Hypoxia-sensitive pathways in inflammation-driven fibrosis

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Manresa MC, Godson C, Taylor CT. Hypoxia-sensitive pathways in inflammation-driven fibrosis. Am J Physiol Regul Integr Comp Physiol 307: R1369–R1380, 2014. First published October 8, 2014; doi:10.1152/ajpregu.00349.2014.—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 coincidental 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.

fibrosis; hypoxia; inflammation; NF-κB; Smad

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. Physiological inflammation is, thus, the first phase of wound healing (5, 46, 47, 89, 93). The infiltrating inflammatory cells and the substances that 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 toward 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 matrix (ECM) components to replace and renew damaged tissue. The newly formed ECM provides the physical scaffold to support subsequent wound closure, remodeling, and repair.

Despite its beneficial role in tissue repair, if uncontrolled or unresolved because of 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 nonhealing wounds with physiological 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 to a chronic, nonresolving process.

In the context of tissue repair following injury, the degree and duration of the inflammatory step are 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 fetal skin, wound healing occurs in a scarless manner. This is, in part, due to the different degrees of inflammation associated with adult or fetal skin wound healing, which is milder in fetal 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 present a threat to the maintenance of tissue structure and function.

Therefore, normal physiological 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, nonresolving 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 physiological 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 interrelated processes.

Physiological 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 physiological function is, therefore, central to directing a successful return to homeostasis in a wounded tissue. This healing response is largely preserved 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 physiological healing is characterized by a hemostatic response directed toward vascular isolation of the wounded area 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 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 that 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 (Fig. 1) (24, 117, 118).

Proliferative phase. Following an effective inflammatory phase, during which injured tissue is sterilized by an effective immune response, the wound-healing process enters the proliferative phase during which the laying down of a new ECM formed from collagen 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 TGF-β, 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 result in a return to tissue normoxia (Fig. 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 preformed 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 physiological conditions, tissues have the ability to respond to injury in a manner that promotes recovery of tissue structure and function and a return to physiological homeostasis (Fig. 1).

Chronic Inflammation: a Nonhealing Wound

The transition from an acute, physiological immune response (evolved to promote the healing of injured tissue) to a state of chronic inflammation reflects the transformation from a physiological to a pathological state. Chronic inflammation leads to a condition resembling a nonhealing wound and is associated with a diverse range of pathologies, including the
The success of the physiological 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 proinflammatory mediator production (5, 44).

The transformation from physiological, healing-associated inflammation to chronic pathologic inflammation occurs when the positive regulators of the inflammatory response outweigh the proresolving 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 nonresolving 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-γ, which further activates proinflammatory macrophages and signals those macrophages to survive (44, 77), thus prolonging the inflammatory process.

While excessive proinflammatory 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 proresolving mediators expressed in different cell types (60, 64). Among these specialized lipid mediators, 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 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 later.

In this context, current evidence points to lipoxin A4 (LXA4) as a negative regulator of TGF-β1, a 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 through 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 (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 are 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 (IPF) (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 leads 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 blocked in the proliferative stage due to nonresolving chronic inflammation. However, it should be noted that fibrosis can also occur as a consequence of defective matrix breakdown during the remodeling 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 nonresolving 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. The NF-κB pathway is a family of transcription factors comprising five subunits that form distinct homodimers or heterodimers. 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 that 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, 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 signaling pathway, the activation of which can be driven by the engagement of multiple receptor subtypes (14, 53). Receptors linked to canonical NF-κB signaling include Toll-like receptors (TLRs), interleukin receptor 1 (IL-R1), tumor necrosis factor receptor (TNFR), and antigen receptors. Typical stimuli for these receptors include LPS and other bacterial components and major inflammatory cytokines, such as TNF-α and 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 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).

TGF-β pathway. The TGF-β family plays a pivotal role in regulating fibrosis. This family of secreted ligands comprises TGFβ 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 bioactivities of TGFβ1 are modulated by additional members of the family, including the BMP agonists and antagonists, whose activities may exacerbate or attenuate TGF-β1-elicted 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 1980s, and its role in collagen production was first reported in 1986 (54). The three TGF-β isoforms are designated TGF-β1, TGF-β2, and TGF-β3. Among these, TGF-β1 is the most prevalent (4, 8, 65) and is the
main isomorh 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 homolog and the related Caenorhabditis elegans Sma. Smad proteins are divided into subtypes depending on their functions: receptor-activated Smads (Smads 1, 2, 3, 5, and 8), common mediator Smads (Smad4), and inhibitory Smads (Smads 6 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 Smad4 and the Smad2, 4 and Smad3, 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 signaling by binding to the TGF-βRI and blocking Smad2, 3 activation, or by promoting Smad degradation (8, 59, 65, 67, 78).

TGF-β1 signals through both canonical (i.e., Smad) and noncanonical pathways. The noncanonical pathway depends on the ability of phosphorylated TGF-βRI and RII to activate various 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 CKD (59) or postmyocardial 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), and 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 (37), or ischemia-reperfusion myocardial infarction (28). In vitro, Smad3 overexpression in hepatic stellate cells caused an increase in α-SMA and collagen-1 (123) and enhanced α-SMA in human fetal 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 signaling 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 that 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 dephosphorylation of TGF-β receptors, and mice lacking this integrin suffered from severe fibrosis in an unilateral urethral obstruction model of kidney fibrosis (20).

The noncanonical 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 upregulation of collagen and CTGF was dependent on Smad1 and ERK 1/2 (90). ERK signaling has also been linked to fibrotic responses in other models of dermal fibrosis, in which the pharmacological antifibrotic effects were shown to be ERK- rather than Smad-dependent (7). Transforming growth factor β-activated kinase 1 (TAK1) is involved in profibrogenic responses (108), and p38 kinase has been proposed to play a role in the development of kidney fibrosis (8), whereas both canonical and noncanonical 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 (O2) 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.

First, 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). Second, 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. Third, 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 of 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 coincidentally 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 upregulated by chronic hypoxia in vivo. In addition, FIZZ1 is highly expressed in bleomycin-induced lung fibrosis models and has 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 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 nonmitochondrial 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 HIFs 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. Whereas 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 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-initiated E3 ubiquitin ligase complex, which targets HIFα subunits to ubiquitin-mediated proteosomal degradation (68). Furthermore, hydroxylation of an asparagine residue within its transactivation 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 that promote inflammation, hypoxia, and fibrosis are primarily driven by NF-κB, HIF, and TGF-β-dependent pathways, 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 (Fig. 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 is often critical for the infiltration and activation of neutrophils 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 stimulus, 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, 119). Furthermore, both HIF and NF-κB are necessary for the regulation of COX-2 and IL-1β, important mediators 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 PHD enzymes (PHD-1, PHD-2, and PHD-3) 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 knockout mice are selectively protected against inflammation (115). Furthermore, several authors have described direct effects of PHDs on the regulation of the 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 hydroxylation of 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. First, HIF-1α is required for macrophage infiltration and activation during the 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 profibrogenic action of HIF-1α, with its ablation attenuating the development of fibrosis (50). Smad3, a key mediator of TGF-β responses, was reported to be upregulated by hypoxia, promoting activation of the TGF-β pathway (98). Furthermore, in CLD HIF-1α-deficient mice developed a milder degree of pulmonary fibrosis compared with wild-type mice, following bile duct ligation (76). On the other hand, a number of studies have highlighted a beneficial role for HIF activation in CKD. Kapitsinou et al. (56) showed that HIF-2α was protective against fibrosis secondary to ischemic kidney injury. Other studies show that inhibition of PHD enzymes using l-mimosine or dimethyloxalylglycine (DMOG) ameliorated fibrosis by reducing important fibrotic markers, such as α-SMA and collagen III (139) and collagen IV (111) in a rat model of CKD. Using the same CKD model, Fang et al. (32) reported that the protective effects of PHD inhibition by l-mimosine were caused by its upregulation of miR-29c, a microRNA that targets fibrotic genes, such as collagen II and thrombospondin (32). 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 on specific proline residues in order to be stable at physiological temperatures (81); 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 (98). A recent study showed that hypoxia induced lung fibroblast proliferation by increasing miR-210 microRNA through a mechanism dependent on HIF-2α (10). Further investigations are required to understand 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 downregulation of PHD-2 gene expression (42), reinforcing important interactions between HIF and TGF pathways.

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

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Interplay between NF-κB and TGF-β pathways. The TGF-β family plays 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 antiapoptotic 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 the progress of fibrotic disease but cannot reverse or arrest it. To date, therapeutic approaches for IPF focus on treating underlying inflammation (18, 63, 104, 130, 131). The failure of anti-inflammatory interventions may support an alternative approach to promoting resolution (106). Ongoing research is directed toward finding therapeutic drugs that 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 cases of mild to moderate disease, these drugs cut the rate of decline in respiratory capacity by 50% over 52 wk relative to placebo (57, 94). The precise mechanism of action of pirfenidone is unclear; however, it inhibits MAP kinases, reduces TGF-β levels, and inhibits responses to profibrotic 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 noncanonical TGF-β1 signaling 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 periods of relapse and remission, most therapies are focused on 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 aminosalicylates such as mesalazine, are the common treatment (24, 92, 100). Immunosuppressors 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 a 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 that specifically target fibrosis.

Current research is focused on developing improved therapeutics for IBD. In this regard, several studies have described beneficial effects of PHD inhibitors, such as DMOG, against inflammation in animal models of IBD (25, 96, 115). Other fibrotic diseases, such as CKD or postmyocardial infarct fibrosis, also lack effective antifibrotic therapeutics. Current CKD therapies are focused in slowing down disease progression, for example, through inhibition of the renin-angiotensin-aldosterone system (29, 109). Other approaches, such as the use of endothelin agonists has proven to give 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 suggest that pirfenidone may be helpful in diabetic nephropathy (107). Intriguingly, lipoxins, proresolving 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 led to proposals that anti-oxidant immune modulators be used 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 pro-fibrotic antagonists, such as gremlin 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 deacetylase 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 in human and economic terms. However, despite this, there are currently very limited effective pharmacologic agents that 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 to characterize new potential targets, which ultimately would allow us to search for specific antifibrotic therapies. Preclinical models indicate that targeting hypoxia-sensitive pathways in fibrosis is of potential therapeutic benefit; however, clinical studies will be required to see whether this can be translated to therapeutic benefit to patients.

GRANTS

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