Neuroinflammatory Mechanisms of Connective Tissue Fibrosis: Targeting Neurogenic and Mast Cell Contributions

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Significance: The pathogenesis of fibrogenic wound and connective tissue healing is complex and incompletely understood. Common observations across a vast array of human and animal models of fibroproliferative conditions suggest neuroinflammatory mechanisms are important upstream fibrogenic events.

Recent Advances: As detailed in this review, mast cell hyperplasia is a common observation in fibrotic tissue. Recent investigations in human and preclinical models of hypertrophic wound healing and post-traumatic joint fibrosis provides evidence that fibrogenesis is governed by a maladaptive neuropeptide-mast cell-myofibroblast signaling pathway.

Critical Issues: The blockade and manipulation of these factors is providing promising evidence that if timed correctly, the fibrogenic process can be appropriately regulated. Clinically, abnormal fibrogenic healing responses are not ubiquitous to all patients and the identification of those at-risk remains an area of priority.

Future Directions: Ultimately, an integrated appreciation of the common pathobiology shared by many fibrogenic connective tissue conditions may provide a scientific framework to facilitate the development of novel antifibrotic prevention and treatment strategies.

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SCOPE AND SIGNIFICANCE

CONNECTIVE TISSUE FIBROSIS is a principal pathogenic process involved in a vast array of human conditions. Unfortunately, many of these conditions are highly recalcitrant to system and local treatment strategies, highlighting the need for an improved scientific and biological understanding of fibrogenesis. Integrating information obtained from clinical studies, animal models, and basic science investigations has identified numerous profibrotic molecular pathways common to many fibrogenic human conditions. Tar-

geted strategies derived from these studies may have profound therapeutic consequences applicable to a large spectrum of similar diseases.

TRANSLATIONAL RELEVANCE

Hypertrophic wound healing and post-traumatic joint fibrosis are examples of two acquired human conditions characterized by aberrant fibrogenic healing responses. Recent evidence from human and animal studies suggests a neuroinflammatory axis mediated by neuropeptides and mast cell signaling

functions as an important upstream fibrogenic stimulus in these conditions. Other models of fibrosis also support these observations. Using validated preclinical models antifibrotic therapies targeting this neuroinflammatory axis are yielding promising results.

CLINICAL RELEVANCE

Novel antifibrotic therapies are under development for human use. Importantly, these therapies may be most efficacious in preventing fibrosis and less effective in those with established disease. This highlights the need to further identify at-risk populations.

OVERVIEW

The connective tissue healing response is a complex myriad of cellular and biochemical events essential to virtually every human structure and organ derived from mesoderm. Connective tissue repair progresses via a series of confluent, yet biologically distinct phases of hemostasis, inflammation, cellular proliferation, matrix synthesis, and tissue remodeling (reviewed in Diegelmann and Evans¹ and Reinke and Sorg). Under normal conditions, equilibrium is established between matrix synthesis, and tissue remodeling, which ultimately enables the injured tissue or organ to heal and regain near normal function. Unfortunately in many fibroproliferative conditions, this equilibrium is lost or never established, and the abnormal connective tissue healing response becomes maladaptive. Strictures, adhesions, keloids, hypertrophic scars, and post-traumatic joint contractures are all common examples of abnormal, excessive, or unwanted connective tissue healing patterns observed after traumatic or surgical insults. Persistent inflammation, tissue hypoxia, and abnormal mechanical forces associated with a variety of acute and chronic conditions are also common atraumatic causes of connective tissue fibrosis typified by renal, pulmonary, and cardiac fibrosis. Despite a detailed understanding of the biological events essential for physiologic connective tissue repair, the mechanisms by which the normal regulatory controls of these processes are circumvented in fibroproliferative conditions remain obscure.

DISCUSSION

Connective tissue fibrosis

Fibrogenic healing pathways are a manifestation of a dynamic interaction between numerous cell types, growth factors, cytokines, mechanical

stimuli, and constituents of the extracellular matrix (ECM) in response to various reactive or reparative stimuli. The terminal consequence of these interactions is a pattern of excessive and disorganized collagen deposition, resulting in permanent tissue and organ dysfunction. Decades of research have consistently demonstrated three central histological components of tissue fibrogenesis: myofibroblast hyperplasia, upregulated fibrogenic growth factors/cytokines, and dysregulated collagen homeostasis.

Myofibroblasts and fibrosis

The myofibroblast is regarded as the principal effector cell in connective tissue fibrosis, responsible for collagen deposition, growth factor liberation, and mechanical wound contraction.^{3,4} Myofibroblast hyperplasia is a common observation in virtually all fibroproliferative conditions such as Dupuytren's contracture of the hand, hypertrophic wound healing, burn scar hypertrophy, post-traumatic joint contractures, scleroderma, idiopathic frozen shoulder, idiopathic pulmonary fibrosis, hepatic, myocardial, and renal fibrosis.³⁻¹²

Myofibroblasts are specialized mesenchymal-derived cells, characterized by the unique expression of the contractile protein, alpha-smooth muscle actin (α -SMA).³ Typically regarded as a differentiated cell type derived from the fibroblast lineage,^{13,14} myofibroblastic differentiation has also been observed from a variety of non-fibroblastic precursors such as hepatic satellite cells,¹⁵ bone marrow-derived fibrocytes,¹⁶ and epithelial cells via an epithelial-mesenchymal transition.¹⁷

The activated myofibroblast is known to remodel the ECM through a combination of three principal mechanisms: mechanical contraction of the ECM via adherent α -SMA populated stress fibers; the synthesis and deposition of additional collagen fibers; and liberation of pro-fibrotic, pro-inflammatory, and pro-angiogenic factors.^{3,18,19} In the context of normal physiologic wound healing, fibroblasts migrate into the wound bed, myofibroblast differentiation and proliferation ensues, which facilitates wound contraction and once epithelialization of the wound is complete, granulation myofibroblasts disappear through a regulated process of apoptosis.^{3,4,20} In fibroproliferative disorders, it remains unclear whether the normal pathways of myofibroblast apoptosis are impeded, or the signals responsible for myofibroblast activation and proliferation are sustained, or both.

Pro-fibrotic growth factors and cytokines

Numerous soluble factors such as transforming growth factor-beta1 (TGF- β 1), connective tissue

growth factor, platelet-derived growth factor (PDGF), angiotensin II, vascular endothelial growth factor, and Th2-type cytokines (IL-4, IL-5, IL-13, and IL-21) are recognized pro-fibrotic mediators.^{21,22} Of these, TGF- β 1 is certainly the most understood and investigated pro-fibrotic growth factor for numerous reasons. TGF- β 1 is an essential regulator of tissue homeostasis, with pro-inflammatory and anabolic growth properties. It is synthesized and liberated by a diverse array of cell types, and also resides within connective tissue in a latent form, activated by mechanical or enzymatic disruption of the ECM.²³ Further, TGF- β 1 is a potent fibroblast chemoattractant and mitogen, is a key signal for the induction of the myofibroblast phenotype, stimulates matrix molecule synthesis, and inhibits myofibroblast apoptosis.^{4,13,24–27} Elevated levels of TGF- β 1 and TGF- β 1 receptors are routinely observed in various human and animal models of hypertrophic scar formation, burn scar hypertrophy, post-radiation skin fibrosis, and post-traumatic joint fibrosis.^{28–37} Similarly, in cardiac and renal fibrosis, upregulation of TGF- β 1 and the renin-angiotensin system has been shown to cooperatively mediate perivascular fibrosis and organ dysfunction (reviewed in Rosenkranz³⁸ and Lan³⁹).

Alternatively, anti-TGF- β 1 strategies (monoclonal antibodies, soluble TGF- β receptor complexes, antisense TGF- β 1 oligonucleotides, small molecule inhibitors, and interferon therapy [IFN- α and IFN- γ]) have also been shown to decrease collagen hyperplasia, inflammatory cell infiltration, wound contraction, neovascularization, and other manifestations of fibrosis across in a variety of preclinical models of hypertrophic wound healing;^{40,41} burn wound hypertrophy;^{36,42} scleroderma;⁴³ and cardiac, pulmonary, and renal fibrosis.^{44–46} Collectively, these data clearly demonstrate an integral role of TGF- β 1 in connective tissue fibrosis, although unfortunately, these promising preclinical results have not been recapitulated in recent human clinical trials,^{47,48} although several trials remain ongoing. Additionally, given the exceptionally broad range of fundamental biological activities ascribed to TGF- β 1 signaling such as wound healing,⁴⁹ fracture repair,⁵⁰ and immunity,⁵¹ upstream mediators of excessive TGF- β 1 synthesis and signaling may serve as more promising post-traumatic antifibrotic therapeutic targets.

ECM organization and remodeling

Irrespective of upstream cellular and pro-fibrotic signaling, connective tissue fibrosis is a direct consequence of excessive and disorganized collagen deposition. Collagen is the principal

organic constituent of the ECM; type I collagen is the most ubiquitous isoform in connective tissue where other collagen types are present in variable amounts in a tissue-dependent distribution. During physiologic connective tissue healing responses, early inflammatory phase healing is characterized by the deposition of collagen types I and III and as healing matures and remodeling proceeds, type III collagen is largely replaced by a more organized and mechanically superior weave of collagen type I.² In hypertrophic wound healing and other fibroproliferative conditions, several abnormalities of collagen homeostasis are observed: there is a net increase in overall collagen content, collagen structure is disorganized, and there is a greater overall contribution of type III collagen.^{35,52–56} Many of the aforementioned upstream cellular and soluble pro-fibrotic messengers likely contribute to this collagen imbalance, however, equally important to this process are matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Collagen turnover is highly dependent on these proteolytic enzymes and in fibrotic disease, collagen hyperplasia is augmented by an anabolic MMP:TIMP ratio.^{54,57–59} Although beyond the scope of this review, exogenous manipulation of local and systemic MMP:TIMP ratios is an intensifying field of research with promising downstream applications in fibrotic disease and other human conditions.

Mast cell contributions to inflammatory disease and fibrosis

For many years now, mast cell hyperplasia has been a common observation within fibrotic tissue, often adherent to or in close apposition to myofibroblasts.^{6,10,60} Increased mast cell numbers have been documented in human and animal models of hypertrophic scar formation, post-traumatic joint fibrosis, burn fibrosis, intraperitoneal adhesions, scleroderma, esophageal strictures, and pulmonary/cardiac/renal fibrosis.^{61–66} However, the events responsible for pathologic mast cell recruitment and the contribution of these cells to the global fibrogenic process are primitively understood.

Mast cell biology. Mast cells originates within the bone marrow and circulate as a CD34 positive cluster cell, only to terminally differentiate after extravasation in peripheral tissues.^{60,67} This is distinctly different from basophils and eosinophils, which mature in marrow and rarely enter peripheral tissues unless appropriately stimulated.⁶⁸ In humans, virtually every organ is populated with a small population of mast cells. Mast cells are

particularly abundant within the mucosal and connective tissues of the skin, lung, and gut. The close proximity of these cells to small venules and capillaries enables the products of mast cell degranulation to have profound consequences on the dynamics of local microcirculation. Activated mast cells induce changes in vascular permeability and cause vasodilatation, but in addition to this, mast cells activate neighboring endothelial cells, leading to the expression of adhesion molecules and cytokines needed for leukocyte rolling, adhesion, activation, and transmigration.^{69,70} Consequently, the mast cell is recognized as a key member in the initiation and propagation of inflammatory responses. The identification of mast cells during the process of wound repair and in sites of matrix degradation supports the role of these cells connective tissue repair and remodeling.^{71–73}

Microscopically, mast cells are easily identified by the appearance of large heterogeneous granules, densely packed within the cell cytoplasm.^{74,75} Within these granules resides a plethora of vasoactive substances, proteases and inflammatory mediators. The most notable of these include histamine and the neutral proteases, tryptase, and chymase. In humans, mast cells are classified according to the presence or absence of chymase within their granules: tryptase- and chymase-positive mast cells (MC_{TC}) and tryptase positive, chymase-negative mast cells (MC_T). MC_{TC} mast cells are commonly localized to the skin, synovium, joint capsule, subcutaneous tissue, and the submucosal layers of the gastrointestinal (GI) tract.^{76–78} MC_T mast cells, on the other hand, populate mucosal surfaces of the GI tract and lung and is also associated with allergic and parasitic disease.^{74,79} Terminal differentiation of CD34-positive mast cell precursors depends on activation of the cell surface receptor (c-kit) via binding of its ligand, stem cell factor (SCF). In humans and rodents, SCF is a potent chemoattractant and mitogen for mast cells and is directly responsible for mast cell proliferation within peripheral tissues.^{80–82} The mechanisms by which mast cells terminally differentiate into MC_{TC} and MC_T phenotypes is still under investigation at present, but is likely governed by the cytokines present within the local environment.⁸³

Mast cell mediators. The complexity of mast cell physiology is exemplified by the vast array of vasoactive, immunogenic, and pro-inflammatory mediators synthesized and liberated by mast cell populations. These mediators can broadly be categorized into preformed and *de novo* synthesized compounds. Histamine accounts for roughly

10–15% of the dry granule weight and is potent mediator of allergic responses.⁸⁴ Its effects are mediated through histamine receptors (H1–H4) and include vasodilatation, bronchoconstriction, pain, and pruritis. Histamine also causes the release of substance P (SP) from type C unmyelinated nerve fibers. This potentiates histamine release, as SP is also a potent mast cell secretagogue.⁸⁵ In addition to histamine, tryptase, and chymase, are major constituents of mast cell granules, with tryptase being the most abundant mast cell synthetic product. Apart from sparse levels in basophils, tryptase is also mast cell specific.^{76,84} The natural *in vivo* substrate of tryptase remains unclear. Actions of chymase include conversion of angiotensin I into angiotensin II, inactivation of bradykinin and degradation of ECM components such as collagen type IV and laminin.^{86,87}

Mast cells are also rich sources of numerous cytokines, chemokines, and growth factors. Notables include IL-1, 3, 4, 5, 6, and 8, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor- α (TNF- α), and IFN- γ . TNF- α can be newly synthesized and is also stored in mast cell granules.⁸⁸ TGF- β appears to be constitutively expressed by mast cells and is also liberated into the local environment after stimulation with calcium ionophores, suggesting it may be associated with intracellular storage.⁸⁹ Other notable growth factors include PDGF and basic fibroblast growth factor (bFGF). Lipid-derived mediators of acute inflammation, such as platelet activating factor (PAF), prostaglandins, and leukotrienes are also rapidly synthesized by mast cells after cellular activation.

Mast cell activation. The traditional pathway of mast cell activation occurs via antigen binding to immunoglobulin E (IgE) antibodies bound to the high-affinity IgE receptor, Fc ϵ RI located on the mast cell membrane. Antigen binding induces IgE cross-linking that results in aggregation and internalization of the Fc ϵ RI receptor. This stimulates a rapid intracellular cascade of events that triggers secretory granule exocytosis, lipid metabolism (used to synthesize prostaglandins, PAF, and leukotrienes), and the production of pro-inflammatory transcription factors. These events are collectively mediated by changes in the release of intracellular calcium stores, an influx of extracellular calcium, a transient rise and fall in cyclic adenosine monophosphate (cAMP), and rapid reorganization of the cytoskeleton.^{74,90,91} Mast cells can also be activated by a variety of other molecules via IgE-independent pathways. This includes activated components of

the complement cascade (C3a),⁹² neuropeptides such as SP, vasoactive intestinal peptide, and calcitonin gene-related peptide (CGRP),^{85,93} superoxide anion; opiates; and various cytokines and chemokines.^{74,94}

Mast cells and connective tissue fibrosis. Mast cells have been implicated in the pathogenesis of fibrotic conditions based on a growing collection of data from *in vitro* studies and numerous human and animal disease models. Mast cell hyperplasia has been observed in fibrotic tissues, often in association with myofibroblast hyperplasia.^{9,10,61,95–100} Mast cells have been visualized to be in intimate contact with fibroblasts and these cells can directly adhere to one another, possibly via c-kit/membrane-bound SCF interactions.^{6,100,101} Myofibroblasts and fibroblasts can also secrete SCF into the local environment, which is a known mitogen and chemoattractant of mast cells.^{102,103} Interestingly, in wounds that heal with minimal scarring, such as fetal wounds and wounds involving the oral mucosa, fewer mast cells are observed.^{104,105}

Several important profibrotic growth factors synthesized and secreted by mast cells include bFGF, PDGF, and TGF- β 1.^{84,106} Fibroblasts cocultured with activated mast cells will contract collagen gels significantly more, synthesize more collagen, and express more α -SMA compared with isolated fibroblast or mast cell populations.^{107,108} In addition to profibrotic growth factors such as TGF- β 1, the abundant mast cell protease tryptase also possesses important profibrotic properties. Like TGF- β 1, tryptase is a potent fibroblast mitogen that can signal the phenotypic transformation of the myofibroblast phenotype and is an upregulator of matrix molecule synthesis.^{107,109} Mast cell tryptase has been shown to mediate fibroproliferative responses in fibroblasts via proteolytic activation of the cell surface receptor, protease-activated receptor 2.¹⁰⁹ In addition to the liberation of pro-fibrotic mediators into the extracellular environment, direct cell-to-cell communication between mast cells and fibroblasts has been documented via communicative gap junctions, thus providing another mechanism by which these cell types can regulate each other.^{101,110,111} Figure 1 illustrates the dynamic interactions between mast cell and fibroblast mediators of inflammation and fibrosis.

Given that mast cell activation and degranulation is an important physiological event in the inflammatory phase of wound healing,¹¹² it is conceivable that in fibroproliferative conditions, persistent pro-inflammatory signals important for

mast cell recruitment and activation are excessive or persist beyond normal regulatory control, establishing a mast cell-rich tissue environment. These intriguing findings have prompted recent assessment of the antifibrotic effects of mast cell inhibition in a variety of disease models. Compounds are available commercially with known inhibitory effects on mast cell degranulation: Ketotifen fumarate (Ketotifen) and sodium cromoglycate (cromolyn) are collectively known as “mast cell stabilizers” as these compounds can stabilize the mast cell membrane and impede mediator release upon stimulation.^{113,114} These compounds have been tested in numerous animal models of connective tissue fibrosis, with promising results.

In a rat model of autoimmune-mediated dilated cardiomyopathy, animals treated with intraperitoneal cromolyn had significant reductions in myocardial collagen hyperplasia, TGF- β 1 levels, and mast cell numbers.⁶⁴ Similarly, in a rat model of chemical-induced pulmonary fibrosis, Hemmati et al., demonstrated that preinjury treatment with cromolyn had protective effects against development of fibrotic changes in lung tissue characterized by decreased inflammatory cell infiltrates, negligible alveolar septal thickening, and reduced collagen mass to near control levels.⁶³ In the tight-skin (tsk) mouse, a genetic mouse model for human scleroderma, treatment with both Ketotifen and cromolyn has been shown to equally decrease mast cell infiltrates, mast cell degranulation, and skin fibrosis compared with untreated animals.¹¹⁵

The red Duroc pig is a breed of pig known to heal full thickness dorsal skin wounds with abnormally fibrotic, hypercontractile scars, characterized by collagen, myofibroblast, and mast cell hyperplasia.^{61,65} Harunari *et al.* has documented a $2.4\times$ increase in the number of mast cells in red Duroc skin wounds compared with normal samples of red Duroc skin.⁶¹ The oral administration of Ketotifen after dorsal skin wound induction using this animal model significantly reduced myofibroblast and mast cell hyperplasia and collagen deposition within scar tissue compared with placebo-treated red Duroc pig scar.⁶⁵ Interestingly, Ketotifen treatment of Yorkshire pigs, a breed that heals skin wounds with a normal phenotype, did not affect wound healing. Importantly, this observation implies Ketotifen treatment ameliorates abnormal healing pathways but does not adversely affect normal healing.

Post-traumatic joint contractures are another thoroughly investigated model of injury-associated connective tissue fibrosis. The human elbow is a

Figure 1. Mast cells mediated inflammation and fibrosis. Mast cells circulate as CD34-positive precursor cells and terminally differentiate in connective tissues. Both IgE dependent and independent mechanisms can activate mast cells causing the release of preformed and newly synthesized pro-inflammatory mediators. Many of these mediators increase vascular permeability and promote the recruitment of other inflammatory cells and additional mast cell precursors. SCF is also secreted by activated fibroblasts and myofibroblasts, further potentiating mast cell recruitment and proliferation. TGF- β is a potent fibroblast mitogen and stimulator of myofibroblast differentiation. It also impedes myofibroblasts apoptosis. bFGF, basic fibroblast growth factor; CGRP, calcitonin gene-related peptide; CTGF, connective tissue growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; SCF, stem cell factor; TGF- β , transforming growth factor-beta; TNF- α , tumor necrosis factor-alpha; VIP, vasoactive intestinal peptide. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

prototypical joint affected by post-traumatic motion loss; in affected patients the connective tissue lining of the joint (joint capsule) is irreversibly modified by fibrogenic connective tissue changes and mast cell hyperplasia.^{7,77,116} Using a validated rabbit model of post-traumatic joint contractures,^{117–119} twice daily subcutaneous administration of Ketotifen given immediately post-injury resulted in significant biomechanical and cellular reductions of joint capsule fibrosis: the biomechanical severity of joint contractures were reduced 50% and joint capsule mast cell and myofibroblast numbers were reduced by up to 70%.¹²⁰ An interesting observation in this study was that not only did mast cell inhibition reduce joint contracture severity and myofibroblast hyperplasia, but also significantly reduced mast cell hyperplasia. These data suggest that activated mast cells may further potentiate joint capsule inflammation and fibrosis by the recruitment of additional mast cells. Growth factors synthesized by

the mast cell, such as bFGF, PDGF, TGF- β 1, and nerve growth factor (NGF) are known to promote mast cell chemotaxis and proliferation^{84,121} and it may be no coincidence that many of these factors are potent fibrogenic mediators as well. In addition to reductions in contracture severity and myofibroblast numbers, Ketotifen treatment in the aforementioned rabbit model also reduced TGF- β 1, collagen type I and α -SMA protein, and gene expression within the affected joint capsule compared with untreated animals.¹²²

Neuroinflammatory pathways and fibrogenesis

The scientific evidence in human and animal models linking mast cell activation to the pathogenesis of connective tissue fibrosis is compelling; however, the mechanisms responsible for persistent mast cell activation and tissue infiltration remain poorly defined. Neuropeptides such as SP and CGRP are a family of extracellular signaling

molecules, which play a fundamental role in connective tissue healing responses. Collectively, these peptides function as neurotransmitters and paracrine factors promoting local vasodilation, tissue edema, cellular proliferation, matrix production, and growth factor synthesis and are important neurotransmitters involved in nociceptive signaling.^{123–125} SP and CGRP are synthesized in the dorsal root ganglia and released by peripheral free nerve endings within tissues such as the skin, muscle, tendons, ligaments, and joint tissue in response to noxious stimuli such as traumatic injury and growth factors such as NGF.^{124,125} Afferent sensory nerves expressing SP are commonly found in close proximity to the microvasculature¹²⁶ and mast cells.⁹³ SP is also a well-known mast cell secretagogue.⁹³ Additionally, SP has been shown to stimulate fibroblast proliferation and impair proapoptotic signaling in myofibroblasts.¹²⁷

Traumatic injury to bone and surrounding soft tissues has been shown to induce local and systemic increases in SP and CGRP.^{128,129} Elevated tissue and plasma levels of SP have been implicated in a variety of fibrotic conditions including Dupuytren's contracture of the hand,¹³⁰ hypertrophic wound healing,^{65,131} keloid scarring,¹³² scleroderma,¹³³ and idiopathic pulmonary fibrosis.¹³⁴ In hypertrophic wound healing models, mechanical wound forces are thought to play an important role in abnormal scar formation and fibrogenesis. In a complex interaction of adhesion molecules, the ECM and cellular mechanoreceptors, mechanical forces can be sensed resulting in cellular proliferation and pro-fibrotic signaling.¹³⁵ It has been shown that mechanical stimuli are sensed by terminal connective tissue nociceptors, which in return increases neuropeptide synthesis and transport to cutaneous free nerve endings and local synthesis by resident skin cells.¹³⁶

In the red Duroc pig model of hypertrophic wound healing, elevated numbers of SP containing nerve fibers within wound specimens were observed during all time points of wound healing compared to control pigs, and in pigs treated with Ketotifen, SP levels within skin wounds were significantly reduced compared untreated pigs.⁶⁵ This evidence suggests that independent of mechanical forces, tissue neuropeptide accumulations can be lessened by impeding mast cell contributions. In post-traumatic joint contractures, immunostaining for SP containing nerve fibers is also markedly increased in fibrotic joint capsule tissue⁷⁷ and similar to the red Duroc pig, Ketotifen therapy reduces SP expression in affected joint capsules (Monument, Hart and Hildebrand; 2009 unpublished observations). Figure 2

illustrates myofibroblast, mast cell, and SP immunoreactivity in joint capsule preparations from the knee in New Zealand white rabbits without a contracture, with a post-traumatic contracture and in rabbits treated with Ketotifen after joint injury.

Integrating this information, a working model of mast cell-mediated, neuroinflammatory post-traumatic joint capsule fibrosis has been proposed (Fig. 3): joint capsule mast cells and fibroblasts become acutely exposed to activating signals and growth factors via the fracture-induced hemarthrosis. Neuropeptides are released as part of the neurogenic response to skeletal injury. Under normal physiologic regulation, as bone and soft tissue healing progress fibroblast, myofibroblast, and mast cell numbers diminish as inflammatory and profibrotic stimuli lessen or anti-inflammatory regulatory mechanisms become engaged. However, in populations of "at-risk" patients, these fibroproliferative stimuli persist and escape regulatory control. Persistent noxious and/or chemical stimuli drive persistent neuropeptide synthesis, providing chronic activation of fibroblasts and mast cells. These upstream events facilitate an environment of sustained myofibroblast hyperplasia resulting in shortening of the ECM and disorganized collagen deposition. As mast cells and fibroblasts become activated, a positive feedback loop is engaged whereby growth factors synthesized and liberated by these cell types function to perpetuate further cell recruitment, proliferation, differentiation, and neuropeptide synthesis. Thus, these cells may form an "axis" consisting of neuropeptides, mast cells, and myofibroblasts. Based on studies of Ketotifen treatment in the rabbit post-traumatic joint contracture model and the red Duroc pig wound healing model, disruption of mast cell contributions to the fibrotic process leads to decreases in SP-containing nerves and reduced mast cell and myofibroblast numbers. Thus, this proposed axis of fibrosis is not likely linear or unidirectional, but rather regulated by multidirectional influences.

Clinical application of mast cell inhibition:

Ketotifen fumarate and sodium cromoglycate

Ketotifen fumarate and sodium cromoglycate are known as "mast cell stabilizers" as both agents prevent vesicle degranulation via interruption of normal intracellular calcium signaling required for degranulation after mast cell activation.^{113,114} Numerous subtle differences between these "mast cell stabilizers" warrant consideration. First, the mechanisms by which these medications blunt the acute rise in intracellular calcium needed for mast

Figure 2. Photomicrographs of joint capsule tissue harvested from the posterior knee joint of New Zealand white rabbits with a post-traumatic joint contracture (**A–D**) and in rabbits treated with Ketotifen after joint injury (**E–H**). (**I–L**) A negative control (omission of primary antibodies) from a contracted joint capsule specimen. Nuclear DAPI staining illustrates cell densities in all three conditions (**A**, **E**, and **I**, respectively). Contracted joint capsules demonstrate high α -SMA, tryptase, and SP immunoreactivity indicative of myofibroblast, mast cell, and SP hyperplasia (white arrows, **B**, **C**, and **D**, respectively). In Ketotifen-treated animals, immunostaining for α -SMA, tryptase, and SP is markedly reduced. Endothelial staining is illustrated by the white block arrows (**D** and **F**). Immunoreactivity of α -SMA, tryptase, and SP is particularly enriched around microvascular elements, which is attributable to α -SMA expressed in pericytes and smooth muscle cells¹⁵⁹ and the normal close anatomic relationship of mast cells (tryptase) and SP-expressing nerve fibers to the microvasculature. The images for each condition are of the same histological area viewed under different light sources and filters (original magnification 200 $\times$). α -SMA, alpha-smooth muscle actin; SP, substance P. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

cell degranulation differs slightly. Sodium cromoglycate is thought to alter intracellular calcium levels by the inhibition of transmembrane chloride channels, while Ketotifen inhibits intracellular calcium influx by stabilizing cAMP levels.^{137–139} Ketotifen is a non-selective H1 receptor antagonist and therefore is considered an antiallergic compound and an antihistamine. Ketotifen also exhibits antiphosphodiesterase activity, thereby impeding the synthesis of prostaglandins and leukotrienes.^{140,141} Another important distinction between Ketotifen and cromolyn is that the bioavailability of orally administered Ketotifen is ~50%,¹³⁸ whereas the oral bioavailability of cromolyn is less than 1%.¹⁴² Because of this, oral Ketotifen has been extensively studied and is an U.S. Food and Drug Administration (FDA) approved medication for patients with mild to moderate asthma. A recent Cochrane review of this literature supports the efficacy of Ketotifen used alone or as an adjunct in this patient population.¹⁴³ Because Ketotifen is a non-selective H1 receptor antagonist, systemic antihistamine side effects such as somnolence, dry mouth, and weight gain have been reported in clinical trials. However, to circumvent these systemic issues with Ketotifen

and cromolyn's negligible oral bioavailability, transdermal delivery techniques for both of these agents are under development.^{144–146} Transdermal delivery of these mast cell stabilizing agents could be an especially advantageous strategy to prevent hand and finger contractures after injury or to prevent hypertrophic wound healing after burns, lacerations, and surgical incisions.

To date, only one clinical study has evaluated the preventative effects of oral Ketotifen in a human disease model of fibrosis. Gruber and Kaufman¹⁴⁷ performed a double-blind randomized controlled trial comparing oral Ketotifen (3 mg twice daily) to placebo in patients with established scleroderma. Apart from relief of pruritis, the administration of Ketotifen did not improve any clinical parameters such as pulmonary function, overall skin scores, and maximal oral aperture. These discouraging results halted the momentum of mast cell inhibition as a treatment strategy for connective tissue fibrosis. Despite these results, Ketotifen has since been tested in various animal models of fibrosis with extremely promising results. A major critique of the results observed in the Gruber and Kaufman study pertain to the timing of drug administration: all patients enrolled in this

Figure 3. Neuropeptide–mast cell–myofibroblast contributions to post-traumatic elbow fibrosis. Traumatic injury to the elbow initiates connective tissue repair processes mediated by mast cell signaling and myofibroblast differentiation. In at-risk patients, this pathway escapes normal regulatory control; excessive SP secretion from terminal nerve endings in the joint and periarticular connective tissue results in persistent mast cell stimulation, propagating the inflammatory cascade of myofibroblast-mediated connective tissue fibrosis. Over time, excessive collagen deposition results in permanent motion loss and joint dysfunction. Modified with permission from Monument *et al.*¹⁶⁰ ECM, extracellular matrix. Adapted from figures 1 and 4 in Michael James Monument, David Hart, Andrew Dean Befus, Paul Salo, and Kevin Hildebrand, Posttraumatic elbow contractures: Targeting neuro-inflammatory fibrogenic mechanisms *Journal of Orthopaedic Science* 2013; 18(6): 869-877. Reprinted by permission. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

study had pre-existing clinical manifestations of fibrosis, with >90% of patients in both study arms having abnormal pulmonary function tests prior to commencing Ketotifen treatment. The distribution and tissue penetration of systemic Ketotifen has not been well characterized and it also possible that Ketotifen was not adequately distributed into the connective tissue of scleroderma patients. This is supported by the observation in the Gruber and Kaufman study where dermal mast cells obtained from post-treatment punch biopsies were just as sensitive to mast cell secretagogues such as compound 48/80 compared to control mast cells.¹⁴⁷ Further, mast cell populations throughout the body are heterogeneous and there is *in vitro* data suggesting that skin mast cells are not overtly sensitive to Ketotifen and cromolyn.^{148,149} However, data from preclinical models of hypertrophic wound healing in the red Duroc pig and post-traumatic knee contractures in the rabbit would suggest that both oral and subcutaneously administered Ketotifen ameliorates abnormal healing responses in the skin and joint capsule, respectively.

Based on preclinical data, Ketotifen and cromolyn therapy should be utilized as a preventative intervention and should not be expected to reverse

existing fibrosis. This conclusion is reinforced by results from the red Duroc pig wound healing model where early administration of Ketotifen after wound induction was effective, whereas delayed administration (28 days after wound induction) did not affect wound contraction,⁶⁵ supporting the argument that early interruption of neuropeptide–mast cell–myofibroblast axis may be crucial to halting the fibrogenic process. Common to all preclinical models showing a treatment effect after Ketotifen or cromolyn administration is the temporal relationship of systemic treatment shortly after the inciting, pro-fibrotic event (wound induction, joint injury, lung injury, etc.). This emphasizes the importance of identifying those at risk for excessive fibroproliferative responses, as pharmacological interventions targeting mast cell signaling will be more effective in preventing fibrosis than treating established disease.

Who is at risk?

Clinically, abnormal fibrogenic healing responses are not ubiquitous to all patients and clearly, the determinants of risk in these patients are not well established. In animal studies, many of the molecular upregulators and manifestations of

Table 1. Quantifiable circulatory biomarkers of connective tissue fibrosis

Biomarker	Source	Half Life	Specificity	Comments
Histamine	Plasma	Short (90–100 s)	Mast cells, Basophils, foreign pathogens	Short half life, low specificity
N-Methylhistamine	Plasma, urine	Long (50–60 min)	Mast cells and Basophils	Longer half life, greater specificity, urine levels dependent on renal function
Tryptase	Serum, plasma	Long (1–2 h)	Mast cells	Specific to mast cells, not affected by blood clotting
Substance P	Plasma, synovial fluid	Short (1–2 min)	Free nerve endings	Low specificity, elevated in various MSK conditions

MSK, musculoskeletal.

tissue fibrogenesis are elevated soon after injury or wound induction.^{33,46,61,77,119} Additionally, in the red Duroc pig model, the antifibrotic effects of Ketotifen were dependent on the timing of drug administration after wound induction.⁶⁵ Consequently, if attempts are made to break the maladaptive axis of neuropeptide–mast cell–myofibroblast regulated fibrogenesis, treatment and prevention strategies should be implemented soon after injury or surgical intervention, highlighting the importance of identifying those at risk.

We have postulated that a neuropeptide–mast cell–myofibroblast axis is vital to the fibrogenic process and the identification of an easily assayed biomarker specific and sensitive to the excessive signaling of this pathway may be highly informative (Table 1). Histamine levels in conditions with suspected mast cell involvement have been examined, such as asthma and physical urticarias demonstrating significant elevations of plasma histamine within affected patients.^{150,151} However, the measurement of plasma histamine as a marker of mast cell activity is complicated by the fact that other circulating cell populations such as platelets⁸⁴ and basophils¹⁵² are also sources of histamine. Further, enzymatic metabolism of plasma histamine is rapid with a half-life of ~90–100 seconds and basal plasma concentrations are usually less than 0.4 ng/mL.¹⁵³ Alternatively, a method of measuring the plasma concentrations of a primary histamine metabolite, N-tau-methylhistamine, which is more stable than the parent histamine peptide, has been developed.¹⁵⁴ Using this technique, plasma concentrations of N-tau-methylhistamine in patients suffering from hypertrophic scar formation after significant full-thickness burn injuries were double those of control patients.¹⁵⁴

Unlike histamine, tryptase is specific to mast cells, is much more stable with a half-life of 2 h and concentrations are not affected by blood clotting.⁷⁶ Therefore, tryptase can be assessed from serum or plasma. Elevated serum and plasma tryptase levels are common in patients suffering from acute anaphylactic reactions and in those with mast cell proliferative disorders such as mastocytosis or

mast cell leukemia.¹⁵⁵ Baseline tryptase levels are also elevated in patients predisposed to anaphylactic reactions.¹⁵⁵ Measurement of circulating SP levels is another consideration. Assays are available to measure SP levels in human plasma and synovial fluid.^{156,157}

Whether detectable differences in the concentrations of histamine, tryptase, SP, or other potential fibrogenic biomarkers can be measured in patients at risk for hypertrophic wound healing, post-traumatic joint contractures, or other fibroproliferative conditions has yet to be determined. Moreover, the profibrotic phenotype may be identified by a constellation of elevated biomarkers rather than a single aberrant measure of an upregulated neuropeptide–mast cell–myofibroblast axis. Further complicating this matter is that activated mast cells are also capable of selective mediator release without degranulating preformed granules and classic mast cell mediators.¹⁵⁸ This stresses the importance of further delineating the precise mast cell mediators involved in fibrogenic healing responses as we search for predictive biomarkers.

SUMMARY

Aberrant connective tissue fibrosis is common to numerous medical and surgical conditions across a broad range of clinical subspecialties and the difficulty in treating these conditions stems from our limited understanding of this important physiological process. These difficulties are further compounded by the lack of therapeutic and preventative measures available for treating affected patients. Recent advances in the development of surrogate animal models of common human fibroproliferative conditions has shed significant light on the pathogenesis of connective tissue fibrosis. Excessive signaling between mast cells, fibroblasts, and myofibroblasts with potential neurogenic modulation of this pathway is supported by various studies and provides encouraging optimism that multiple avenues of augmentation to this pathway may exist. Further characterization

of this neuroinflammatory pathway, the receptors involved and the identification of those at risk for progressive and maladaptive fibrosis will be fundamental to the development of effective treatment and preventative strategies.

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TAKE HOME MESSAGES

- Connective tissue fibrosis is characterized by myofibroblast proliferations and disorganized collagen hyperplasia.
- Preclinical models of numerous fibroproliferative disorders have enabled the identification of key fibrogenic molecular pathways.
- Mast cell and neuropeptide signaling are dysregulated during the acute phases of fibrogenesis.
- FDA approved medications are available to regulate mast cell activity, which have demonstrated promising antifibrotic effects in preclinical models of fibrosis.
- Successful, systemic antifibrotic therapies require early intervention and the identification of "at risk" populations.

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Abbreviations and Acronyms

bFGF = basic fibroblast growth factor
 C3a = complement component 3a
 cAMP = cyclic adenosine monophosphate
 CGRP = calcitonin gene-related peptide
 c-kit = cellular homolog of the feline sarcoma viral oncogene v-kit
 CTGF = connective tissue growth factor
 DAPI = 4',6-diamidino-2-phenylindole
 ECM = extracellular matrix
 FcεRI = Fc fragment of IgE, high affinity receptor 1
 FDA = U.S. Food and Drug Administration
 GI = gastrointestinal
 IFN-α = interferon alpha
 IFN-γ = interferon gamma
 IgE = immunoglobulin E
 IL = interleukin
 MC_T = tryptase- and chymase-positive mast cells
 MC_{TC} = tryptase positive and chymase-negative mast cells
 MMP = matrix metalloproteinase
 NGF = nerve growth factor
 PAF = platelet activating factor
 PDGF = platelet-derived growth factor
 SCF = stem cell factor
 SP = substance P
 TGF-β = transforming growth factor-beta
 TIMP = tissue inhibitor of matrix metalloproteinases
 TNF-α = tumor necrosis factor-alpha
 α-SMA = alpha-smooth muscle actin