Proinflammatory and Profibrotic Mediators: Principal Effectors of Leiomyoma Development as a Fibrotic Disorder

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Abstract

Leiomyomas are believed to derive from the transformation of myometrial smooth muscle cells/ connective tissue fibroblasts. Although the identity of the molecule(s) that initiate such cellular transformation and orchestrate subsequent growth is still unknown, conventional evidence indicates that ovarian steroids are essential for leiomyoma growth. Ovarian steroid action in their target cell/tissue is mediated in part through local expression of various growth factors, cytokines, and chemokines. These autocrine/paracrine molecules with proinflammatory and profibrotic activities serve as major contributing factors in regulating cellular transformation, cell growth and apoptosis, angiogenesis, cellular hypertrophy, and excess tissue turnover, events central to leiomyoma growth. This review addresses the key regulatory functions of proinflammatory and profibrotic mediators and their molecular mechanisms, downstream signaling that regulates cellular events that result in transformation, and commitments of specific cells into forming a cellular environment with a possible role in development and subsequent growth of leiomyomas.

Leiomyomas are benign uterine tumors of unknown etiology believed to arise from myometrial cellular transformation. Despite the presence of multiple tumors in the same uterus, they occur independent of metastasis and display a limited capacity of malignant transformation (<1%). Histologically, leiomyomas are well-encapsulated tissues that primarily consist of smooth muscle cells and connective tissue fibroblasts, with a limited vascularization network. Cytogenetic analysis consistently identified several nonrandom chromosomal rearrangements in leiomyomas that are considered to account for their tumorigenesis; however, inconsistency in their occurrence implies an indirect participation in the development of leiomyomas.[1–4] Currently, the identity of factor(s) that initiate the transformation of myometrial cells into leiomyomas and orchestrate their subsequent development remains unknown. However, the development of leiomyomas during the reproductive years and regression following menopause implicate a direct role for ovarian steroids as their key growth-promoting factors. Substantial evidence has also been generated in support of the expression and function of various locally expressed growth mediators, including oncogenes and tumor suppressor genes, in regulating leiomyoma growth and regression. Whether these mediators participate in initial cellular transformation or development of leiomyomas is unknown; their differential expression in leiomyomas and

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regulation of leiomyoma cells under in vitro conditions implicate their potential function in growth and regression.

Despite the presence of multiple tumors in the same uterus, a significant percentage of women with leiomyomas are symptom free. However, the presence of symptomatic leiomyomas, due to their rapid growth, uterine bleeding, pelvic pain, and pelvic masses, is the leading reason for hysterectomy.[5–7] Clinical and epidemiological observations indicate that symptomatic leiomyomas account for more than a third of all hysterectomies performed. [7,8] Whether performed by laparotomy, laparoscopy, or robot-assisted laparoscopy, hysterectomies are major abdominal surgical procedures that carry an increased risk of postoperative morbidity. Intraoperative/postoperative complications due to injuries to the bowel, bladder, blood vessels, or other sites are the leading cause of postsurgical scar formation initiated by various proinflammatory and profibrotic mediators at the site of injury following hysterectomy and/or myomectomy.

In recent years, considerable progress has been made toward several new nonsurgical alternatives to hysterectomy. These interventional procedures include minimally invasive uterine artery embolization (UAE), using particulate emboli to occlude the uterine arteries and to disrupt the blood supply to fibroids, as well as magnetic resonance (MR) imaging-guided focused ultrasound surgery to reduce leiomyoma volume. These procedures are effective in alleviating fibroid-related symptoms and are gaining popularity,[9,10] although MR imaging-guided focused ultrasound is only effective in the management of large tumors. However, particulates used in UAE and excessive cellular damage following the conclusion of MR imaging-guided focused ultrasound leaves debris and cellular fragments within the uterine environment that can cause tissue reactivity and a local inflammatory response, respectively. Tissue reactivity to foreign materials and cellular damage are the main cause of local inflammation, and it remains to be determined whether such procedures, through the generation of local inflammatory mediators, result in other site-specific complications. Although it may be rare, aberrant implantation of leiomyoma fragments in the peritoneal environment or abdominal wall after laparoscopic-assisted surgeries can result in the growth of the tumors outside the uterus.[11]

MEDICAL MANAGEMENT OF LEIOMYOMAS

In addition to the surgical procedures just described, considerable progress has been made toward the medical management of leiomyoma growth through therapeutic interventions. These efforts have been centered on the development of therapeutics that interfere with ovarian steroid biosynthesis or steroid receptor-mediated actions, due to the fact that leiomyoma growth is sensitive to ovarian steroids. Among these agents are the long-acting analogues of gonadotropin-releasing hormone agonist (GnRHa) therapy, which act through the pituitary-ovarian axis and create a hypoestrogenic condition. GnRHa therapies have proven effective in causing leiomyoma regression, but with considerable side effects, thus prolonged therapy is not advised. Because discontinuation of GnRHa therapy results in the rapid return of the tumor to its original size, cotherapy with low doses of cyclic or continuous estrogen "add back" has been effective in reducing GnRHa side effects and allows prolonged therapy. However, cotreatment with medroxyprogesterone acetate reverses the beneficial effect of GnRHa therapy.[7] In addition to GnRHa, preclinical and clinical trials with therapeutics that are selective estrogen receptor modulators (SERMs) and, more specifically, selective progesterone receptor modulators (SPRMs), have shown potential as additional therapeutic avenues for managing the growth of leiomyomas.[12–16] Several of these trials are investigating the effects of the SERMs raloxifene and tamoxifen and the SPRM's CDB2914, asoprisnil (J865), and CDB4124, as well as RU-486.[12–21] Similar to

GnRHa therapy, prolonged therapy with these agents is not advised beyond a few months due to associated negative endometrial and liver side effects.[21]

There is limited information on how current hormonal therapeutic interventions act at the molecular level and why their use results in the regression of some leiomyomas for some women, but not all, who receive therapy. It is clear that GnRHa therapy acts at the level of the pituitary-ovarian axis to regress leiomyoma growth; however, evidence also supports the direct action of GnRHa on various peripheral tissues, including the uterus, through direct interaction with GnRH receptors. Considerable evidence also exists to support that GnRHa acts directly on cell growth and apoptosis in myometrial and leiomyoma cells by influencing the expression and function of a large number of genes, including proinflammatory and profibrotic mediators.[22–25] Several studies have also provided evidence for the direct regulatory action of raloxifene, tamoxifen, RU-486, ZK98299, CDB2914, and asoprisnil on the expression of many genes involved in inflammatory response, fibrosis, and apoptosis, which results in leiomyoma regression.[14,26–35] However, unlike RU-486 and ZK98299, which affect both leiomyoma and myometrial cells, CDB2914 and asoprisnil have been reported only to affect leiomyoma cell growth and the expression of several growth factors, cytokines, and proteases, without affecting the same factors in myometrial cells.[26,27,29,31,33–35] Although the in vitro evidence is convincing, clinical observations do not support such cell-specific actions for these agents because both leiomyomas and myometrium and their isolated primary cell cultures express progesterone receptors (PRs), and these compounds are developed based on their potential interactions with PRs. The most likely explanation may lie in our limited understanding of the molecular mechanisms underlying ovarian steroid receptor activation and mediated signaling in leiomyomas as compared with other steroid-sensitive cells and tissues. Studies with crystal structures of the progesterone receptor (PR) ligand binding domain complexed with asoprisnil and the corepressors nuclear receptor corepressor (NCOR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMAET) indicated that asoprisnil differentially recruits coactivators and corepressors when compared with RU-486 or progesterone (P4) in a breast cancer cell line; this specific cofactor interaction profile is apparently insufficient to oppose estrogenic activity in the rat uterus.[36] In ELT3 leiomyoma cells, asoprisnil demonstrated partial P4-like inhibition of cyclooxygenase expression and enzymatic activity. Considering that leiomyoma and myometrium express several nuclear receptor coactivators and corepressors,[37] it is conceivable that common molecular mechanisms may explain the actions of ovarian steroids in all their target cells and tissues, although accumulated evidence supports their unique functions in cell- and tissue-specific manners. This is particularly important because all cell types contain the same genome but only use a selective portion of it to implement their specific function in a time- and cell-dependent context. As such, the biological relevance of ovarian steroid actions in leiomyoma may become highly relevant for gaining a better understanding of the modes of action of current therapeutics if we identify their specific target genes, specifically proinflammatory and profibrotic genes, and the molecular mechanisms of their actions that result in the growth and regression of leiomyomas.

CELLULAR ORIGIN, PHENOTYPIC TRANSFORMATION, AND MEDIATORS OF LEIOMYOMAS

In addition to having benign uterine tumorigenic characteristics, leiomyomas are fibrotic disorders commonly characterized by excess accumulation of extracellular matrix (ECM), which is similarly observed in a wide spectrum of organ-specific fibrotic disorders such as dermal, renal, pulmonary, hepatic, and myocardial fibrosis. Although the etiology of fibrosis is either unknown or poorly understood, it is clear that a complex and multifaceted interactions among various cell types, including epithelial, endothelial, smooth muscle,

infiltrating immune/inflammatory related cells, and, most importantly, fibroblasts, are critical for the establishment and progression of the disease. In this context, a strong association between inflammation and an increased risk of fibrinogenesis and tumorigenesis has been well established.

Inflammation involves a well-coordinated response to cellular injuries caused by infection, chemicals, mechanical insult, radiation, and chemotherapeutic drugs, resulting in either local or systematic activation of an innate and adaptive immune response. The innate immune cells (e.g., macrophages, mast cells, dendritic cells, and natural killer cells) are involved in initiating the inflammatory response by releasing various cytokines, chemokines, matrix-remodeling proteases, eicosanoids, and reactive oxygen and nitrogen species at the site of injury. Inflammatory reactions and maintenance of precise cellular homeostasis are critical for normal defense mechanisms and tissue repair processes to proceed; any failure in regulatory mechanisms leading to chronic inflammation could result in establishment of a conductive microenvironment favoring the initiation and progression of fibrinogenesis and tumorigenesis (Figs. 1 and 2).

Uterine tissue is susceptible to fibrinogenesis, as seen in rare occasions in women affected by Asherman's syndrome, or in response to mechanical injury in women undergoing endometrial ablation and cesarean delivery.[38–41] However, no evidence exists that uterine mechanical injuries result in development of leiomyomas, although histologically, they resemble fibrotic tissues that develop in response to such injuries at various sites throughout the body. Unlike leiomyomas, fibrinogenesis in other tissues occurs in an age-independent manner and from a common end point of defective wound repair. Fibrinogenesis requires a sustained but not chronic inflammatory condition that results in defective wound repair and tumorigenesis. It is intriguing that similar mechanical injury that causes fibrosis in adults results in near-normal tissue regeneration in fetal skin and is a gestational-dependent event. [42] However, graft transplantation of adult skin flaps into fetal skin results in scar formation, whereas homologous transplantation of fetal skin flaps do not, despite well-developed neovascularization in both cases. The molecular mechanism that results in fetal skin regeneration is not well understood; however, the fibroblastic phenotype and genetic imprinting are considered potential mechanisms responsible for the differential outcome of fibrosis in fetal versus adult skin.[42]

CELL TYPES WITH POTENTIAL OF MYOFIBROBLASTIC TRANSFORMATION

Tissue fibrosis occurs throughout the body as part of a complex defensive mechanism against injuries to isolate surrounding cells from undergoing further cellular damage. Whether they develop as a result of systemic connective tissue diseases or in response to injuries, cellular events leading to fibrosis involve delicate inter-play among many distinct cell types and mediators. In recent years, our understanding of the biology of fibrosis has increased precipitately, primarily through various in vitro and in vivo models, including transgenic and knockout mice models. These studies implicated the existence of a common cellular component among all tissues undergoing fibrosis involving epithelial cell injury, activation and survival of fibroblasts, alternatively activated macrophages, and the infiltration of bone marrow–derived progenitor cells.[43–51] Although in specific cases the activation of the epithelium is a key mediator of initial fibrinogenic signaling, in an overwhelming number of cases fibroblasts are the principal cell type involved in the establishment and progression of tissue fibrosis. In addition to resident fibroblasts, evidence implicates possible participation of circulating fibroblast precursors, or fibrocytes, in events leading to fibrinogenesis. Circulating fibrocytes have been detected during wound healing and are considered to ultimately participate in fibrotic disorders such as hypertrophic scars

and keloids, scleroderma, renal fibrosis, airway remodeling in asthma, and experimental models of lung fibrosis.[44–46,48–50,52,53]

The myometrium consists of mainly myometrial smooth muscle cells (MSMCs), connective tissue fibroblasts, vascular cells, and a detectable number of bone marrow–derived progenitor cells.[54,55] Although the roles of stem cells, fibrocytes, and vascular-derived cells in the development of leiomyomas are yet to be proven, in the setting of local inflammatory-mediated cellular injury, MSMC and resident connective tissue fibroblasts, because of their sheer numbers, are arguably the most likely cell populations to undergo cellular damage and phenotypic transformation and participate in the process of leiomyoma development. Cellular transformation into a myofibroblastic phenotype is key to the establishment and progression of fibrinogenesis (Fig. 1).[43,46,56,57] In the case of tissues undergoing epithelial injury, epithelial to mesenchymal transition (EMT), is a determining factor in the outcome of fibrinogenesis.[43,46,56,57] Considerable evidence exists with respect to the molecular mechanisms of EMT, endothelial-MT, and mesenchymal to epithelial (MET) transition; more specifically, during the developmental stage in which cells display a remarkable phenotypic plasticity. Under this context, loss of cell-cell adhesion, increased motility, cytoskeletal and morphological changes, and resistance to apoptosis are among changes that occur as a result of EMT during the development, events that also occur during wound healing and tissue fibrosis. In tissues such as lung, kidney, liver, and skin, all with a prominent epithelial cell layer, EMT, endo-MT, and MET are integrated parts of the tissue repair process and fibrosis, and appear to involve similar cellular changes and molecular pathways.[43,46,56,57] In the absence of EMT, alternative cellular transition from other cell types into the fibroblastic phenotype, potentially including endo-MT and macrophage transformation into fibroblasts, can also participate in this process. In either case, fibroblasts require an enriched microenvironment to proliferate and undergo differentiation before developing into reparative or fibrotic mechanisms at the site of tissue damage.

Cellular insult caused by various injuries often results in the infiltration of platelet-rich plasma into the tissue, forming a fibrinous clot at the site of injury. The extent of fibrinous clot formation, also referred as provisional matrix, depends on the degree of tissue damage. [58] The provisional matrix is a rich source of many active molecules and an essential component of the normal tissue repair process (Fig. 1). The infiltration of inflammatory- and immune-related cells from peripheral blood into the provisional matrix and induction of a local inflammatory response are important features of soft tissue repair because removal of this matrix or depletion of macrophages at the site of injury results in defective tissue debridement, fibroblast proliferation, and wound repair.[58] The recruitment of inflammatory-related cells, more specifically, macrophages, to the site of injury is further sustained by platelet-derived cytokines, as well as other chemoattractant factors. These factors include fibrinopeptides cleaved from fibrinogen by thrombin, degradation products of fibrin produced by plasmin, platelet factor 4, eicosanoids (leukotriene (LT)B4, LTC4, and prostaglandin E2 [PGE2]), and platelet activating factor released from endothelial cells or activated neutrophils.[59,60] The recruitment of inflammatory cells to the site of injury is also facilitated by adhesive molecules such as fibrin, fibronectin, vitronectin, and other ECM that are present in the provisional matrix, as well as integrins that recognize these molecules.[59–64] The infiltrating monocytes that become activated and differentiate into macrophages are a major source of growth factors, cytokines, chemokines, eicosanoids, and proteases. In addition to macrophages, neutrophils, T cells and mast cells, smooth muscle cells and connective tissue fibroblasts are the source of many of these molecules, or one may consider them as nonprofessional inflammatory related cells.

Fibroblasts are derived from the surrounding connective tissue and migrate into the provisional matrix, proliferate, and simultaneously undergo differentiation, acquiring a

myofibroblastic phenotype defined by the expression of α-smooth muscle actin.[65,66] Unlike connective tissue fibroblasts, fibrocytes express α-smooth muscle actin, which is characteristic of fibroblasts after transition into the myofibroblastic phenotype. Ultimately, fibroblasts, fibrocytes, and progenitor stem cells derived from circulation, as well as other cell types, are transformed and differentiate into the myofibroblastic phenotype. In the case of smooth muscle cells, their transformation into the myofibroblastic phenotype results in the expression of vimentin, a somewhat common characteristic of fibroblasts. Myofibroblasts, through the production of ECM, form a myofibroblast-enriched tissue with the ability to respond to various growth-promoting factors and to produce more ECM that can develop into fibrotic tissue such as leiomyoma. However, under normal tissue repair processes with limited inflammatory response, remodeling of the provisional matrix by various proteases, removal of fibroblasts through apoptosis, and re-epithelialization of the damaged cells results in near-normal tissue repair.[67]

The development of leiomyoma and its transformation into fibrotic benign tumors could also occur in response to local inflammatory reactions and the involvement of similar cellular components, despite the lack of evidence of the existence of chronic inflammation in the myometrium. There are reasons to believe that a micro-inflammatory environment with a constant supply of a variety of inflammatory mediators, by maintaining interplay between the extrinsic and intrinsic pathways, could promote myometrial cellular transformation into tumorigenesis and fibrinogenesis, as in leiomyomas. Such local inflammation could occur in response to cellular stress caused by either exogenous or endogenous agents, and by cells undergoing apoptosis. Sustained local inflammation can cause the generation of reactive oxygen and nitrogen species, which can function as chemical effectors in such a micro-environment. Inflammatory-and immune-related cell migration into the inflamed site, as well as local myometrial smooth muscle cells, fibroblasts within the connective tissue, and other vasculatures, all express various types of inflammatory mediators including a large number of cytokines, chemokines, eicosanoids, oxygen and nitrogen radicals, and proteases. [22,68–73]

The individual and combined actions of these mediators function to initiate, amplify, and control many molecular events that ultimately lead to resolution of the inflammatory response. At the same time, excess generation of these mediators and prolonged inflammation also serve as plausible mechanisms that initiate cellular transformation of the neighboring cells through DNA damage, leading to the activation of oncogenes and/or inactivation of tumor suppressor genes. In the case of myometrial cellular transformation into leiomyoma cells, the outcome is the generation of the myofibroblastic phenotype, which is the main cellular component of fibrinogenesis in all fibrotic tissue. Thus, conceptually, many cellular and molecular events that initiate progression of tissue fibrosis may occur during leiomyoma growth, but modalities that result in their development place leiomyomas into a different category, being unique tumors with fibrotic characteristic.

PROINFLAMMATORY MEDIATORS AND THEIR POTENTIAL ROLE IN LEIOMYOMA DEVELOPMENT AND GROWTH

Considerable evidence exists to support the role of proinflammatory and profibrotic mediators in normal cellular activities and in the pathophysiology of many diseases, including tumorigenesis and fibrinogenesis. In this context, localized cellular exposure to inflammatory mediators often results in cell surface activation of phospholipids that initiate the formation of many intrinsic substrates, including eicosanoids such as prostacyclin, thromboxane, leukotrienes, antithrombin III, protein C, plasminogen activators, and plasminogen activator inhibitors (Fig. 3).[60–63,74–78] The expression and activities of these substances are regulated by the local release of cytokines, chemokines, and oxygen radicals

produced by activated inflammatory and immune cells (Fig. 3). In addition, some cytokines and chemokines released and retained in the extracellular compartment before local cellular injury can become activated and serve as chemotactic factors, further potentiating the migration of inflammatory cells into the area.

Many cytokines and chemokines regulate the production of eicosanoids and proteases, which are essential factors that mediate the inflammatory response.[60–63,74–78] Eicosanoids act as intracellular mediators of ovarian steroids, cytokines, chemokines, and growth factor signaling in various cell types, and their elevated production has been associated with an increased incidence of tissue fibrosis; inhibition of their actions using nonsteroidal anti-inflammatory drugs reduces the effect. Leukotrienes are potent chemoattractant factors for many inflammatory-related cells and have been implicated in the pathogenesis of a variety of inflammatory processes.[60,79–83] Myometrium and leiomyomas express all of the components of cyclooxygenase and lipo-oxygenase pathways, and they contain prostaglandin, thromboxane, and leukotriene receptors that mediate their actions in the tissues.[69,84–87] In addition, it is generally accepted that the actions of ovarian steroids, specifically the mitogenic action of estrogen, are mediated in part through the expression of autocrine/paracrine growth factors, cytokines, and chemokines. Interaction of these mediators with their receptors activate various signaling pathways, resulting in consequent regulation of cell growth and differentiation, apoptosis, and expression of ECM and adhesion molecules, proteases, and proteases inhibitors that regulate the outcome of tissue turnover. In the absence of ovarian steroids, many of these events are directly influenced by individual and combined actions of growth factors, cytokines, chemokines, and other active molecules. These molecules collectively regulate the local myometrial micro-inflammatory condition as it proceeds through either a reparative stage or a degradative phase, ultimately resulting in the development of leiomyoma and fibrotic characteristics.

Cytokines act in a predominantly paracrine/autocrine manner and are produced and act at a local micro-environment.[88,89] Some of the key characteristics of cytokines are their wide pleiotropy and their element of redundancy in biological function; all cytokines possess many overlapping functions that potentially could be mediated by other cytokines. Many cytokines are rarely produced individually; rather, they are expressed along with other cytokines. Thus the effects of one cytokine may be influenced by others released simultaneously by the same cell or by neighboring cells following activation, resulting in either synergistic or antagonistic effects. What distinguishes cytokines from growth factors is their ability to activate selective intracellular-signaling pathways that are not linked to mitogenesis. These distinctions are not always clear because some downstream cytokine targets are also linked to cell growth, such as MAPK/ERK. Cytokines act through specific cell surface receptors that are grouped into superfamilies based on the presence of homologous regions. These receptors are often present in low numbers; however, they become upregulated following cell activation (for review, see O'Garra and Robinson[88] and Stetson et al[89]). Cytokines are produced in response to cellular stress caused by either exogenous or endogenous agents, and they function to control and minimize cellular damage. However, an uncontrolled and sustained generation of cytokines can lead to altered cell growth, differentiation, and apoptosis.

Chemokines represent a large superfamily of small peptides that are divided into several subgroups according to the spatial arrangement of the first two cysteine residues in the NH2-terminal region, including CXC or α, CC or β, C or γ, and CX3C or δ.[90–92] Functionally, chemokines are divided into two groups: inducible inflammatory chemokines, which are produced at sites of inflammation and function to recruit various inflammatory- and immune-related cell types, and constitutive chemokines, which are produced in bone marrow and secondary lymphoid organs where they regulate leukocyte trafficking under

noninflammatory physiological conditions. Regulation of these processes is complex and involves both differential secretion and presentation of chemokines in target tissues, as well as variably regulated expression of chemokine receptors during differentiation and activation of inflammatory cells. Chemokines also play regulatory functions in angiogenesis, hematopoiesis, infectious response, and fibrosis (Fig. 4).[90,91,93,94] The biological actions of chemokines are mediated through a distinct family of G-protein-coupled receptors that have been identified in various cells types and include 10 CC chemokine receptors (CCR1-CCR10), 6 CXC chemokine receptors (CXCR1-CXCR6), and one CX3C receptor (for review, see Gerard and Rollins,[90] Lau et al,[91] and Zlotnik and Yoshie[92]). Although the origin of cytokines and chemokines is broadly known, the precise contribution of each cell type to their production in acute and chronic inflammation remains elusive.

Considerable progress has been made in understanding the molecular etiology of leiomyomas during the past decade. Using various conventional as well as large-scale gene expression profiling and proteomic analysis has led to the identification of a large number of genes and proteins with altered expression, including many growth factors, cytokines, chemokines, eicosanoids, and proteases that exhibit patterns of expression, which, to an extent, may allow the differentiation of myometrium into leiomyomas.[95–100] Despite the discrepancies among the results generated from various expression profiling strategies, each individual study allowed, to an extent, the sorting of genes in leiomyomas from matched myometrium based on the expression profile of a set of differentially expressed genes. Thus far, the gene array studies have indicated that the levels of expression, rather than the presence or absence of a selective number of genes, including many growth factors, cytokines, and chemokines, differentiate leiomyomas from myometrium, independent of tumor size.[95–100] This phenomenon is not unique to leiomyomas and myometrium because studies with gene array analyses in many other cell types and tissues have revealed similar results, including the comparative analysis between leiomyomas and keloids.[24,101–103]

Because leiomyomas develop more frequently among African Americans as compared with other ethnicities, subjecting paired leiomyoma and myometrium from African Americans and whites to genomic and proteomic analyses yielded considerable similarity, relative to the molecular environments regulating fibroids, with differences seen in a gene-specific manner rather than in an ethnic-specific manner.[98,104,105] Because many of the differentially expressed genes identified in different cohorts are known to regulate inflammatory response, angiogenesis, cell cycle, apoptosis, and ECM turnover, their products may account for the rapid growth of leiomyomas and their associated symptoms in African Americans as compared with other ethnic groups.

Despite these advances, we are still far from the identification of specific genes and the molecular mechanisms of action of their products that initiate the development and subsequent growth of leiomyoma. Rather, such efforts, along with rapid progress in various aspects of gene functional analysis in other fields, has led to the identification of genes whose products act as mediators of cell growth and differentiation, apoptosis, angiogenesis, inflammation, oxidative stress, ECM accumulation and tissue turnover, oncogenes and tumor suppression, and so on. These processes, either directly or indirectly, have been associated with the establishment and progression of many pathological disorders, including tumor development and fibrinogenesis, two well-established characteristics of leiomyomas.

IMPLICATION OF INFLAMMATORY-RELATED MEDIATORS

Although the direct contribution of constitutive inflammation to the development of leiomyoma remains speculative, considerable evidence exists in support of the involvement of proinflammatory and profibrotic mediators in the pathogenesis of leiomyomas. The

results generated from conventional and gene expression profiling strategies have demonstrated the expression of a large number of growth factors, cytokines, and chemokines with proinflammatory and profibrotic properties in the myometrium and leiomyomas. Among the major cytokines, the expression of interleukin (IL)-1, IL-6, IL-11, IL-13, IL-15, interferon (IFN)γ, tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and erythropoietin have been documented in leiomyomas, with some evidence implicating their biological relevance to leiomyoma patho-physiology. [72,106–108] These and other cytokines are recognized as key regulators of inflammation, angiogenesis, and tissue remodeling; events that are central to leiomyoma growth. Of particular interest, overexpression of IL-11, a member of the IL-6 family, and IL-13, a Th2 cytokine with homology to IL-4, serve as key regulators of subepithelial airway fibrosis, particularly through interaction with transforming growth factor (TGF)-β. [89,109–117] IL-11 and IL-13, similar to TGF-β, are overexpressed in leiomyomas and are differentially regulated by ovarian steroids, GnRHa, RU-486, and TGF-β. GM-CSF has been shown to induce its own expression and the expression of TGF-β1 in myometrial and leiomyoma smooth muscle cells. [106] TGF-β is a potent chemoattractant factor for macrophages and fibroblasts, whereas GM-CSF promotes macrophage uptake of apoptotic neutrophils. [118–127] The interactions between TGF-β and GM-CSF may act as an important regulator in maintaining a balance between the inflammatory and immunosuppressive activities of GM-CSF and TGF-β, respectively. Furthermore, GM-CSF markedly increases the expression of TGF-β, and IL-1α, IFN-γ, IL-6, and TNF-α induce the expression of platelet-derived growth factor (PDGF), resulting in progression of tissue fibrosis. [118–127]

The expression profiles of many chemokines have also been characterized through large-scale gene expression profiling of leiomyomas and matched myometrium. Further evaluation of chemokine expression indicates local production of macrophage inflammatory protein (MIP)-lα, MIP-1β, regulated upon activation, normal T-cell expressed and secreted (RANTES), eotaxin, IL-8, CCR1, CCR3, CCR5, CXCR1, and CXCR2, in both leiomyoma and myometrium, with lower content of eotaxin, MIP-1α, MIP-1β, and CCR5 mRNA in leiomyomas, all with some degree of dependency on the menstrual cycle. [128] Eotaxin mRNA expression in myometrial tissue from patients with single nodes was found to be much higher than in those with multiple nodes and higher than in those with proliferating leiomyomas. It has been observed that IL-8 expression in leiomyoma, as well as MIP-1α and CCR3 expression in myometrium, increases in patients with submucosal nodes, with a direct correlation between tumor size and the level of IL-8 and MIP-1β expression. [128] The expression of IL-8 and monocyte chemoattractant protein 1 (MCP-1) mRNA levels in myometrial cells were also higher than observed in leiomyoma cells, with highest expression observed during the secretory phase of the menstrual cycle; [70,129] expression decreased in GnRHa-treated tissues. Treatment with IL-1α and TNF-α caused a time- and dose-dependent increase in IL-8 production by myometrial cells; treatment with E_2 and P_4, alone or in combination, resulted in a decrease in MCP-1 production. [70]

The possibility of cytokines and chemokines participating in cellular events that result in myometrial cellular transformation into leiomyoma cells is conceivable. Plasma-derived fibrinogen released in response to cellular injury at the site of inflammation has been shown to elicit the release of several chemokines, including MIP-1α, MIP-1β, MIP-2, and MCP-1. [130,131] MCP-1, in turn, stimulates local cellular LTB-4 production, and the LTB-4 receptor antagonist CP-105–696 inhibits the recruitment of neutrophils and macrophages, which is accompanied by a reduction in MCP-1 expression. [60,90,132–141] These results suggest that induction of chemokines following the initiation of cellular and/or tissue injury mediates inflammatory response, occurring directly or indirectly through the production of LTB-4. Interestingly, MIP-2 expression was found to be limited to localized inflamed regions, independent of the expression of proinflammatory cytokines such as TNF-α or

IL-1.[142] IFN-γ treatment has been shown to selectively inhibit LPS-induced MCP-1, MIP-1α, MIP-1β, and MIP-2 expression and to induce IP-10 expression, which is an antiangiogenic chemokine. IL-3, IL-4, GM-CSF, IL-10, and IL-13 are expressed by myometrial and leiomyoma cells, and they are known to induce the expression of C10, a CC chemokine that regulates the chronic stage of host defense reaction. In addition, MCP-1 and MIP-1α expression is induced by IL-3 and GM-CSF, and it is inhibited by IL-4 and IFN-γ. In contrast, MCP-1 and MIP-1α inhibit IL-3- and GM-CSF-induced C-10 expression, which consequently induces rapid production of TNF-α and MCP-1, and later, an increase in IL-13.[139,143−146] IL-13, in addition to its role as a potent anti-inflammatory molecule, serves as a key profibrotic cytokine through its induction of TGF-β. Another cytokine with anti-inflammatory properties is IL-10, which effectively inhibits the expression of MCP-1, MCP-5, MIP-1α, MIP-1β, MIP-2, IP-10, KC, and RANTES. These observations indicate the importance of complex interactions among cytokines and chemokines in inflammatory responses suggest their possible influence in myometrial inflammatory reactions that lead to development of and growth of leiomyomas. Furthermore, polymorphisms in several cytokine genes, including those encoding IL-1β, IL-6, and TNF-α, have been associated with an increased incidence of leiomyoma development.[147−150] Further studies correlating polymorphisms in IL-1, IL-2, IL-4, IL-8, IL-12, and IL-18 genes with leiomyoma susceptibility indicated that proportions of the IL-12Rβ1 codon 378, but not other genes, was associated with the disease.[148]

The production of proteolytic enzymes in response to inflammation is also fundamental to angiogenesis, not only for the degradation of perivascular matrix, but also for the migration and proliferation of fibroblasts and endothelial cells (Figs. 3 and 4). During angiogenesis, the initial migration and proliferation of endothelial cells occurs in a fibronectin-rich ECM, whereas in vascular maturation, which takes place at later stages, it is rich in laminin.[58] These processes also involve integrins, the essential components of ECM-cell and cell-cell interactions, which promote cell migration, gene expression, cell differentiation, and other cellular activities.[63,75,151−156] At the initial stage of angiogenesis, the production of proteases such as matrix metalloproteinases (MMPs) and serine proteases (fibrinolytic system; plasminogen activators) by endothelial cells is necessary to degrade components of the ECM, including fibronectin and laminin.[157−162] However, these proteases are produced in inactive forms and must become activated to initiate their local actions. The proteolytic activities of these enzymes are regulated by naturally occurring physiological inhibitors, tissue inhibitors of MMPs (TIMPs), and plasminogen activator inhibitors (PAIs). Cytokines, chemokines, and growth factors, such as IL-1, IL-8, TNF-α, GM-CSF, vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), endothelial growth factor (EGF), TGF-α, TGF-β, PDGF, and IGF-I, all serve as angiogenic enhancing factors due to their ability to regulate the expression of MMPs, the fibrinolytic system and their inhibitors, and endothelial cell proliferation and migration. MMPs, tissue plasminogen activator, urokinase plasminogen activator, TIMPs, and PAIs are expressed by myometrial and leiomyoma cells and, similar to observed in other systems, their expression is regulated by various cytokines, chemokines, and growth factors.[22,103,163−168]

Hypoxia, a condition that promotes cellular stress and the inflammatory response, alters the expression of several cytokines, chemokines, and eicosanoids, as well as the expression of several proteases and angiogenic factors that enhance the development and progression of fibrosis.[169−176] VEGF, FGF, EGF, and TGF-β, either alone or through synergistic interactions, stimulate plasminogen activator (PA) expression, which converts latent TGF-β into its active form.[169−176] The activation of TGF-β by inhibiting PA expression provides a feedback loop that modulates FGF and VEGF expression, and, whereas FGF-2 and TGF-β have opposing effects on PA activity, FGF acts as a potent inducer of uPA expression with a relatively modest effect on PAI-I synthesis, and TGF-β downregulates uPA and upregulates

PAI-I synthesis.[169–176] Several cytokines and chemokines also regulate the expression of the fibrinolytic system, including several interleukins, M-CSF, GM-CSF, and MCP-1.[63,64,66,74,75,90,93,111,132,153,158,169,177–182]

Angiogenesis also depends on the balance between angiogenic factors and their inhibitors. Among the angiogenic suppressors are cytokines, such as TGF-β, TNF-α, and the interferons.[77,93,115,169,171,173,175,176,183–187] Other factors with angiogenic inhibitory activities include collagen synthesis modifiers, protamine that inhibits the mitogenic action of FGF, PF-4, HA, thrombospondin, and angiostatin. The expression of these mediators in the myometrium and leiomyoma implies that both their direct and indirect actions can alter angiogenesis during leiomyoma development and growth. Several antiangiogenic factors known to control and terminate the multistage process of angiogenesis, including protease inhibitors, inhibitors of growth factors, cytokines, and chemokines, are being investigated for their potential in controlling angiogenesis and inflammation. These compounds may be useful as potential agents in the management of leiomyoma growth.

TRANSFORMING GROWTH FACTOR-β: THE PRINCIPAL MEDIATOR OF LEIOMYOMA FIBROSIS

TGF-β is universally recognized as a key profibrotic cytokine due to its multifunctional role in cell growth and differentiation, angiogenesis, apoptosis, inflammation, regulation of ECM, and in regulating the expression of adhesion molecules, proteases, and protease inhibitors. TGF-βs consist of three isoforms: TGF-β1, TGF-β2, and TGF-β3, produced by many cell types as latent proteins and intracellularly cleaved to form two mature homodimeric peptides. The mature TGF-β is noncovalently associated with latency-associated peptide, which is required for efficient secretion and prevents the binding of TGF-β to cell surface receptors, maintaining bioavailability in the ECM compartment. ECM proteins such as latent TGF-β-binding protein 1 (LTBP-1), LTBP-2, -3, and -4 and fibrillin-1 and –2 are involved in sequestration of latent TGF-βs in ECM and functionally regulate their activation.[188–202] Activation of latent TGF-β is a prerequisite for binding to specific cell surface receptors, which consist of TGF-β type I, type II, and type III receptors. TGF-β type IIIR, also known as betaglycan, exists in a soluble form and associates with TGF-β in the ECM, serving as a regulatory mechanism in maintaining bioavailability of TGF-β at the tissue level.[188,189,203–205] TGF-β mediates its actions through binding to and activation of TGF-β type IIR, resulting in activation of TGF-β type IR and recruitment and activation of several signaling pathways, specifically Smad signaling. Smads are composed of pathway-specific regulatory Smads (Smad1, 2, 3, 5, and 8), the common Smad (Smad4), and the inhibitory Smads (Smad6 and 7). TGF-β type IR activates Smad2/3, which associates with Smad4, causing translocation of the signaling molecules into the nucleus where they direct specific transcriptional responses to TGF-β actions. The inhibitory Smad7 interacts with TGF-β type IR and prevents phosphorylation of regulatory Smads, interrupting TGF-β-induced signaling. TGF-β also activates the MAPK pathway; as such, activation of Ras/MEK signaling acts as a positive regulator of cell growth, whereas activation of the p38 MAPK/JUK pathways elicits negative regulation of cell growth.

TGF-β both stimulates and inhibits cell growth, and its mitogenic activity appears to be indirect and due to the induction of growth factors such as EGF, PDGF, and PDGF α receptor.[188,204,205] The role of TGF-β in angiogenesis, similar to its action on cell growth, displays a biphasic effect; at low doses, it synergistically enhances, whereas at high concentrations it decreases vascular invasion of cultured endothelial cells induced by VEGF and FGF. TGF-β inhibits the activities of other angiogenic factors in endothelial proliferation and migration under most cell culture conditions and can stimulate the production of ECM and proteinase inhibitors that are involved in angiogenic activity.[190–202]

Deletion of the TGF-β1 gene through homologous recombination suggests a fundamental role for TGF-β in regulating inflammatory response, and studies conducted with mice heterozygous for TGF-β1 (+/−) implicate TGF-β's role in initiation and progression of fibrosis. As such, overexpression of TGF-β is widely accepted as a key factor in several fibrotic disorders, including pulmonary fibrosis, glomerulonephritis, cirrhosis of the liver, and dermal scarring. In various fibrotic disorders, the apoptotic process has been associated with progression of this abnormality.[188,203–206]

Apoptosis is a mechanism by which cells undergo programmed cell death and is recognized by its characteristic morphological and biochemical changes. In general, apoptosis is mediated through extrinsic (death receptor) and intrinsic (mitochondrial) pathways.[207,208] The extrinsic pathway involves binding of a death ligand to its cognate cell surface receptor, where the ligand may be an integral membrane protein on a neighboring cell (e.g., Fas ligand) or a soluble extracellular protein (e.g., TNFα). The intrinsic pathway involves several proteins released from the mitochondrial intermembrane into the cytoplasm (i.e., cytochrome c). TGF-β receptor signaling directly or indirectly participates in the apoptotic process by regulating various components of extrinsic and intrinsic apoptotic pathways.[206–208] For instance, the apoptotic process is accompanied by a decrease in the level of Bcl-X L protein and a low Bax/Bcl-X L ratio. TGF-β downregulates Bcl-2 and Bcl-X L while it increases the expression of p53 and Bax; this action of TGF-β occurs through interactions with TNF-α, a key regulator of Bax and p53 expression. TGF-β also activates caspases 3, 8, and 9. TGF-β exerts inhibitory actions on cell growth primarily by arresting cells at the G1 stage of the cell cycle(a stage where cells undergo apoptosis), as well as by downregulation of proliferative proteins, such as c-myc, coupled with upregulation of cell cycle inhibitory proteins p15INK4b, p21CIP1, or p27KIP.[203,206] Enhanced Smad2 expression and Smad7 nuclear translocation has been implicated as a mechanism of TGF-β-induced apoptosis in vitro and in vivo.[203] Because the gene promoters for TGF-β2 and -3 contain TATA boxes and a common proximal CRE-ATF site, they are subject to hormonal and developmental regulation; however, the TGF-β1 promoter lacks the classic TATA box and has multiple regulatory sites that can be immediately activated by early genes, such as c-jun, c-fos, and EGR-1, and by several oncogenes, such as Abl, Fos, Jun, Ras, and Src.[188,203–206] Several different gene products participate in mediating the apoptotic process, inflammatory response, and fibrinogenesis, and they are regulated downstream from these immediate early genes and oncogenes.

The expression, regulation, and potential role of the TGF-β system in leiomyoma has been well documented.[17,19,26,28,106,164,165,209–215] TGF-β isoforms, TGF-β receptors, TGF-β-binding proteins, and all of the components involved in their signaling pathways, including Smads, are expressed in leiomyomas and myometrium and their isolated smooth muscle cells. To an extent, the expression of TGF-β systems are elevated in leiomyomas, with a menstrual cycle–dependent occurrence, implicating their regulation by ovarian steroids. In addition to ovarian steroids, TGF-β self-regulates its own expression in leiomyoma and myometrial cells, and GM-CSF, a key cytokine known to regulate fibroblast transition into the myofibroblastic phenotype, regulates the expression of TGF-β as well. Leiomyoma cells were characterized by their ability to release more TGF-β when compared with myometrial cells, which retained more TGF-β, a mechanism that could account for the difference in the local bioavailability of TGF-β in leiomyoma.[106] Under culture conditions where leiomyoma and myometrial smooth muscle cells were maintained in a quiescent state, TGF-β isoforms do not act as mitogens; however, they increased the rate of DNA synthesis displaying a higher response to TGF-β1 and TGF-β3 when compared with TGF-β2.[106] Other studies have demonstrated a higher level of TGF-β3 mRNA in leiomyomas and a higher mitogenic response to TGF-β3, as compared with TGF-β1 in leiomyoma smooth muscle cells and to myometrial smooth muscle cells.[17,19] Comparatively, TGF-β1 has been identified as a

dominant isoform in other fibrotic tissues, and TGF-β3-associated fibrotic activity has been found to be due to, and mediated through, the induction of TGF-β1,[216–219] although controversial observations in rat dermal wound healing implicate antifibrotic activity for TGF-β3. Because TGF-β isoforms are subject to posttranscriptional regulation, differences in the levels of protein expression may reflect their tissue-specific functions, which are mediated through the same receptor system.

Leiomyoma is characterized by overexpression of TGF-β type IIR when compared with the myometrium and pretreatment of leiomyoma smooth muscle cells (LSMC) with TGF-β type IIR antisense oligomers and/or neutralizing antibodies prevents TGF-β receptor-mediated actions.[26] In addition, E2 and TGF-β interactively regulate LSMC growth, and inhibition of endogenous TGF-β or blocking TGF-β receptor action (TGF-β exogenous action) reduces E2 action, TGF-β self-regulation, and PAI-1 expression. These results suggest that cross talk between TGF-β and downstream ER signaling may regulate these and other cellular events in leiomyoma. In addition, the expression of MMPs and their tissue inhibitors, TIMPs, in leiomyoma and myometrium was differentially regulated by TGF-β in MSMCs. MMPs and TIMPs play a central role in inflammatory response, angiogenesis, apoptosis, and ECM turnover, and their regulation by TGF-β further points out the importance of TGF-β in the pathophysiology of leiomyoma. Microarray studies have provided additional evidence of the importance of TGF-β regulatory actions in the expression of many genes, some of which have not been known previously to be a target of TGF-β action.[107] GnRHa therapy and treatment of isolated LSMCs and MSMCs with GnRHa has been shown to inhibit TGF-β, TGF-β receptor expression, as well as the expression of several other cytokines and chemokines. In addition to GnRHa, available evidence also indicates that treatment with antiestrogens, P4, SERMs, and SPRMs alters the growth and expression of several proinflammatory and profibrotic mediators in leiomyoma and myometrium, as well as in their isolated smooth muscle cells.[26,220,221] Treatment with asoprisnil decreases the expression of proliferating cell nuclear antigen-positive, EGF, IGF-I, TGF-β, EGF receptor, IGF-IRa, TGF-β RII, and Bcl-2 expression, and it increases cleaved caspase-3 in leiomyoma cells but not in myometrial cells. These results suggest that asoprisnil selectively inhibits proliferation by downregulating the expression of growth factors and their receptors and induces apoptosis in leiomyoma cells without affecting proliferation and apoptosis in normal myometrial cells.[29] It has also been reported that blockade of TGF-β signaling with the TGF-β type IR kinase inhibitor SB-525334 significantly decreases tumor incidence and multiplicity, and reduces the size of these tumors in the EKR rat model, although pharmacological inhibition of TGF-β signaling was observed to promote the development of epithelial tumors,[222] a phenomenon that is also observed in other systems.

TGF-β regulates the expression of Smad3 and Smad7, and this self-regulatory mechanism controls TGF-β receptor-mediated action. We have reported that TGF-β and GnRHa activate Smad in LSMCs and MSMCs, and their cross talk alters the expression, induction, and activation of these and other pathways.[215] Because GnRHa also targets the expression of TGF-β-, TGF-β receptors, and, more selectively, Smad7 expression, this suggests that downstream genes regulated by TGF-β receptors are also the targets of GnRHa action. Smad signaling results in the transcriptional activation of ECM, adhesion molecules, proteases and protease inhibitors, as well as cell cycle regulators; alteration in the cellular distribution of the expression of Smads has been associated with tumorigenesis.[188,203–206] TGF-β receptors also use components of other pathways, including mitogen-activated protein kinase (MAPK), as part of their intracellular signaling, with functional interactions with PKC, Ca^{2+}/CaM, and Smads. Functional interactions between MAPK and Smad signaling pathways mediate the biological actions of GnRH, TGF-β, and activin (a member of the TGF-β family), which, in turn, regulate the expression of gonadotropins, GnRH, and GnRH receptors.[223,224] GnRHa and TGF-β1 treatment increases nuclear translocation of Smads

and ERK1/2 in LSMCs and MSMCs, and the differences observed in the mRNA expression of immediate early response genes c-fos and c-jun in LSMCs and MSMCs suggests a postreceptor divergence of signaling in these cells. GnRHa and TGF-β1, through these signaling pathways, alter the expression of type I collagen, fibronectin, and PAI-1 mRNA in LSMCs and MSMCs in a cell- and gene-specific manner.[28] Because the products of these genes regulate diverse biological activities, and specifically, ECM turnover, their differential expression may indicate a critical role for these genes in leiomyoma growth and regression. [28] In recent microarray studies, several genes in this category were identified displaying differential expression in leiomyoma and myometrium, and they were targeted by GnRH therapy and TGF-β treatment in vitro.[23,107,213,225–229]

Results generated predominantly from in vitro studies have led to the hypothesis that TGF-β action in tissue fibrosis is indirect and is mediated through the induction of connective tissue growth factor (CTGF or CCN2). Similar to TGF-β, elevated expression of CCN2 has been documented in several fibrotic tissues and is considered a key regulator of connective tissue formation, angiogenesis, and tissue fibrosis.[230,231] However, the expression of CCN2 in leiomyomas is inversely correlated with not only TGF-β1, but also with TGF-β3 expression. [102] Such an inverse correlation was also observed between the expression of TGF-β1 and TGF-β3, and that of CCN3. However, under in vitro conditions, TGF-β1 increased the expression of CCN2 and CCN4 in LSMCs and MSMCs, whereas it inhibited the expression of CCN3 in the same cells. TGF-β3 has been shown to increase CCN2 expression in LSMCs,[232] suggesting that both TGF-β isoforms may have similar functions in regulating the expression of CCNs in LSMCs and MSMCs. Gene expression profiling studies have also revealed a lower expression of CCN2 in hypertrophic scars, which express elevated levels of TGF-β1 as compared with that observed in normal skin.[233] These observations in leiomyoma and hypertrophic scars suggest that CCN2 may not serve as a common downstream mediator of TGF-β action in all fibrotic disorders. To understand the biological significance of the variable expression profiles of CCNs and their regulation in leiomyoma and myometrium requires a more detailed investigation, considering the wide range of biological activities for these signaling molecules observed in other cell types.

OTHER PROFIBROTIC MEDIATORS

The EGF family members, including EGF, TGF-α, and heparin-binding (HB)-EGF, serve as mitogenic and differentiation factors for several cell types of ectodermal, mesodermal, and endodermal origins, and they play a key role in the wound healing process and in tissue fibrosis.[234–237] Leiomyoma and myometrium express EGF, TGF-α, HB-EGF, and EGF receptor with relatively stable expression throughout the menstrual cycle, although it has been shown that higher levels of EGF are expressed during the luteal phase in patients with leiomyoma.[108] The local production of EGF, TGF-α, and HB-EGF, as well as the presence of their receptors, imply an autocrine/paracrine role for these growth factors in regulating biological processes in the myometrium and leiomyomas. The EGF family of growth factors participates in many cellular activities associated with tissue fibrosis, including cell growth and ECM expression by fibroblasts and myofibroblasts, more specifically, through interaction with other growth factors such as PDGFs and IGFs, in both leiomyoma and myometrial smooth muscle.

The PDGF family includes PDGF-AA, PDGF-BB, and PDGF-AB, which are derived from disulfide-bonded homo- and heterodimers of two structurally similar A- and B-polypeptide chains (PDGF-A and PDGF-B), as well as two additional isoforms, PDGF-C and PDGF-D, which are derived from protease activation of other isoforms.[238–240] Although PDGF is expressed by a variety of cell types, including inflammatory-related cells, smooth muscle cells, fibroblasts, and myofibroblasts, platelets and macrophages serve as the major source

of PDGF.[241,242] Macrophage-derived PDGFs act as chemotactic factors for myofibroblasts, and abundant evidence supports the idea that PDGFs play a role in the progression of fibrotic disorders by promoting myofibroblast proliferation, angiogenesis, and ECM production (such as collagen, fibronectin, and glycosaminoglycans).[238−240,243] Only limited data are available regarding the expression of PDGFs and their receptors in leiomyomas. The expression of PDGFs and PDGF receptors in myometrium and leiomyoma displays relatively similar abundance throughout the menstrual cycle. Myometrium and primary cultures of MSMCs express PDGF-AB as well as PDGF β receptor, but they express a low level of PDGF α receptors.[108] Leiomyoma has been reported to contain more PDGF receptor binding sites than observed in the myometrium, but with the receptors they exhibit lower affinity for PDGF. Direct evidence is currently lacking that confirms that the expression of PDGFs and their receptors is regulated by the ovarian steroids, although reduction in their expression in leiomyoma and myometrium from women who have received GnRHa therapy indirectly supports the possibility for steroid-related regulation of their expression.[244]

PDGFs regulate their own expression and through their interactions with other growth factors and cytokines, they can induce various cellular activities that promote tissue fibrosis. Adenoviral overexpression of PDGF-B only causes a mild fibroproliferative response, whereas combination with TGF-β1 results in significant mesenchymal cell proliferation and collagen deposition. In addition, PDGF and PDGF receptors serve as mediators of the mitogenic actions of TGF-β and IL-β in fibroblasts and smooth muscle. IFN-γ is reported to alter the expression of PDGF-B,[238−240] and evidence exists that that it also regulates IL-13 action in the pathogenesis of lung fibrosis, stimulating the production of PDGF through a STAT-6-dependent mechanism.[115] This suggests that the paracrine actions of cytokines serve as important mechanisms for the maximal growth of and chemotactic responses of myofibroblasts. FGF-2 also induces the expression of PDGF receptor in bronchial smooth muscle cells. Soluble PDGF binding proteins such as α2-macroglobulin, PDGF-associated protein, and the extracellular part of the PDGF-a receptor, as well as ECM components, such as secreted protein acidic and rich in cysteine (SPARC), regulate the activity and availability of PDGF isoforms. PDGF receptor tyrosine kinase inhibitors have been used for controlling PDGF-induced tissue fibrosis.[243] Administration of the PDGFR selective tyrosine kinase inhibitor AG1296, a tyrphostin analog, has been shown to reduce pulmonary fibrosis in a rat model. Imatinib is a PDGF receptor tyrosine kinase inhibitor that has been shown to ameliorate chronic allograft nephropathy.[245]

The insulinlike growth factor (IGF) system is composed of IGF-I and -II, the IGF receptors (types I and II), and IGF binding proteins (IGFBPs), as well as a range of IGFBP proteases, [1,246,247] and are expressed in temporal and tissue-specific manners. The family is regulated by numerous factors, including hormones, growth factors, and physiological parameters, including nutrition, injury, and disease. IGF-I and IGF-II exhibit considerable homology in amino acid sequence, and both carry significant homology to insulin. IGFBPs exhibit widespread serum, tissue, and extravascular fluid distribution, and through high affinity binding, they regulate the biological activities of IGF. Many components of the IGF family also bind to the ECM where, upon release, they can bind to IGF receptors present on surrounding cell, specifically, to connective tissue fibroblasts. IGF and other growth factors are released from the ECM through proteolytic actions of various proteases, such as neutrophil proteases, cathepsin G, and elastase. Although IGFs have growth-promoting activity in specific cell types, they serve as strong antiapoptotic and differentiation factors for many cell types, including myoblasts.[1,246,247] Similar to the mitogenic activity of EGF, IGF-1 mitogenic activity requires interaction with other growth factors, such as EGF and PDGF. PDGF, TGF-α, HB-EGF, and IGF-1, which are expressed and released by many cell types, can potentially act in this manner and influence cell migration, proliferation, and

angiogenesis, even at the earliest stages of leiomyoma growth. IGFs, IGFBPs, and IGF receptors are expressed in both leiomyoma and myometrium throughout the menstrual cycle. However, no difference in expression of IGFs, IGFRs, and IGFBPs has been observed in leiomyoma when compared with myometrium.[108]

The VEGF and FGF families of growth factors are known as key regulating factors for angiogenesis, an important step in the pathogenesis of tissue fibrosis. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PlGF). Alternative exon splicing of VEGF genes results in the generation of several isoforms of each factor, each with multiple and diverse biological functions.[176,183] Through alternative splicing of pre-mRNA of the VEGF-A gene, several protein isoforms are generated, which include VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206. VEGF165 is the predominant isoform; VEGF145 and VEGF183 are less frequent isoforms. Most VEGF-A isoforms contain a heparin-binding domain, and VEGF121 is predominantly detected as a soluble form, whereas VEGF189 and VEGF206 are relatively insoluble and are located in association with cells or are sequestered in ECM, binding heparin with high affinity. VEGF-C and VEGF-D induce capillary endothelial cell migration and proliferation, and they act as vascular permeability factors and are abundantly expressed in the heart, lung, skeletal muscle, colon, and small intestine. VEGF's mediate their actions by binding to the tyrosine kinase receptors Flt-1 (FMS-like tyrosine kinase) and KDR (kinase domain region) receptors, although they may employ other different signal transduction properties. VEGF121, VEGF165, and VEGF189 are expressed in leiomyoma and myometrium and have been characterized by in situ hybridization and found to be localized smooth muscle cells. It has also been observed that levels of VEGF mRNA are significantly higher during the secretory phase than in the proliferative phase of the menstrual cycle in normal myometrium, and that these levels do not change significantly when compared with leiomyoma. Leiomyoma tissue collected from women treated with a GnRH analog did not express significantly different levels of VEGF mRNA as compared with leiomyoma tissue collected from untreated women.[108,248] Although ovarian steroids regulate VEGF expression in other steroid-sensitive cells, the lack of direct evidence, as well as the observation that VEGF expression remains unaffected in GnRH tissues, indicates that factors other than ovarian steroid may regulate VEGF expression in leiomyoma and myometrium.

The FGF family is classified into subgroups according to structure, biochemical properties, and expression.[249] The biological activity of FGFs is mediated through FGF receptors, which are members of the cell surface receptor tyrosine kinase family. These receptors contain Ig-like domains, which regulate binding affinity and ligand specificity, and a stretch of acidic amino acids (acidic box domain) located between Ig-like domains I and II, followed by a heparin binding region and a cell adhesion homology domain. FGFs are characterized by the lack of an extracellular export sequence, which has become an impediment of their role in angiogenesis. The alternative model of how bFGF can be transported out of the cell is based on binding of bFGF to a heparin sulfate proteoglycan that is located at the cell surface; the heparan sulfate-bound bFGF could then be activated and regulated in a manner through which the action of heparanase bypasses the need for a transport signal.[249,250] The expression bFGF, FGF receptor 1, and FGF receptor 2 has been characterized by immunohistochemistry and Western blotting in leiomyomas and in myometrium, with detectable changes observed between leiomyomas and myometrium in staining intensity following GnRHa treatment. No tissue differences were observed when stained for FGF receptor 2 and no significant differences were observed between the untreated and GnRHa-treated groups. It was concluded that the expression and localization of bFGF and, to some extent, its receptors, are influenced by sex steroid hormones in leiomyomas and in myometrium, and lack of differences in expression between leiomyomas

and myometrium favors the theory that bFGF may not necessarily contribute to the differences in growth regulation in these tissues.[251]

Signal transduction cascades activated by growth factor, cytokine, and chemokine receptors are mediated predominantly through Ras/MAPK, signaling, including phosphoinositol kinase, STATs, PKA, PKC, Src family tyrosine kinase, phospholipase C, and antiapoptotic kinase Akt signaling pathways, as well as several other transcriptional regulators. Translocation of the activated signals into the nucleus results in phosphorylation of several transcription factors, including AP-1, Elk-1, and SAP, which, in turn, regulate the expression of target genes. MAPK activation also results in activation of intranuclear proteins, including cyclin D1, which is important for the progression of the cell cycle from G1 to S-phase (for review, see[234–240,246,249]). MAPK is also activated by integrins, which are reported to be expressed in leiomyoma;[252] however the biological significance of this finding remains to be investigated. Focal adhesion kinase (FAK) serves as an integrin signaling molecule and participates in promoting angiogenesis and cell migration, and it is expressed in both leiomyoma and myometrium, exhibiting altered expression during GnRHa-induced tumor regression.[253] Integrins have also been associated with processes leading to activation of latent TGF-β. TGF-β receptor has also been implicated in IGFBP-3 cell signaling through Smad2 and Smad3, resulting in IGFBP-3-induced gene transcription and an inhibitory growth response.[254] In addition, IGF-II/M-6-PR play a role in TGF-β1 activation by binding to latent TGF-β1.[189]

REGULATORY FUNCTIONS OF MICRO-RNAS IN INFLAMMATION AND FIBROSIS

Throughout the past several years, micro-RNAs (miR-NAs) have emerged as key regulators of gene expression stability. miRNAs are small (22 nucleotide) RNA that bind to the 3′ untranslated region of target genes, ultimately leading to mRNA cleavage and degradation. miRNAs are transcribed as precursor form from genes that constitute ~3% of the human genome, followed by extensive processing by Drosha, DGCR8/Pasha, and Dicer; in the nucleus and cytoplasm, precursor miRNAs form into mature miRNAs (19 to 24 nucleotides). The mature miRNAs, through interaction with RNA-induced silencing complex (RISC), regulate the expression of their specific target mRNAs.[255,256] Evidence generated based on computational algorithms and conformational experiments indicate the presence of thousands of miRNAs, carrying potential to target the expression of 30% of all human genes.

Considerable evidence based on expression profiling supports the role of miRNA in the initiation and progression of many human cancers.[257] Expression profiles of a few hundred miRNAs in leiomyomas and matched myometrium, as well as their isolated smooth muscle cells, also support their potential regulatory functions in leiomyoma gene expression stability.[258,259] Among the miRNAs whose expression was significantly altered in leiomyomas as compared with myometrium are the let-7 family, miR-17, miR-21, miR-23b, miR-29b, miR-34a, miR-26a, miR-18a miR-125b, miR-139, miR-155, miR-206, miR-181a, and miR-142–5p. Altered expression of some of these miRNAs was also observed to be specifically different in leiomyomas from African Americans when compared with whites and other ethnic groups.[258,259] These miRNAs are predicted to target the expression of many genes, including ovarian steroid receptors, TGF-β, TGF-β receptors, and several proinflammatory and immune-related genes. The regulatory functions of miRNAs in inflammation and immune system regulation occur through the well-coordinated control of specific genes, and this regulation is critical for initiation and termination of inflammatory and immune-related processes. Different patterns of expression of specific miRNAs have been observed in hematopoietic cell lineages, and their association with proliferation and

differentiation, along with their altered expression in cancer and immune and inflammatory disorders, are evidence that miRNAs play important roles in these processes. Several miRNAs, specifically let7, miR-17–5p, miR-20a, miR-106a, miR-125b, miR-146, and miR-155, have been identified to influence the expression of inflammatory and immune response mediators.[256,260] The expression of miR-155, miR-125, miR-17–5p, miR-20a, and miR-106a has been associated with inhibition of the differentiation and maturation of monocytes by regulating the expression of RUNX1 and macrophage colony-stimulating factor receptor (M-CSFR). Inflammation that results in nuclear factor (NF)-κB-dependent activation of monocyte activity results in the induction of miR-146, which, in turn, inhibits TNF receptor–associated factor 6 (TRAF6) and IL-1 receptor–associated kinase 1 (IRAK1) genes, the two key molecules downstream to the Toll-like receptor and cytokine signaling. Activation of murine macrophages by IFN-β also resulted in upregulation of miR-155 through the induction of TNF-α and JNK pathways, whereas miR-155-deficient mice are immunodeficient.[261] Furthermore, IL-6 has been reported to induce the expression of let-7a and contribute to survival through the expression and activation of STAT3 phosphorylation.[260–264] Hypoxia, which promotes angiogenesis, has been shown to reduce miR-15b and miR-16 expression; miR-221 and miR-222 indirectly reduce the expression of endothelial nitric oxide synthase (eNOS), which is an angiogenic inducer and contributor to other endothelial cell functions.[217] The miR-17–92 cluster contains miR-17–5p, miR-17–3p, miR-18a, miR-19a, miR-20a, miR-19b, miR-92–1, and miR-18 and miR-19 have been found to target the expression of CTGF and thrombospondin-1 (TSP-1), respectively.[94] The biological significance of miRNA-mediated CTGF regulation is due to the ability of CTGF in mediating the profibrotic actions of TGF-β in tissue fibrosis (for review, see Luo and Chegini[258]).

Further elucidation of the expression, regulation, and function of miRNAs in leiomyoma as compared with myometrium may allow for providing markers for diagnosis, mechanisms of inflammation-associated regulation of miRNA expression, and miRNA-mediated gene targeting, for therapeutic intervention to regulate leiomyoma growth. Furthermore, establishing the relationship between inflammatory response and miRNA expression profiles in the uterus could assist us in identifying novel links between inflammation, tumorigenesis, and tissue fibrosis.

SUMMARY AND CONCLUSIONS

Symptomatic uterine leiomyomas cause chronic pelvic pain and abnormal uterine bleeding. Medical therapies, which include analgesics or hormones for both pain and abnormal bleeding, are initially used to relieve these symptoms. The most common hormonal therapies involve the use of GnRH agonists. Recent clinical and preclinical investigation has provided evidence supporting the use of SERMs and SPRMs as possible alternative therapies to suppress leiomyoma growth. These therapies are not only expensive but have associated side effects that can be either intolerable to the patient or are capable of causing further morbidity, such as hot flashes and bone loss. However, many patients end up requiring hysterectomies when conservative medical therapy becomes poorly tolerated or ineffective. Hysterectomies are one of the most common operations, and many are directly due to the presence of symptomatic leiomyomas. Hysterectomies are major operations that carry potential risks, such as bleeding, infection and adhesion formation, and possible damage to other vital anatomical structures that can further add to the pain and suffering of the patient while increasing the overall cost to society. It is paradoxical that so little is known about the biology of a tumor that has such a costly morbidity.

In recent years, substantial progress has been made toward the identification and understanding of the molecular environment surrounding leiomyomas. Based on these

studies, alongside comparative assessments with other systems, it appears that leiomyoma growth and regression are determined by the rate of cell proliferation, differentiation, apoptosis, angiogenesis, protease production, and ECM deposition. Ovarian steroids and various growth factors, cytokines and chemokines, peptide hormones and stress factors, and so on, are key regulators of many of these processes. However, the biological functions of many of these molecules in leiomyoma remain to be determined. Classically, ovarian steroid action is mediated through interactions of ER and PR with different DNA response elements that regulate transcriptional activation of specific genes that regulate these processes. Growth factors, cytokines, chemokines, however, mediate their actions through rapid recruitment and activation of intracellular signaling proteins that translocate into nucleus and regulate similar/different sets of genes. Ovarian steroids can also activate signaling pathways activated by growth factors/cytokines/chemokines, referred to as "nongenomic action" and is now recognized as a novel pathway that could be activated by these mediators even in the absence of E_2. Investigation is needed to test many of the factors/pathways that are used by ovarian steroids, including both genomic and nongenomic pathways, which lead to inflammatory and fibrotic responses and, subsequently, to leiomyoma growth. In addition to leiomyoma smooth muscle cells, activation of many of these pathways and their downstream targeted genes are involved in regulating a wide variety of processes in other leiomyoma cell types that regulate matrix accumulation, angiogenesis, and local inflammatory response.

Leiomyomas develop from transformation of myometrial smooth muscle cells and/or connective tissue fibroblasts. However, the identity of molecule(s) that initiate myometrial cellular transformation and subsequently regulate their growth remains unknown. In vitro studies show that smooth muscle cells are highly plastic and can respond to changes in environmental factors by changing their phenotype. These changes depend on the integration of a large number of molecules that can aggregate and determine the patterns of gene expression appropriate for transformation of myometrial cells into leiomyoma. Recent microarray studies have provided evidence that many genes are differentially expression the myometrium and in leiomyoma, and they can be functionally categorized into transcription factors, as well as regulators of signal transduction pathways, apoptosis, cell and tissue structure, and other metabolic activities. In addition, isolated cells can participate in the transformation of myometrial cells into leiomyoma. However, the expression of many of the putatively identified genes requires confirmation, and definitive studies are needed to elucidate their precise molecular mechanisms of action in initiating the fibrotic response. Definitive studies in this area depend on use of sophisticated conditional gene targeting systems, where understanding their molecular mechanism regulating these genes would assist us to design new therapies complementing GnRHa, SERM, and SPRM that are being used as therapeutic medical management of leiomyoma but with many limitations.

Acknowledgments

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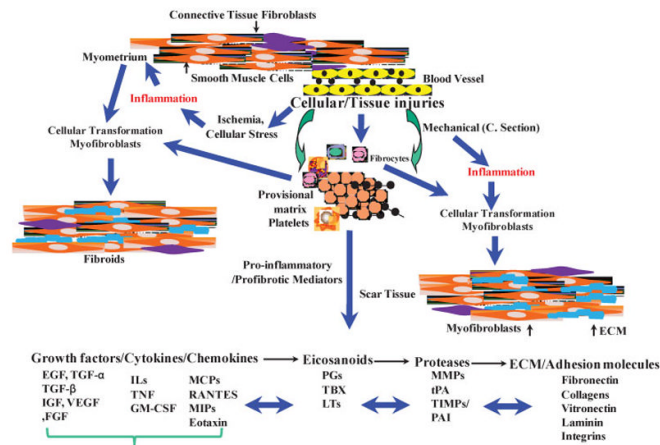
Figure 1.
Schematic diagram illustrating an overview of cellular injury and inflammatory-mediated steps in development of leiomyoma as compared with uterine scar formation caused by mechanical injury (i.e., myomectomy and cesarean delivery). Cellular/tissue injuries and generation of an inflammatory response, either at a small scale (microenvironment) or an extended area (myomectomy/cesarean delivery), results in individual and combined regulatory interactions among several proinflammatory and profibrotic mediators. These mediators, including cytokines, chemokines, growth factors, eicosanoids, proteases, and extracellular matrix (ECM), activate and cause myoblasts and resident fibroblasts differentiation into myofibroblastic phenotype. In addition, possible participation of fibrocytes, which are derived from bone marrow and through circulation reside at the site of inflamed/injury, as well as transformation of vascular endothelial cell into mesenchymal cells, also exist to transform into myofibroblastic phenotype. Collectively, myofibroblasts are highly responsive to the action of various mediators, including cytokines and chemokines, and they produce and deposit various components of ECM that are essential for tissue repair process. Continuous inflammation, excess myofibroblastic transformation, and production of large quantity of ECM with concurrent reduced degradation represent a pathway that leads to development of either leiomyoma and/or scar tissue formation. EGF, endothelial growth factor; FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IGF, insulinlike growth factor; IL, interleukin; LT, leukotriene; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MMP, metalloproteinase; PAI, plasminogen activator inhibitor; PG, prostaglandin; RANTES, regulated upon activation, normal T-cell expressed and secreted; TBX, thromboxane; TGF, transforming growth factor; TNF, tumor necrosis factor; TIMP, tissue inhibitor of metalloproteinase; tPA, tissue plasminogen activator; VEGF, vascular endothelial growth factor.
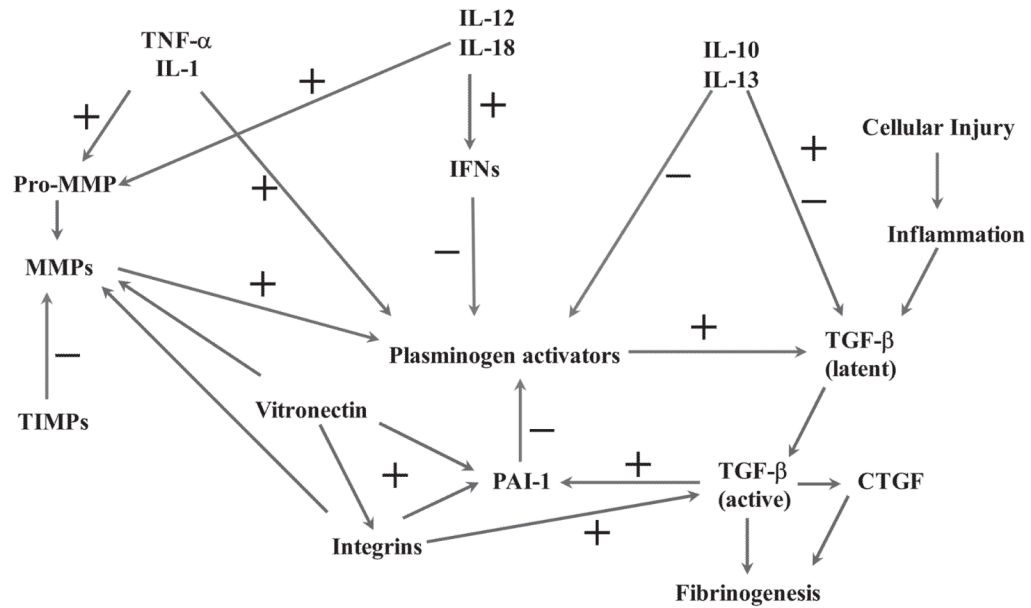
Figure 2.
Schematic diagram of pathways involving cellular injury and inflammatory-mediated regulation of fibrinolysis through the individual and combined actions of several cytokines and chemokines. Because fibrinolysis is an essential component of provisional matrix establishment and resolution, and angiogenesis and a key part of cellular/tissue repair involving the activation of transforming growth factor (TGF-β), any alteration in this process can often lead to defective fibrinogenic activity and tumorigenesis, including leiomyomas. IFN, interferon; IL, interleukin; MMP, metalloproteinase; PAI, plasminogen activator inhibitor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor.
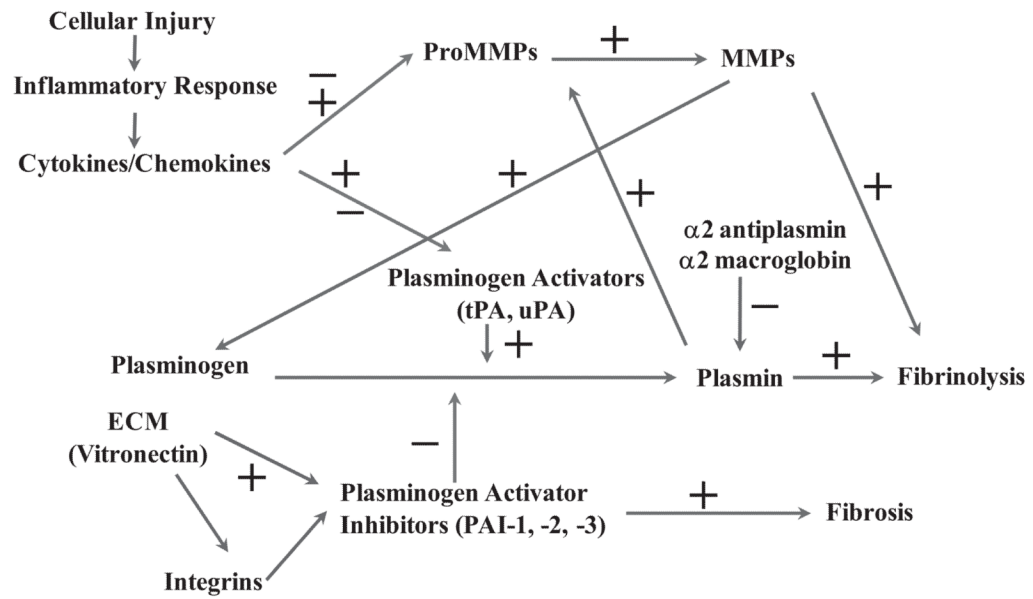
Figure 3.
Schematic diagram of pathways involving cellular injury and inflammatory-mediated regulation of proteolytic activities involving matrix metalloproteinases (MMPs) and their regulation by several cytokines and chemokines. MMPs and their tissue inhibitors play a central role in provisional matrix degradation, cellular migration, angiogenesis, as well as tissue turnover, processes required for normal cellular/tissue regeneration and repair. Aberrant expression and activation of MMPs has been associated with various fibrinogenic and tumorigenic disorders, including leiomyomas. ECM, extracellular matrix; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.
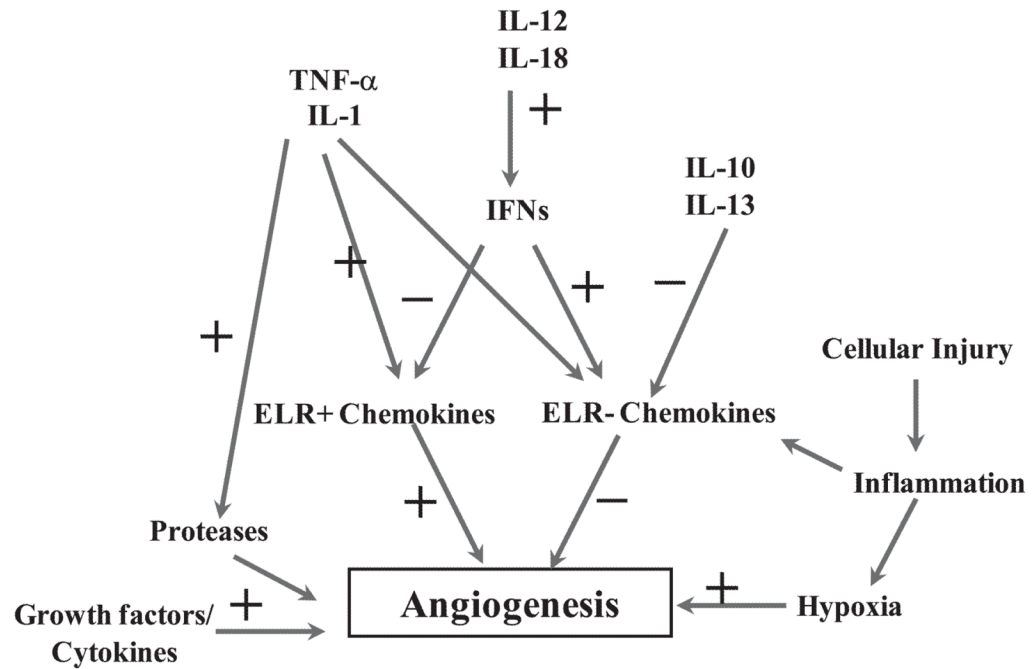
Figure 4.
Schematic diagram of pathways involving cellular injury and inflammatory-mediated regulation through the action of individual or combined interaction of several cytokines and chemokines, including ELR- and ELR+chemokines. Because angiogenesis is a key step in neovacuolization and the reparative process of cellular/tissue injury, any alteration in this process can often lead to increased fibrinogenesis and tumorigenesis, including leiomyomas. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.