The Oxygen sensitivity of the HIF pathway is conferred by a family of dioxygenases termed the HIF prolyl hydroxylases (PHDs). PHDs catalyze a hydroxylation reaction requiring Oxygen and 2-Oxoglutarate as substrates. Figure 2 outlines key aspects of the HIF pathway and its Oxygen-dependent regulation by PHD enzymes. In a normoxic environment, constitutively expressed HIF-α subunits are hydroxylated on two prolyl residues within its Oxygen-dependent degradation domain, which allows its recognition by the von Hippel Lindau (VHL)-initiated E3 ubiquitin ligase complex which targets HIFα subunits to ubiquitin-mediated proteosomal degradation(68). Furthermore, hydroxylation of an asparaginase residue within its trans-activation domain by the asparaginyl hydroxylase factor inhibiting HIF (FIH), blocks HIF binding to CBP/p300 and other cofactors, preventing the formation of active transcription complexes(73, 83). In hypoxia, the lack of Oxygen prevents HIF-α hydroxylation,
allowing its translocation to the nucleus, where it binds to HIF-β and forms an active transcription factor(16). As well as driving an adaptive response to hypoxia, it has recently become clear that HIF also plays an important role in the regulation of inflammation(102).

Crosstalk between inflammatory, fibrotic and hypoxic pathways.

In chronically inflamed and fibrotic tissues, perfusion is compromised and Oxygen consumption is elevated(98, 118). Therefore hypoxia accompanies inflammation and fibrosis(79, 102) in chronic inflammatory diseases such as inflammatory bowel disease(24, 95, 114), chronic kidney disease(56, 139), chronic liver disease(76) and pulmonary idiopathic fibrosis(124). As outlined above, transcriptional responses which promote inflammation, hypoxia and fibrosis are primarily driven by NF-κB, HIF and Smads respectively. However, rather than acting independently, an extensive degree of crosstalk between these three pathways likely exists in the microenvironment of a chronically inflamed and fibrotic tissue (Figure 3). Examples of such crosstalk are given below:

- **Crosstalk between HIF and NF-κB**: given that chronically inflamed tissues become hypoxic, HIF and NF-κB are often activated at the same time(13, 101, 102). HIF-1α has been shown to be highly expressed in macrophages, which are key cells in both acute and chronic inflammation(9, 79). Indeed, HIF-1α activation has been found to be critical for the infiltration and activation of PMN cells and macrophages, as demonstrated using conditional knockout mice for HIF-1α in immune cells, thus playing a central role in inflammation(22). LPS, a classical proinflammatory stimuli, induces HIF-1α mRNA expression even under normoxic conditions(9). This positive regulation of HIF by LPS is likely to be mediated through NF-κB activation, as NF-κB has been found to bind to, and positively regulate the HIF-1α promoter(39, 88, 102,
Furthermore, both HIF and NF-κB are necessary for the regulation of COX-2 and IL-1β, important mediators implicated in inflammation(13, 16). This evidence suggests that HIF-NF-κB crosstalk is crucial in inflammatory responses.

A number of studies have highlighted the role of the HIF prolyl hydroxylase enzymes (PHD-1, 2 and 3) in in vivo models of inflammatory disease. Pharmacological inhibition of PHD enzymes is protective in multiple animal models of colitis(25, 52, 96) and can also attenuate endotoxic shock(48). The protective effects of PHD inhibition in murine DSS induced colitis are primarily dependent upon PHD-1 inhibition as PHD-1 knock-out mice are selectively protected against inflammation(115). Furthermore, several authors have described direct effects of PHDs on the regulation of NF-κB pathway(23, 36, 101, 145). Hypoxia activates NF-κB in vivo(36) while PHD-1 regulates the activation of NF-κB, possibly by hydroxylating IKKβ(23). More recently, it has been shown that PHD-1 and FIH combinatorially regulate IL-1β induced activation of NF-κB(101). Therefore, a significant body of evidence now supports an intimate degree of crosstalk and interdependence between HIF and NF-κB in chronically inflamed environments.

- **Interplay between HIF and TGF-β pathways**: Our understanding of crosstalk between the HIF and TGF / Smad pathways is less well developed, however some evidence for this exists. Firstly, HIF-1α is required for macrophage infiltration and activation during inflammatory response(22). As macrophages are primary producers of TGF-β, this indirectly correlates hypoxia and HIF to the production of TGF. Other work suggests that hypoxia induces production of TGF-β1 by macrophages(98) and increases the transcription of TGF-β1 in dermal fibroblasts(42).

The role of HIF in TGF-β driven fibrosis has been studied in different systems, however its role remains controversial. Studies using models of CKD point to a pro-fibrogenic action of HIF-1α, with its ablation attenuating the development of
fibrosis. Smad-3, a key mediator of TGF-β responses, was reported to be upregulated by hypoxia, promoting activation of the TGF-β pathway. Furthermore, in CLD HIF-1α-deficient mice, which developed a milder degree of pulmonary fibrosis compared to wild type mice, following bile duct ligation. On the other hand, a number of studies have highlighted a beneficial role for HIF activation in CKD. Kapitsinou et al shown that HIF-2α was protective against fibrosis secondary to ischemic kidney injury. Other studies show that inhibition of PHD enzymes using L-mimosine or DMOG ameliorated fibrosis by reducing important fibrotic markers such as α-SMA and collagen-III and collagen IV in a rat model of CKD. Using the same CKD model, the protective effects of PHD inhibition by L-mimosine were reported to be caused by its up-regulation of miR-29c, a microRNA which targets fibrotic genes such as Collagen-II and thrombospondin. The reduction of collagen deposition caused by PHD inhibition may also be explained to some extent by the fact that collagen fibers need to be hydroxylated in specific proline residues in order to be stable at physiologic temperature, therefore, collagen hydroxylase inhibition, (which can be achieved with pan-hydroxylase inhibitors) may reduce collagen stability and accumulation.

The effects of HIF in other fibrotic diseases such as IPF are less clear. HIF-1 was found to be upregulated in alveolar epithelial cells from mouse models and patients of IPF. A recent study showed hypoxia to induce lung fibroblast proliferation by increasing miR-210 microRNA through a mechanism dependent on HIF-2α. Further investigations are required to reveal the role of HIF and hypoxia-sensitive targets in fibrotic diseases and their potential for therapeutic manipulation.

On the other hand, TGF-β1 might induce HIF-1α stabilization through down-regulation of PHD-2 gene expression reinforcing important interactions between HIF and TGF pathways.
• Interplay between NF-κB and TGF-β pathways: The TGF-β family play an important role in the inhibition of inflammatory responses (138). This is likely associated with a switch from inflammatory to proliferative and resolving phases. As outlined above, the NF-κB pathway is the master regulator of inflammatory responses, and is also implicated in apoptosis (17).

Several studies have described how the regulation of apoptosis by NF-κB can be regulated through TGF-dependent signaling. For example, the treatment of hepatocytes with TGF-β1 mediates repression of NF-κB and subsequently induces apoptosis (3, 17). In contrast, other studies suggested that TGF could induce NF-κB-dependent anti-apoptotic effects (31, 41). TAK1 and Smad7 have also been implicated in crosstalk between NF-κB and TGF-β in epithelial carcinomas (40).

Current therapies in fibrotic disease:

Current therapeutic options for fibrosis are extremely limited and at best slow progression of disease but cannot reverse or arrest it. To date therapeutic approaches for interstitial pulmonary fibrosis (IPF) focus on treating underlying inflammation (18, 63, 104, 130, 131). The failure of anti-inflammatory interventions may support an alternative approach of promoting resolution (106). Ongoing research is directed towards finding therapeutics which target aberrant wound healing in response to chronic injury. Recent progress in therapies for IPF have demonstrated efficacy of two small molecule drugs: Pirfenidone and the kinase inhibitor nintedanib. In mild to moderate disease these drugs cut the rate of decline in respiratory capacity by 50% over 52 weeks relative to placebo (57, 94). The precise mechanism of action of perfinidone is unclear, however it inhibits MAP kinases, reduces TGF-β levels and inhibits responses to pro fibrotic cytokines such as PDGF (63). Nintedanib is an intracellular inhibitor of multiple tyrosine kinases including VEGF, PDGF and
FGF receptors. The tyrosine kinase inhibitor imatinib/Gleevec blocks non canonical TGF-β1 signalling in experimental models of fibrosis, however it failed to achieve improvements when tested in a randomized controlled trial in IPF(63). In IBD-associated fibrosis, tissue scarring can lead to intestinal malfunction and obstruction, usually resulting in the need for surgical resection of the affected tissue(55, 114). Since IBD is characterized by the periods of relapse and remission, most therapies are focused in achieving and maintaining the remission state through immune suppression which ultimately might prevent fibrotic progression. In this context glucocorticoids such as prednisolone, alone or in combination with aminosalicilates such as mesalazine, are the common treatment(24, 92, 100). Immunosupresors such as Azathioprine or Methotrexate have been used in an attempt to control the autoimmune component of this disease and as an alternative to corticosteroids in maintaining remission(24, 100). However, both treatments have limited effectiveness and high risk of adverse effects. Alternative maintenance therapy can be achieved using anti-TNF-α antibodies(24, 100), which can be administered alone or in combination with Azathioprine(97, 100). However, as outlined above there is a dearth of drugs available which specifically target fibrosis.

Current research is focused in developing improved therapeutics for IBD. In this regards, several studies have described beneficial effects of PHD inhibitors such as dimethyloxalylglycine (DMOG) against inflammation in animal models of IBD(25, 96, 115). Other fibrotic diseases such as CKD or post myocardial infarct fibrosis also lack effective anti-fibrotic therapeutics. Current CKD therapies are focused in slowing down disease progression, for example through inhibition of the renin-angiotensin-aldosterone system (RAAS)(29, 109). Other approaches, as the use of endothelin agonists had given very limited efficacy in clinical trials due to adverse
cardiovascular events(29), while anti TGF-β therapy or anti CTGF therapy are also being investigated(29). Recent data from a human trial suggests that pirfenidone may be helpful in diabetic nephropathy(107). Intriguingly, lipoxins, pro-resolving lipid mediators have shown fibrosuppressant activities in experimental models of kidney and lung fibrosis (11, 66). The growing appreciation of oxidative stress as a driver of fibrosis has lead to proposing AIMs (anti-oxidant immune modulators) as a useful therapeutic intervention in renal fibrosis as exemplified by bardoxolone methyl, an inducer of Nrf-2 notwithstanding its recent failure in a clinical trial(51, 74, 142). The exquisite balance in activities between the fibrosuppressant BMP agonists and profibrotic antagonists such as grelin and USAG-1 suggests that amplifying the BMP actions(135, 142) or suppressing antagonists may be beneficial in experimental models of renal fibrosis(26, 126, 127). Efforts to develop peptide agonists of the BMP system as fibrosuppressants for kidney disease have generated highly controversial data(112, 113, 128). In the case of the heart, the development of a fibrotic healing after myocardial infarct leads to the loss of function of the infarct area and can lead to heart failure. Recent preclinical research identified histone de-acetylase inhibition as a potential therapy to revert cardiac fibrosis(84).

Conclusions

Inflammation-driven fibrosis is a common and severe clinical complication in several important chronic inflammatory diseases resulting in high costs both in human and economic terms. However, despite this, there are currently very limited effective pharmacologic agents which target the development of fibrosis. Most efforts to date have been focused on targeting underlying inflammation. Therefore, future research should be focused on achieving a better understanding of the main molecular mechanisms involved in fibrosis in order to characterize new potential targets, which
ultimately would allow us to search for specific anti-fibrotic therapies. Pre-clinical models indicate that targeting hypoxia-sensitive pathways in fibrosis is of potential therapeutic benefit, however clinical studies will be required if this can be translated to patient benefit.
References:


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FIGURE LEGENDS:

**Figure 1.** Comparison between physiologic wound healing and inflammation-driven fibrosis.

Physiologic wound healing (left hand side) involves a controlled and resolving inflammatory response followed by proliferative and maturation phases leading to successful healing of the injured tissue. This is associated with a transient period of tissue hypoxia. In cases of inflammation-driven fibrosis (right hand side), a persistent non-resolving inflammatory signal drives an overactive inflammatory response leading to an accumulation of macrophages and prolonged stimulation of fibroblasts, with a resultant excessive production of ECM, leading to fibrosis and scar formation. Unlike the transient hypoxia associated with physiologic wound healing, fibrotic tissue which remains inflamed produces a chronically hypoxic microenvironment.

**Figure 2.** The HIF pathway: Under normoxic conditions (left hand panel), hydroxylation of key proline residues within the HIF Oxygen-dependent degradation domain (ODD) by HIF hydroxylases directs HIF-α subunits to proteosomal degradation. HIF is further repressed by Oxygen-dependent hydroxylation of an asparagine residue in the NAD which prevents its binding to transcriptional cofactors. Under conditions of hypoxia (right hand panel), reversal of these hydroxylation reactions leads to the stabilization and transactivation of HIF leading to the expression of a large cohort of genes which promote cell adaptation to hypoxia.

**Figure 3.** Critical pathways in chronic inflammatory disease. In chronically inflamed tissues, inflammation, fibrosis and hypoxia are co-incidental events. This results in generation of cytokines and TGF-β as well as inhibition of HIF hydroxylases respectively. This in turn results in the co-incidental activation of NF-kB, Smads and HIF. Understanding the nature of crosstalk between these three aspects of inflammation-driven inflammation will be key to the development of new therapeutic targets for the control and possibly reversal of inflammation-driven fibrosis.
Figure 1
Figure 2

Normoxia

Hypoxia

O₂

O₂

O₂

O₂

O₂

PHDs

HIF-α

FIH

HIF-α

OH

HIF-α

OH

HIF-β

p300

HIF-β

p300

Proteosome

Proteosome
Chronic inflammatory disease

Inflammation

Cytokines

NF-κB

Fibrosis

Smads

TGFβ

Hypoxia

HIF

Hydroxylase

Figure 3