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Molecular Biomarkers of Graves' Ophthalmopathy

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Abstract

Graves' ophthalmopathy (GO), a complication of Graves' disease (GD), is typified by orbital inflammation, ocular tissue expansion and remodeling and, ultimately, fibrosis. Orbital fibroblasts are key effectors of GO pathogenesis exhibiting exaggerated inflammatory and fibroproliferative responses to cytokines released by infiltrating immune cells. Activated orbital fibroblasts also produce inflammatory mediators that contribute to disease progression, facilitate the orbital trafficking of monocytes and macrophages, promote differentiation of matrix-producing myofibroblasts and stimulate accumulation of a hyaluronan-rich stroma, which leads to orbital tissue edema and fibrosis. Proteomic and transcriptome profiling of the genomic response of ocular and non-ocular fibroblasts to INF-γ and TGF-β1 focused on identification of translationally-relevant therapeutic candidates. Induction of plasminogen activator inhibitor-1 (PAI-1, SERPINE1), a clade E member of the serine protease inhibitor (SERPIN) gene family and a prominent regulator of the pericellular proteolytic microenvironment, was one of the most highly up-regulated proteins in INF-γ- or TGF-β1-stimulated GO fibroblasts as well as in severe active GD compared to patients without thyroid disease. PAI-1 has multifunctional roles in inflammatory and fibrotic processes that impact tissue remodeling, immune cell trafficking and survival as well as signaling through several receptor systems. This review focuses on the pathophysiology of the GO fibroblast and possible targets for effective drug therapy.

Keywords

Graves' disease; orbitopathy; biomarkers; inflammatory cytokines; TGF-β; PAI-1; SERPINs; fibrosis; plasmin cascade; tissue remodeling

1. Introduction and clinical manifestations of orbitopathy

Graves' ophthalmopathy (GO), which affects approximately 50 percent of Graves' disease (GD) patients, exhibits a prominent female gender bias (Wiersinga and Bartalena, 2002).

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Conflict of interest

The authors declare no conflict of interest.

Ocular involvement varies occurring before, with or after the onset of overt thyroid disease and presents as swelling, inflammation, redness, dryness, proptosis, eyelid retraction, foreign body sensation and stromal remodeling which can be extensive (Dik et al., 2016; (Wiersinga and Bartalena, 2002; Wiersinga et al., 1998; Bahn, 2010; Piantanida et al., 2013; Chng et al., 2012). Severely affected individuals (3-5% of those with ocular pathology) experience pain, diplopia, compression of the optic nerve and subsequent loss of vision largely as a consequence of connective tissue and muscular expansion within the confines of the boney orbit (Bahn, 2015, 2016). The most common ocular symptom of early mild GO is upper eyelid retraction coincident with complaints of foreign body sensation, photophobia and tearing. Lagging of the upper eyelid in downgaze (Von Graefe's sign) and inability to close the eyelids completely (lagophthalmos) are further diagnostic hallmarks as is the presence of corneal erosions and superior limbic keratoconjunctivitis (Dolman, 2018). In moderate GO, inflammation and edema may lead to gaze abnormalities and myopathy; the patient may experience vertical diplopia in upgaze with compromised extraocular muscle (EOM) movement secondary to fibrosis (reviewed in Khalilzadeh et al., 2011). In severe disease, EOM/orbital fat expansion and progressive scarring within the orbit, concomitant with connective tissue and glycosaminoglycan accumulation, manifests a worsening proptosis and predisposes the optic nerve to compression (Dik et al., 2016). Chronic complaints of acute dry eye and pain, elevated intraocular pressure, visual field deficits or vision loss are common when the optic nerve is impinged (Bahn 2010) and may warrant surgery to prevent irreversible blindness (Dolman, 2018). The management of mild GO consists of artificial tears and other topical lubricating options; Tarsorrhaphy is considered an alternative for chronic dry eye complaints. Steroids or radiotherapy can reduce inflammation in patients with more advanced GO and ophthalmic surgery is an option in cases of severe or emergent orbitopathy.

2. Cellular pathophysiology

Most patients with GO have EOM and/or adipose tissue enlargement (Dik et al., 2016). Patients under the age of 40 usually exhibit fat expansion while those over 60 present with muscle involvement (Forbes et al., 1986; Anderson et al., 1989). The EOM fibers in GO are separated by an amorphous, granular material consisting of collagen fibrils and non-sulfated glycosaminoglycans (GAGs), with the most abundant and highly-hydrophilic GAG being hyaluronan (HA) (Smith et al., 1989; Dik et al., 2016). HA synthesis is up-regulated in the GO fibroblast in response to IL-2 and TGF-β1 where it accumulates in the orbit connective tissue (Smith et al., 1989; Bahn, 2003; Khalilizadah et al., 2011). In active disease, the polyanionic charge and high osmotic pressure of this hydrated HA-rich matrix results in edema while likely exacerbating cellular growth, migration and inflammatory cell influx (Bahn, 2010; Hufnagel et al., 1984; Natt and Bahn, 1997; Smith et al., 1989; Bahn, 2010; Guo et al., 2011; Evanko et al., 2012; Itano et al., 2002). Although the specific HA synthase involved in orbital disease is uncertain (Galgoczl et al., 2016), earlier findings implicated HAS2 (Kaback and Smith, 1999; Zhang et al., 2009) and ocular fibroblasts derived from a mouse model of GO express significant levels of HAS2 (Gortz et al., 2016) supporting an association between HAS2 and GO pathogenesis.

Orbital fibroblasts are both the major targets of inflammatory cytokines released by infiltrating immune cells as well as active participants in pathological progression (Bahn, 2010, 2015, 2016; van Steensel et al., 2012a,b; Feng et al., 2017; reviewed in Bahn, 2015; Dik et al., 2016). In early GO, diffuse infiltration of primarily CD4+ T cells are the predominant microenvironmental effectors in the orbit but CD8+ T cells, macrophages, plasma cells and B cells are also evident in the EOM and adipose tissue (Pappa et al., 1997; Eckstein et al., 2004). Type 1 helper T cells produce inflammatory cytokines including IL-2, interferon-γ and TNFα the initial stages (Bahn, 2010). As disease progresses to a more chronic stage, type 2 helper T cells produce additional inflammatory cytokines including IL-4, IL −5, IL-6 and IL-10 (Aniszewski et al., 2000) while macrophages, fibroblasts and adipocytes synthesize and release IL-1, IL-6, IL-16 and TGF-α (Kumar and Bahn, 2003; Hiromatsu et al., 2000; Bahn 2010; Pawlowski et al., 2014). In GD-associated CD34+ T cell dysfunction, recent findings suggest that elevated levels of miR-4443 results in the increased expression of IL-1, IL-6, IL-17 and INF-γ (Qi et al., 2017). The increased expression of this cohort of proinflammatory effectors would be expected to impact virtually all orbit-resident cells including stromal fibroblasts and the vascular system. Orbit fibroblasts exhibit exuberant inflammatory responses when compared with fibroblasts from other anatomical sites as well as contribute to immune cell recruitment and activation (Smith et al., 2008; Dik et al., 2016). Indeed, stimulation of orbital fibroblasts with IL-1β, leukoregulin, INF-γ, or TNF-α results in a greater induction of cytokines, HA, prostaglandins and profibrotic factors compared to dermal cells (reviewed in Dik et al., 2016). It is not immediately obvious, however, whether this reflects their anatomical or developmental uniqueness relative to mesenchymal-derived fibroblasts. An increased number of T helper17 cells, moreover, which synthesize IL-17A in response to stimulation with IL-23, traffic to the orbit during development of thryoid-associated orbitopathy where the secreted IL-17A amplifies the proinflammatory and fibrotic response of resident fibroblasts promoting their differentiation into matrix-producing myofibroblasts and exacerbating disease progression (Fang et al., 2016, 2017; Zhao et al., 2018).

Human ocular cells can also differentiate into high thyrotropin-expressing mature adipocytes (Sorisky et al., 1996; Valyasevi et al., 1999; Starkey et al., 2003) which may, in part, explain the enlargement of orbital fat in GO. A significant fraction of fibroblasts isolated from the EOM of GO patients express the surface marker Thy-1 (Khoo et al., 2008), as opposed to GO orbital fat fibroblasts in which only 50% are Thy-1^+ (Khoo et al., 2008). Elevated expression of Thy-1 (CD90) defines a fibroblast subpopulation that produces prostaglandin E2, IL-8 and HA (Khoo et al., 2008). When exposed to TGF-β which is strongly expressed in the orbit of patients with mild and severe GO (Pawlowski et al., 2014), these fibroblasts differentiate into myofibroblasts which participate in repair and fibrosis (Smith et al., 2002; Bahn, 2010). The duration and level of TGF-β exposure, coupled with the intrinsic heterogeneity of the ocular fibroblast cohort, may well dictate disease severity (Smith et al., 2002). TGF-β also increases expression of sphingosine-1-phosphate (S1P), a profibrotic effector for various cell types including GO fibroblasts (Ko et al., 2017). Since S1P receptor blockade attenuates expression of fibrotic and tissue remodeling factors in GO cells (Ko et al., 2017), TGF-β signaling may activate several pathways that contribute to ocular inflammation and fibrosis (e.g., Fang et al., 2016; Dik et al., 2016).

Numerous CD34+ fibrocytes are evident in the orbit of GO patients but not in healthy individuals (Douglas et al., 2010; Peng et al., 2012). These cells may play a pivotal role in the pathogenesis of GO, secondary to their expression of IGF-1 and thyroid stimulating hormone receptors, two well-known autoantigens in GO (e.g., Smith, 2015; Bahn, 2015). Orbital fibroblasts in GO patients, in fact, express higher levels of IGF-1R than non-diseased controls (Smith 2003). Receptor-activating antibodies stimulate signaling in orbital fibroblasts to release inflammatory cytokines, including IL-6 and TNF-α (Douglas et al., 2010). Therapeutic targeting of the IGF-1R with teprotumumab, an inhibiting antibody, may provide a therapeutic option for patients with active GO (Mohyi and Smith, 2017).

3. Molecular basis of orbital disease

Expression profiling of orbital tissue and ocular fibroblasts from GO patients revealed significant up-regulation of several immediate-early genes including those encoding the inflammation/fibrosis inducers CYR61, connective tissue growth factor (CTGF) and the serine protease inhibitor plasminogen activator inhibitor-1 (PAI-1) (Lantz et al., 2005; Tsai et al, 2015; Smith et al., 1992; Higgins and Smith, 1993) suggesting involvement in disease initiation and/or progression. Since the proinflammatory cytokines interferon-γ (INF-γ) and IL-1α and the potent profibrotic factor TGF-β1 are implicated in Graves' orbitopathy (Wakelkamp et al., 2003), proteomic and transcriptome analysis of the genomic response of ocular and non-ocular fibroblasts to INF-γ and TGF-β1 focused on identification of potential disease-relevant targets. Of 129 individual proteins resolved in 2-D gel separations of cutaneous fibroblasts suitable for quantitative analysis, the relative abundance of 14% changed in response to INF-γ (Smith and Higgins, 1993a,b). In contrast, 38% of the *de novo*-synthesized proteins resolved in 2-D gel separations of GO fibroblasts were significantly influenced by exposure to INF-γ (Smith et al., 1992; Higgins and Smith, 1993) with an approximately equal number partitioning between the up- and down-regulated sets (Figure 1). This differential sensitivity to INF-γ reprogramming evident between GO and dermal fibroblasts underscores the exacerbated response of diseased orbital cells to proinflammatory stimuli. Although alterations in specific proteins involved in inflammation and remodeling were also resolved by MALDI mass spectrometry of orbital tissue obtained from GO patients compared to non-thyroid involved controls, more significant up-regulations were evident in GO patients not previously treated with steroids (Matheis et al., 2015). Induction of plasminogen activator inhibitor-1 (PAI-1, SERPINE1), a clade E member of the serine protease inhibitor (SERPIN) gene family and a prominent regulator of the pericellular proteolytic microenvironment (Figure 2), was one of the most highly up-regulated proteins in INF-γ-stimulated GO fibroblasts (Smith et al., 1992). By comparison, INF-γ only modestly increased (5-fold) or attenuated PAI-1 levels in dermal fibroblasts. PAI-1 was virtually undetectable in unstimulated orbital cells compared to expression levels under basal conditions in all dermal fibroblast strains (Smith et al., 1992; Higgins and Smith, 1993). Similarly, large-scale mRNA expression profiling of confirmed that PAI-1 transcript abundance was markedly increased (28-fold) in the intraorbital adipose/connective tissue collected from severe active Graves' disease patients by lateral decompression surgery compared to that obtained from patients without thyroid disease undergoing cosmetic procedures (Planck et al., 2011). Tear PAI-1 levels, moreover, were also significantly

increased in GO patients relative to GD patients without orbitopathy or to normal controls (Ujhelyi et al., 2012). Data analysis confirmed, in fact, that PAI-1 was the only protein to exhibit a statistically increased release in GO relative to GD patients with non-ocular involvement. These findings collectively suggest that the regulation of pericellular proteolysis may be fundamentally different between cutaneous and ocular fibroblasts (Smith et al., 1992).

While TGF-β1 is highly-expressed in the GO orbit (Pawlowski et al., 2014), analysis of differentially-expressed genes indicates that the fibroblast response to TGF-β1 is considerably more complex compared to the rather restricted reprogramming induced by INF-γ (Gardner et al., 2004; Planck et al., 2011). Moreover, as is the case for INF-γ, the TGF-β1-stimulated increase in PAI-1 mRNA and protein levels was significantly greater in GO vs. dermal fibroblasts (Cao et al., 1995) and likely contributes to the matrix expansion characteristic of active Graves' disease. Whether this is impacted by the elevated HA levels in the GO orbit (Wang et al. 2005, Guo et al. 2011) is unknown, however HA increases PAI-1 expression in human vascular smooth muscle cells (Marutsuka et al. 1998) and a positive correlation exists between HA and PAI-1 produced by human aortic endothelial cells exposed to inflammatory stimuli (Devaraj et al. 2009). In human umbilical vein endothelial cells, moreover, high molecular weight HA both activates the type I TGF-β receptor and induces PAI-1 expression (Park et al. 2012). It is reasonable to assume that similar vascular consequences of HA exposure may occur in the microenvironment of Graves' orbitopathy.

4. Multifunctional roles of PAI-1 in inflammatory/fibrotic disease

Among its varied functions, PAI-1 negatively regulates the plasmin-dependent pericellular proteolytic cascade, largely through inhibition of the urokinase/tissue-type plasminogen activators (uPA/tPA), effectively limiting ECM degradation and fibrinolytic activity (Figure 2) contributing, thereby, to the initiation and/or progression of fibrotic disease (Ghosh and Vaughan, 2012; Flevaris and Vaughan, 2017). Plasmin targets several ECM proteins directly while also activating various proenzymes of the matrix-metalloproteinase (MMP) family creating a proteolytic stromal remodeling cascade. PAI-1 restricts this process of proteinase activation, thus controlling the locale and extent of ECM degradation by (1) direct inactivation of PAs attenuating, thereby, plasmin generation/MMP activation which increases matrix deposition and promotes fibrosis and (2) targeting uPA receptor-bound uPA complexes for endocytotic clearance via members of the LDL-receptor family (Ghosh and Vaughan 2012). Development of gene-deficient animals confirmed that PAI-1 null mice are, in fact, protected from excessive ECM accumulation as well as lung, liver, kidney and vascular fibrosis and PAI-1 uPA/tPA domain decoys reduced both injury-initiated and established interstitial fibrosis (Gonzalez et al., 2009). Plasmin levels and activity, however, are not affected by PAI-1 deficiency in certain tissues (e.g., kidney) suggesting the involvement of other pathways impacted by PAI-1 knockout (e.g., Flevaris and Vaughan, 2016). Indeed, illumina-based microarray analysis revealed that a number of genes involved in diverse biological processes (e.g., immune system processing, stress response, cytokine and growth factor signaling, cell growth, migration and death, ECM organization and transcriptional regulation) were up- or down-regulated in several organ systems by the

genetic absence of PAI-1 (Ghosh et al., 2013). Clearly, the role of PAI-1 in fibrotic disease is complex and likely transcends its function as a regulator of the pericellular proteolytic microenvironment. In this regard, increased PAI-1 levels in the Graves' disease orbit may well impact various cell types (e.g., endothelial and immune cells, activated pericytes) as well as resident fibroblasts. PAI-1 is, in fact, a key contributor to intravascular coagulopathy, endothelial dysfunction and metabolic syndrome (Aso, 2007) each of which may be exacerbated by the effects of thyroid disease on glucose/insulin metabolism, Insulin is a potent inducer of PAI-1 expression *in vivo* (Nordt et al., 1995) and hyperinsulinemia is a major factor in PAI-1 elevation (Aso, 2007; De Taeye et al., 2005) where this SERPIN may promote vascular luminal fibrin accumulation. and creation of a procoagulant state (Cozma et al., 2007). Color doppler imaging revealed significant vascular anomalies in dysthyroid ophthalomopathy involving the superior ophthalmic vein (SOV), likely a consequence of optic nerve compression by the expanding EOM (Walasik-Szemplinska et al., 2015; Nakase et al., 1994; Kurioka et al., 2001; Yanik et al., 2005; Perez-Lopez et al., 2011). SOV thrombosis, while uncommon, is a pathophysiologically important complication of GO (Sorrentino et al., 2018) and may well reflect a state of PAI-1-induced coagulopathy and vessel pathology. Small molecule PAI-1 inhibitors (e.g., TM5441, TM5007, Tiplaxtinin) significantly attenuate the development of insulin resistance, intravascular coagulopathy, vascular thrombosis, and fibrosis in several mouse model systems (Lee et al., 2017; Isuhara et al., 2008; Rouch et al., 2015; Hennan et al., 2008; Baxi et al., 2008; Smith et al., 2006). Given the relative ease of PAI-1 inhibitor systemic administration, these findings suggest that the use of anti-PAI-1 functional therapeutics may have efficacy in the management of the orbital consequences of GD.

Recent findings also highlight an unexpected involvement of PAI-1 in innate immunity. PAI-1-deficient mice develop an attenuated inflammatory/fibrotic response following tissue injury while transgenic PAI-1 over-expressing animals exhibit increased macrophage and T-cell infiltration and/or immune cell tissue residence time (Oda et al., 2001; Gupta et al., 2016). In the aorta, monocyte adhesion to the intima is significantly reduced in streptozotocin-treated PAI-$1^{-/-}$ mice reflecting decreases in the inflammatory mediators TNF-α and monocyte chemotactic protein-1 (Zhao et al., 2017). Since PAI-1 provides a "don't eat me" signal, effectively inhibiting neutrophil efferocytosis (Park et al., 2008; Chao et al., 2011), it appears that this SERPIN may affect cellular influx as well as the intensity and/or duration of the injury-initiated inflammatory phase. Indeed, elevated PAI-1 levels closely mirror systemic and localized inflammation while exogenously-delivered PAI-1 stimulates expression of proinflammatory cytokines (e.g., TNFα and macrophage inflammatory protein-2) in primary bone marrow macrophages (Gupta et al., 2016). The protease inhibitory as well as the vitronectin- or LRP1-binding properties of PAI-1, however, are not necessary for macrophage activation but TLR4 is required, at least in part, since TLR4 neutralizing antibodies or the genetic depletion of TLR4 attenuated PAI-1-induced tissue inflammation (Gupta et al., 2016) suggesting that PAI-1 may function as a matricellular damage-associated molecular pattern (DAMP) TLR ligand (Marquerlot et al., 2006; Cartier-Michaud et al., 2012). PAI-1 appears involved, in fact, in lipopolysaccharide (LPS) signaling and PAI-1 knockdown attenuates LPS-induced increases in macrophage TLR4, MD-2, MyD88, TNF-α, IL-1β and NF-κB levels while vector-driven PAI-1 over-

expression enhanced these responses (Ren et al., 2015; Wang et al., 2014). Recent findings implicate specific TLR4 and TLR9 polymorphisms in the pathogenesis of GD and thyroid-associated ophthalmopathy (Cho et al., 2017). Although the mechanism is unclear, PAI-1 participates in host inflammatory responses via TLR4, at least in macrophages (Gupta et a., 2016). This is likely to have a significant impact on fibrogenic outcomes following tissue injury and/or prolonged inflammation as exogenous PAI-1 treatment increased TGF-β1, collagen 1α1, collagen 1α2 and MCP-1 transcripts in non-ocular cells (Nicholas et al., 2005; Jeong et al., 2016; Seo et al., 2009) and may well do so in the orbit. The TLR4/RAGE DAMP-type ligand HMGB1 also activates a subset of genes in the TGF-β1 profibrotic signature that includes PAI-1, CTGF and TGF-β1 (Cheng et al., 2015) suggesting that DAMPs and LPS utilize common and unique signaling pathways that may be exploited in the design of interventional strategies. Collectively, it appears that TLR4 may function as a molecular “switch”, activated by endogenous DAMPs to initiate repair while stimulating TGF-β1 signaling (by down-regulating the TGF-β pseudoreceptor BAMBI) promoting the persistent expression of TGF-β target genes to create and maintain a progressive fibrotic microenvironment (Bhattacharyya and Varga, 2015; Bhattacharyya et al., 2013). This is particularly relevant to the cytokine-driven inflammatory microenvironment and extensive matrix remodeling that typifies the onset and progression of orbitopathy in GD patients. DAMPs, including various fragments of proteoglycans and ECM components, are released from damaged tissues and subsequently activate TLR2, 4, 6, and 9 to initiate downstream signaling triggering and prolonging the inflammatory response (Frevert et al., 2018). Low molecular weight HA, moreover, is a potent stimulator of both TLR2 and TLR4 resulting in the activation of the NF-κB pathway and mobilization of a matrix-active remodeling cascade that includes increased expression of PAI-1 (Frevert et al., 2018). The marked up-regulation of HA in GO fibroblasts may exacerbate disease progression, through up-regulation of TGF-β signaling and PAI-1 expression (Wang et al., 2005; Guo et al., 2011; Marutsuka et al., 1998; Devaraj et al., 2009; Park et al., 2012), facilitating creation of a fibrotic stroma in the confines of the orbit.

5. Conclusions

Expression profiling of orbital tissue and ocular fibroblasts from GO patients revealed significant up-regulation of several potentially disease-relevant genes in response to INF-γ or TGF-β including that encoding the serine protease inhibitor PAI-1, a downstream effector of the fibrotic response. PAI-1 limits matrix degradation by negatively impacting the plasmin-activated pericellular proteolytic cascade to promote tissue fibrosis while promoting the duration and amplitude of the inflammatory response by inhibiting neutrophil efferocytosis. Exogenously-delivered PAI-1, moreover, stimulates TGF-β1 synthesis in several cell types which could be attenuated by small molecule PAI-1 functional inhibitors, suggesting the existence of a PAI-1/TGF-β1-positive feedback mechanism. These findings suggest that PAI-1 may initiate, perhaps maintain, a potential pro-fibrogenic “loop” (Nicholas et al., 2005; Seo et al., 2009) consistent with recent observations that engineered PAI-1 over-expression is sufficient to promote the development of a fibrogenic phenotype (Lian et al., 2018). PAI-1 also stimulates myofibroblast differentiation, a transition blocked by pretreatment with small molecule functional inhibitors (Omori et al., 2016). Several such

compounds (e.g., SK-216, TM5275) similarly attenuated bleomycin- and TGF-β1-induced lung fibrosis in mice (Huang et al., 2012; Omori et al., 2016). It is tempting to speculate, therefore, that targeted pharmacological down-modulation of PAI-1 expression or function (Rouch et al., 2015) may provide multi-level therapeutic opportunities to inhibit the onset and progression of tissue inflammatory and fibrotic disease, particularly in the context of the accessible GO orbit.

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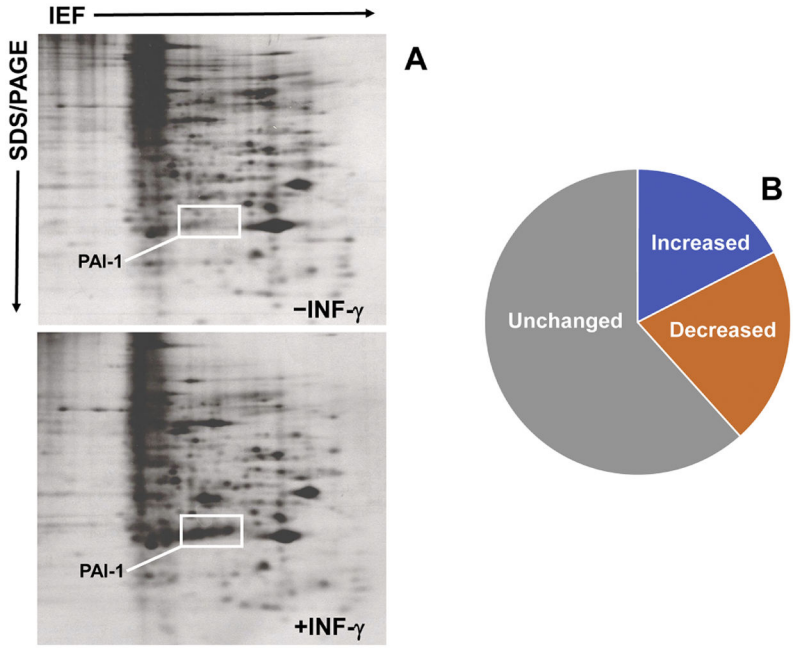

Figure 1.

Two-dimensional electrophoretic separations of *de novo*-synthesized ^{35}S-methionine-labeled cellular proteins from control and INF-γ-stimulated GO fibroblasts **(A)** confirmed a significant up-regulation (>16- to >40-fold) in the induced expression of the various distinct isoelectric variants of PAI-1 described previously (Higgins and Smith, 1993). PAI-1 map coordinates were confirmed using combined immunoblotting and 2-D gel separation criteria established previously (Higgins and Ryan, 1992). Individual protein spots were detected by fluorography and quantified with a Zeiss MOPS III digital image analyzer (Smith et al., 1992). I An approximately equal number of the resolved INF-γ-responsive GO fibroblast protein complement partitioned between the up- and down-regulated sets **(B)**.2IndividuMM-dimensional electrophoretic protein maps derived from 2 individual strains of gamma- spot 55s and 65s-69s induction by interferon gamma in human orbital fibroblasts. Cells were incubated in control

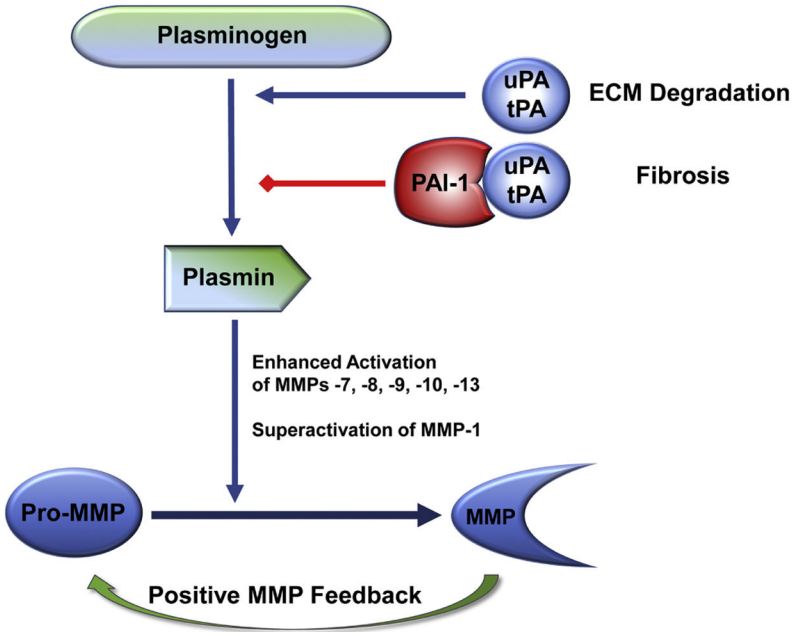

Figure 2.

PAI-1 is a critical factor in the regulation of pericellular proteolysis and tissue fibrosis. Plasminogen activators (urokinase, uPA; tissue-type, tPA) are the physiologically relevant plasmin-generating proteinases that impact ECM homeostasis through a complex and interdependent proteolytic cascade. uPA-stimulated conversion of plasminogen to plasmin leads to an increased downstream activation of matrix metalloproteinases (MMPs). Collectively, plasmin and MMPs dictate the locale and extent of ECM remodeling. Increased PAI-1 expression and/or activity facilitates ECM accumulation and attenuates ECM degradation which, if prolonged or chronic, results in the onset and progression of fibrotic disease (reviewed in Flevaris and Vaughan, 2016; Rabieian et al., 2018; Milenkovic et al., 2017; Higgins et al., 2018).