

Theme Issue Article

Integration of non-SMAD and SMAD signaling in TGF- β 1-induced plasminogen activator inhibitor type-I gene expression in vascular smooth muscle cells

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Summary

Overexpression of plasminogen activator inhibitor-1 (SERPINE1, PAI-1), the major physiological inhibitor of pericellular plasmin generation, is a significant causative factor in the progression of vascular disorders (e.g. arteriosclerosis, thrombosis, perivascular fibrosis) as well as a biomarker and a predictor of cardiovascular-disease associated mortality. PAI-1 is a temporal/spatial regulator of pericellular proteolysis and ECM accumulation impacting, thereby, vascular remodeling, smooth muscle cell migration, proliferation and apoptosis. Within the specific context of TGF- β 1-initiated vascular fibrosis and neointima formation, PAI-1 is a member of the most prominently expressed subset of TGF- β 1-induced transcripts. Recent findings implicate EGFR/pp60^{c-src}→MEK/ERK 1/2 and Rho/ROCK→SMAD2/3 signaling in TGF- β 1-stimulated PAI-1 expression in vascular smooth muscle cells. The EGFR is a direct upstream regulator of

MEK/ERK 1/2 while Rho/ROCK modulate both the duration of SMAD2/3 phosphorylation and nuclear accumulation. E-box motifs (CACGTG) in the PE1/PE2 promoter regions of the human PAI-1 gene, moreover, are platforms for a MAP kinase-directed USF subtype switch (USF-1→USF-2) in response to growth factor addition suggesting that the EGFR→MEK/ERK axis impacts PAI-1 expression, at least partly, through USF-dependent transcriptional controls. This paper reviews recent data suggesting the essential cooperativity among the EGFR→MAP kinase cascade, the Rho/ROCK pathway and SMADs in TGF- β 1-initiated PAI-1 expression. The continued clarification of mechanistic controls on PAI-1 transcription may lead to new targeted therapies and clinically-relevant options for the treatment of vascular diseases in which PAI-1 dysregulation is a major underlying pathogenic feature.

Keywords

SERPINE1, PAI-1, TGF- β 1, epidermal growth factor receptor, EGFR, Rho kinase, SMADs, MAP kinases, transcription, pp60^{c-src}, cardiovascular disease

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PAI-1 and cardiovascular disease

PAI-1 (SERPINE1) is the major physiologic regulator of the plasmin-based pericellular proteolytic cascade, a modulator of vascular smooth muscle cell (VSMC) migration and a causative factor in tissue fibrotic and cardiovascular disease (1–9) (Fig. 1). In-vivo studies in PAI-1-null mice confirmed the role this SERPIN in arteriosclerosis and vascular fibrosis (2, 3, 10–12). Moreover, transgenic animals engineered to overexpress PAI-1 spontaneously develop arterial thrombosis and perivascular fibrosis as a function of age (13) consistent with the emergence of PAI-1 as a significant biomarker and predictor of cardiovascular disease-related death (14, 15). PAI-1 expression is also linked to neointimal expansion, as development of a VSMC-rich neointi-

ma is significantly reduced in PAI-1^{-/-} mice (compared to wild type counterparts) in response to oxidative stress-mediated vessel injury and in the balloon-catheterized carotid artery (6, 16, 17). The decrease in neointima formation is particularly striking in the context of combined ApoE/PAI-1 deficiency (8, 16). Such findings in animal models of vascular injury have relevance to recent clinical observations. Indeed, post-transluminal coronary angioplasty PAI-1 activity was significantly greater in patients with restenosis compared to those without clinical recurrence (18). The actual role of PAI-1 in VSMC accumulation, however, is likely to be complex. Transgenic overexpression of PAI-1 in VSMC (using a SM22 promoter) promotes smooth muscle proliferation through FLIP-mediated activation of the ERK1/2 and NF- κ B pathways (19). There is also considerable

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evidence that PAI-1 expression actually protects VSMC from plasmin-induced apoptosis/anoikis (e.g. [20, 21]), likely by suppression of caspase-3 activation (22, 23), suggesting that PAI-1 modulation of neointimal growth is a consequence of both increased proliferation and reduced apoptosis. Plasmin-mediated VSMC apoptosis both within the aortic wall or in culture is initiated by plasminogen activation (by either tPA or uPA) and is effectively impaired by PAI-1 (24, 25). Furthermore, VSMC isolated from PAI-1^{-/-} mice are extremely sensitive to plasminogen-induced apoptosis compared to wild-type, tPA^{-/-} or uPA^{-/-} VSMC reflecting a >10-fold increase in conditioned medium plasmin activity (23, 24). This anti-apoptotic effect of PAI-1 is not restricted to VSMC or to plasminogen-initiated cell death. PAI-1 effectively inhibited both spontaneous and camptothecin-induced apoptosis in human prostate cancer and promyelocytic leukemia cell lines (26) and rescued human keratinocytes from plasminogen-mediated loss of cell viability (27). PAI-1 may also have anti-adhesive and pro-apoptotic activities, at least in the setting of vascular cell attachment to vitronectin, a matrix constituent on which PAI-1 affects adhesion via the proximity of uPAR/ integrin binding sites in the SMB domain (28, 29).

TGF- β 1 and PAI-1: Links to vascular disease progression

The available data in vascular and non-vascular cells strongly suggest that induced PAI-1 expression occurs as part of a primary response to fibrogenic growth factors, among the most prominent of which is TGF- β 1 (30–33) (Fig. 1). Indeed, TGF- β family members are fundamental in the pathogenesis of several cardiovascular and vascular fibrotic diseases including hypertension, pathogenic restenosis, atherosclerosis and cardiac hypertrophy/fibrosis by impacting the expression of disease-relevant genes (e.g. PAI-1, connective tissue growth factor) (34–37). TGF- β 1-induced neointimal growth is effectively suppressed by PAI-1 ablation implicating PAI-1 as a major target of TGF- β -associated vascular pathology *in vivo* (31, 38, 39) stimulating interest in the TGF- β /PAI-1 expression control axis as a potential therapeutic opportunity. TGF- β ligand neutralizing antibodies, soluble TGF- β RII receptor constructs, TGF- β type-1 receptor (ALK5) inhibitors, TGF- β /PAI-1 antisense/siRNA-based therapies and small molecule PAI-1 inhibitors (e.g. TM5007, ZK4044, PAI-039) are currently in either preclinical or phase I evaluations (e.g. [40–46]). The consistent implication of PAI-1 and TGF- β 1 in neointima formation and vascular fibrosis (6, 16, 30, 31, 47–49) supports the likelihood that clarifying the signaling network underlying TGF- β 1-induced PAI-1 expression may well provide novel, perhaps selective, targets to address TGF- β /PAI-1-dependent cardiovascular disease.

Role of *src* kinase/EGFR signaling in TGF- β 1-induced PAI-1 expression in vascular smooth muscle cells

TGF- β 1 stimulation of quiescent VSMC results in phosphorylation (at Y416) of the non-receptor tyrosine kinase pp60^{c-src} and

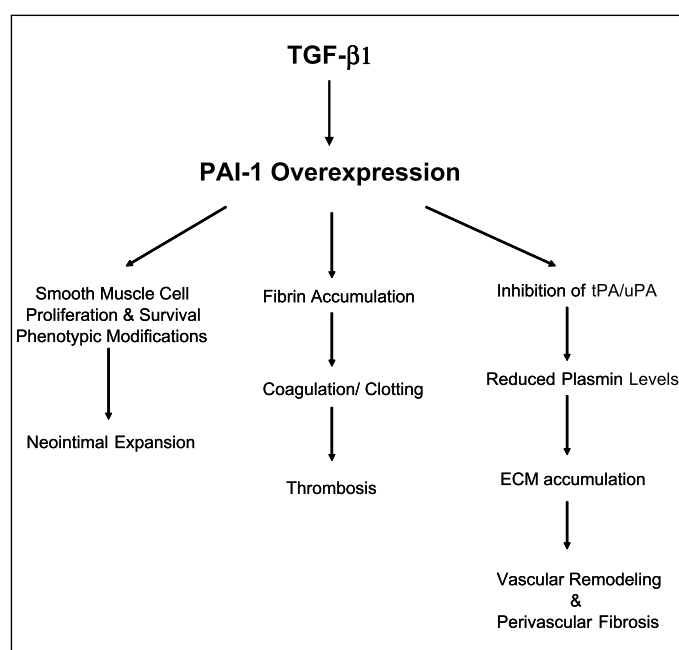


Figure 1: Potential contribution of overexpression of PAI-1 to cardiovascular disease (CVD). TGF- β 1, a major physiological regulator of PAI-1 expression, promotes extracellular matrix accumulation largely through the regulation of plasmin generation and MMP-mediated matrix degradation (via direct induction of PAI-1) as well as by inducing synthesis of matrix proteins (e.g. fibronectin, collagen) which, collectively, facilitate creation of a profibrotic state in vascular tissues. Increased PAI-1 expression by profibrotic and inflammatory factors (e.g. TGF- β , angiotensin) contributes to vascular thrombosis, by inhibition of fibrin degradation, neointimal expansion and arteriosclerosis, at least in part, by increasing VSMC proliferation and reducing VSMC apoptosis. PAI-1 elevation also attenuates plasmin-mediated matrix remodeling resulting in excessive extracellular matrix accumulation, a hallmark of perivascular fibrosis.

the rapid activation of the epidermal growth factor receptor (EGFR) at Y845 (a *src*-target residue) (37, 50). EGFR^{Y845} phosphorylation is specifically dependent on the catalytic activity of c-*src* (51). Indeed, pretreatment with the highly specific *src* family kinase inhibitor SU6656 effectively blocked TGF- β 1-stimulated EGFR^{Y845} phosphorylation; pEGFR^{Y845} activation in response to TGF- β 1, moreover, was detected in wild-type fibroblasts but not in their counterparts genetically deficient in the *src* family kinases c-*src*-, c-*yes*-, c-*fyv* (SYF^{-/-} cells) (37). Demonstration of c-*src*/EGFR complexes in the EGFR-overexpressing A431 cell line as well as in TGF- β 1-stimulated VSMC established linkage between *src* family kinases and the EGFR (50, 52, 53). The functional significance of such interactions, at least with regard to the PAI-1 response to TGF- β 1, was confirmed using a molecular genetic approach. A DN- pp60^{c-src} construct completely blocked TGF- β 1-initiated PAI-1 induction while TGF- β 1 failed to stimulate PAI-1 expression in SYF^{-/-} fibroblasts; importantly, PAI-1 expression was restored in SYF^{-/-} cells engineered to re-express a wild-type pp60^{c-src} construct (37). While the mechanism of *src* regulation in response to TGF- β 1 is uncertain, p130^{CAS} is involved in *src* kinase signaling (54) and the adaptor protein Shc, specifically the p66 and p52 isoforms, is important for both *src* activation and formation of (Shc-depend-

ent) EGFR/*c-src* complexes (55–57). Another model suggests that *c-src* associates with the EGFR upon ligand binding via interactions between the *c-src* SH2 domain and the EGFR^{Y992} residue resulting in EGFR^{Y845} phosphorylation and initiation of downstream events (51). Regardless of the precise mechanism, pharmacologic blockade of EGFR signaling (with AG1478), use of site-specific dominant-negative (DN) or mutant EGFR constructs (e.g. kinase-dead EGFR^{K721A}, EGFR^{Y845F}) and genetic ablation of EGFR1 effectively inhibited TGF- β 1-initiated PAI-1 transcription confirming participation of the EGFR in PAI-1 gene control (37). Although the EGFR^{Y845F} mutant is an EGF-responsive kinase with retention of at least some downstream signaling ability (58), it is, nevertheless, an effective inhibitor of EGF-/transactivating agonist-induced DNA synthesis, indicating that Y845 is required for mitogenesis (51, 59). Since the EGFR^{Y845} site regulates several distinct signaling pathways (reviewed in [51]), the requirement for both a functional EGFR and, in particular, an intact Y845 residue in TGF- β 1-initiated signaling strongly suggests that the EGFR^{Y845} residue constitutes a platform for bifurcation of downstream events with specific impact on TGF- β 1-induced PAI-1 transcription. TGF- β 1 stimulated ERK1/2 phosphorylation, moreover, in EGFR^{+/+} but not EGFR^{-/-} cells consistent with prior observations that TGF- β 1-dependent ERK1/2 activation is downstream of EGFR signaling (32).

Involvement of Rho/ROCK signaling in PAI-1 expression in TGF- β 1-stimulated vascular smooth muscle cells

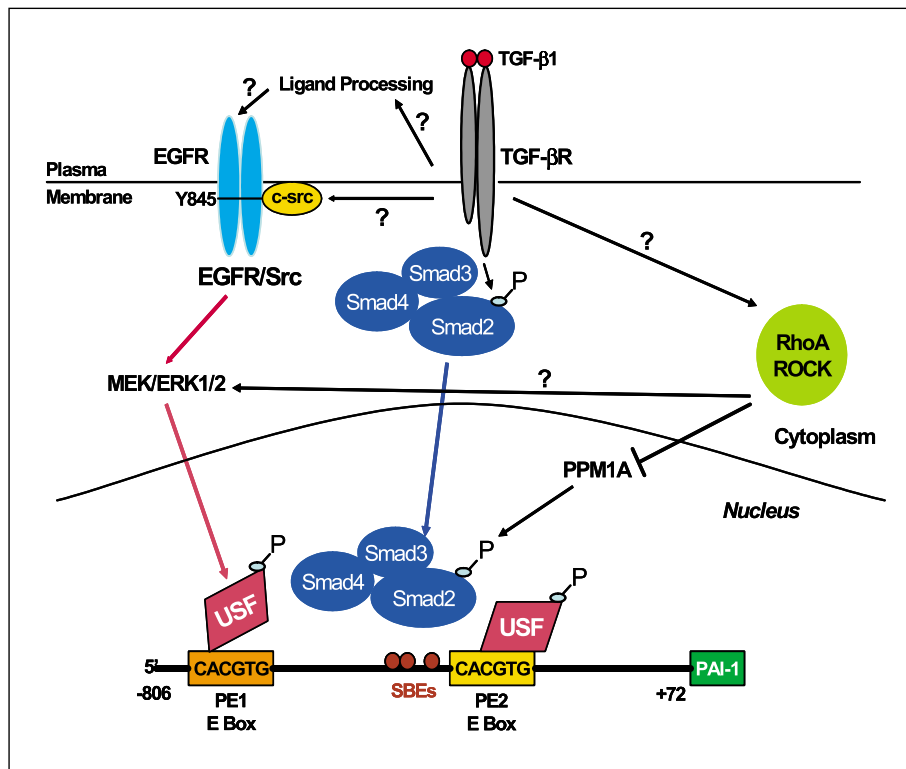
EGFR^{-/-} as well as SYF^{-/-} fibroblasts are fully capable of responding to exogenous TGF- β 1 as SMAD2/3 are effectively phosphorylated in both wild-type and EGFR^{-/-} fibroblasts. Similarly, TGF- β 1-induced SMAD2 phosphorylation is not altered by EGFR blockade either pharmacologically (with AG1478) or molecularly (by expression of EGFR^{KD} or EGFR^{Y845F}) (37). While the MEK inhibitors U0126 and PD98059 completely blocked TGF- β 1-induced PAI-1 expression as well as ERK1/2 phosphorylation (32), SMAD2 activation was not impacted (37). Collectively, these data indicate that SMAD2/3 are efficiently phosphorylated in response to TGF- β 1 in both EGFR^{+/+} and EGFR^{-/-} fibroblasts as well as SYF^{-/-} cells, suggesting that TGF- β 1-directed SMAD2 phosphorylation (at the carboxy terminus) is EGFR/MEK-independent. Indeed, recent data clearly indicates that TGF- β 1 stimulates PAI-1 expression through two distinct but cooperating pathways that involve EGFR/pp60^{c-src}→MEK/ERK signaling and EGFR-independent, but Rho/ROCK-modulated, TGF- β 1-directed SMAD activation (37). Rho/ROCK are critical elements in the progression of cardiovascular disease (reviewed in [60–62]) particularly in the context of TGF- β 1-induced vascular fibrosis (34). Balloon injury-induced neointima formation is, in fact, suppressed by Rho/ROCK inhibitors (63) and angiotensin II-induced perivascular fibrosis in ROCK^{+/-} mice is significantly reduced compared to wild-type littermates (64). Importantly, PAI-1 expression in response to various other fibrogenic stimuli (e.g. C-reactive protein, hyperglycemia) is also largely Rho/ROCK mediated sug-

gesting that targeting this pathway may have multi-level therapeutic implications (65–67). Activation of RhoA in response to TGF- β 1 preceded optimal PAI-1 induction; pretreatment with C3 transferase, transfection of a dominant-negative RhoA (DN-RhoA^{N17}) construct or incubation with the ROCK inhibitor Y-27632 ablated PAI-1 induction in response to TGF- β 1 (37) similar to the requirements for smooth muscle α -actin and connective tissue growth factor expression (68, 69). Recent findings suggest a more complex control of SMAD function by members of the small GTPase family than may have been previously appreciated. While TGF- β 1 receptors phosphorylate SMADs downstream of growth factor engagement, the Rho/ROCK pathway modulates the duration of SMAD2/3 phosphorylation (37). How Rho/ROCK impact TGF- β 1-initiated SMAD2/3 activation and sub-cellular localization is not known but this pathway may function to provide efficient SMAD2/3 activation for extended periods impacting, thereby, SMAD-dependent transcriptional regulation of target genes including PAI-1 (70–72). Alternatively, Rho/ROCK signaling may be required to inhibit negative regulation of SMAD2/3 function via the inhibitory SMAD7 or by inactivation of SMAD phosphatases (e.g. PPM1A) sustaining, thereby, SMAD2/3 transcriptional actions (e.g. [73, 74]).

Cooperative SMAD and non-SMAD factors mediate PAI-1 gene induction by TGF- β 1 in vascular smooth muscle cells

While SMAD2/3 activation may be necessary it is not sufficient for TGF- β 1-stimulated PAI-1 expression in the absence of EGFR signaling. One model consistent with the available data (29, 32, 37, 50, 75, 76) suggests that SMADs and specific MAP kinase-targeted transcription factors occupy their separate binding motifs at the critical TGF- β 1-responsive PE2 region E box in the human PAI-1 promoter (29, 32, 77–79). Indeed, the available data strongly suggest that complex formation at the PE2 site requires cooperative signaling by the EGFR→ERK (USF) and Rho/ROCK (SMAD) pathways (Fig. 2). A similar required E-box motif (CACGTG) maps to the expression-regulating HRE-2 region in the rat PAI-1 promoter (80). Extract immunodepletion and super-shift/complex-blocking experiments confirmed one PAI-1 E box-binding protein to be USF, a member of the basic helix-loop-helix-leucine zipper (bHLH-LZ) family of MYC-like proteins (75, 77). Dominant-negative interference with USF DNA-binding ability significantly reduced TGF- β 1-mediated PAI-1 transcription (29, 32, 79). Since MAP kinases regulate the DNA-binding and transcriptional activities of USF (32, 76), TGF- β 1 signaling through SMAD2/3 may actually cooperate with EGFR/MEK-ERK-activated USF to attain high level PAI-1 expression (50, 76). SMADs can interact with other E box-binding HLH-LZ factors such as TFE3 at the PE2 site in the PAI-1 gene at least in one cell type (72). There is evidence, in fact, to suggest that such interacting complexes impact PAI-1 gene control since USF occupancy of the PAI-1 PE2 region E-box site, which is juxtaposed to three SMAD-recognition elements, modulates transcription in response to TGF- β 1 or serum (29, 32, 37, 79). Such potential promoter-level co-oper-

Figure 2: Model for TGF- β 1-induced PAI-1 expression. Current data indicates that TGF- β 1 activates two distinct signaling pathways that initiate transcription of PAI-1. Rho/ROCK are required for maintenance of SMAD phosphorylation as well as ERK activation (through yet to be defined mechanisms) while the pp60^{c-src}-activated EGFR (at the Y845 site) signals to MEK-ERK initiating likely ERK/USF interactions resulting in USF phosphorylation and a subtype (USF-1 \rightarrow USF-2) switch at the PAI-1 PE1/PE2 E box sites. Collectively, these two promoter-level events stimulate high levels of PAI-1 in response to TGF- β 1 occupancy. The actual mechanism underlying EGFR activation in response to TGF- β 1 is unknown but may involve direct recruitment of src kinases to the EGFR or the processing and release of a membrane-anchored EGFR ligand (e.g. HB-EGF). Similarly, events associated with TGF- β 1 stimulation of the RhoA/ROCK pathway are presently unclear. Rho/Rock may regulate the activity and/or function of the SMAD phosphatase PPM1A impacting, thereby, the duration of SMAD-dependent transcription of target genes such as PAI-1.



activity is supported by the realization that SMADs interact rather weakly with their SMAD-binding elements and that interactions with other factors are necessary to initiate gene expression. Recruitment of this multicomponent complex likely requires participation of the TGF- β 1-stimulated EGFR \rightarrow MEK/ERK and Rho/ROCK pathways for the optimal response of the PAI-1 gene to TGF- β 1. This has significant cardiovascular implications as USF levels increase early after balloon injury to the carotid artery (81) where it regulates expression of pro-atherogenic genes including osteopontin (81, 82) and PAI-1 (6, 18) as well as genes involved in the etiology of familial combined hyperlipidemia and the metabolic syndrome (83).

Recent findings have highlighted the increasing complexity of SMAD-non-SMAD protein interactions in TGF- β 1-dependent PAI-1 gene control. Members of the p53 family are critical elements in a subset of TGF- β 1 responses due, at least in part, to the ability of MAP kinase-phosphorylated p53 to bind SMAD2, forming transcriptionally active multi-protein complexes (84–86). DNase I footprinting/methylation interference and oligonucleotide mobility shift analyses confirmed that p53 binds to a recognition motif in the PAI-1 promoter (87). DNA-binding activity was associated with both p53 sequence-driven reporter gene transcription and induced expression of the endogenous PAI-1 gene likely utilizing the two p53 half-sites (AcA-CATGCCT, cAGCAAGTCC) at –224 to –204 bp relative to the transcription start site (88). TGF- β 1-induced expression is significantly attenuated in cells in which p53 levels are reduced by siRNA (85) and, in p53-deficient lung tumor cells that express little or no PAI-1, engineered re-expression of p53 rescues both basal and inducible PAI-1 expression (89). One mechanism sug-

gests that p53 interacts with SMAD2 (90). In TGF- β 1-stimulated cells, binding of USF to the PE2 site, which is juxtaposed to three SMAD-binding elements, may facilitate DNA bending. Phasing analysis revealed that certain bHLH-LZ of the MYC family (including USF) orient the DNA bend toward the minor groove (91), which could potentially promote interactions between p53, bound to its downstream half-site motif, with SMAD2 tethered to the upstream PE2 region SMAD site. Similarly, the transcriptional coactivator p300/CREB-binding protein (CBP), a histone acetyltransferase, interacts with and acetylates SMAD2/3 in response to TGF- β 1 resulting in enhanced PAI-1 transcription (92–94). RAP250, a protein with no intrinsic enzymatic activity but effectively recruits histone acetyltransferases and methylases to chromatin complexes, also interacts with SMAD2/3 and is essential for maximal PAI-1 induction in response to TGF- β 1 (95). At least one recent review provides insights into the determinants of TGF- β -Smad signaling including a critical analysis of selective interactions between the SMADS and other signaling pathway components (96).

E-box motifs are platforms for TGF- β 1 regulation of PAI-1 expression

There are at least five E box-like sequences in the human PAI-1 promoter; only E boxes E4 and E5 are classic consensus sites with E4 (the PE2 E-box motif) and E5 (the PE1 E box) flanked by the 5' adjoining ATT trinucleotide "spacer"/SMAD-binding elements and the 3' 4G/5G polymorphism, respectively (77). USF proteins are major PAI-1 E box-binding factors (97) and com-

petitive occupancy of the PE1(E5)/PE2(E4) sites by distinct USF homo- or heterodimer pairs has transcriptional consequences (e.g. [98]). Indeed, chromatin immunoprecipitation (ChIP) confirmed that the PAI-1 gene PE2 E box is, in fact, a USF target *in vivo* and that function-disrupting USF mutants inhibit PAI-1 induction (79). Site occupancy and transcriptional activity, furthermore, require conservation of the PE2 core E-box structure as the CACGTG→CACGGA and TCCGTG dinucleotide substitutions (in the rat gene) and a CACGTG→CAATTG or TCCGTG replacement (in the human gene), with retention of PAI-1 flanking sequences, resulted in loss of both competitive binding and growth factor-dependent reporter activity (77, 80). ChIP assessment of the E-box site in the PE2(E4) region of the human PAI-1 gene, moreover, indicated a dynamic occupancy by USF subtypes (USF-1 vs. USF-2) as a function of growth state (79). An exchange of PE2(E4) E box USF-1 homodimers with USF-2 homo- or USF-1/USF-2 heterodimers, moreover, closely correlated with PAI-1 gene activation. Indeed, USF-2-stimulated human PAI-1 promoter activity in HepG2 cells required the PE2(E4) and PE1(E5) sites and, importantly, the E box-like hypoxia response element (HRE) CACGTACA at nucleotides -194/-187; PE2(E4) or PE1(E5) sequence mutations attenuated PAI-1 promoter activity while HRE mutation completely abolished reporter signal (98). While both USF-1 and USF-2 bound to PE2(E4) and PE1(E5) target probes (32, 98), USF proteins did not bind directly to the HRE target sequence; the PAI-1 HRE was bound by an ATF-1/CREB-like protein suggesting that HRE binding factor(s) may interact cooperatively with USF-occupied E4/E5 to affect transcriptional output (98). DNASTAR program alignment of the human and rat PAI-1 promoters indicated that the PE1(E5) and PE2(E4) E-box sites differed in homology by 1 and 2 bases, respectively (99). Mutational analysis, moreover, confirmed that the PE2, as compared to the PE1, E box was important in TGF- β 1-directed PAI-1 promoter activity (77). Such sequence differences in the rat versus human PAI-1 gene likely dictate expression levels as a consequence of the nature of the associated transcriptional complexes and the particular conditions of stimulation (e.g. 100–102).

The CACGTG hexanucleotide “core” is a target for occupancy by at least seven members of the bHLH-LZ transcription factor family (USF-1, USF-2, c-MYC, MAX, TFE3, TFEB, TFII-I). USF proteins, however, have a MgCl₂-dependent preference for C or T at the -4 position (103). The human PAI-1 gene, in fact, has a T at the -4 site of the PE2 region E box as well as a purine at +4 and -5 and a pyrimidine at +5 (A₋₅T₋₄C₋₃A₋₂C₋₁G₊₁T₊₂G₊₃G₊₄C₊₅), all of which facilitate USF binding (103). In this regard, the CACGTG→TCCGTG mutation is particularly relevant, since bHLH-LZ proteins with E-box recognition activity have a conserved glutamate important for interaction with the first two nucleotides (CA) in the E-box motif (91). These data are also consistent with the known hexanucleotide motif preference (CACGTG or CACATG) of USF proteins (104–106). Successful PAI-1 probe competition by a CACGTG “core” flanked by non-PAI-1 sequences (but with retention of T at -4 and a purine at +4) and the failure of specific E-box mutants to similarly compete (or to produce band shifts when used as targets) further indicate that a consensus hexanucleotide E box at the PE2(E4) site in the PAI-1 gene is both necessary and sufficient for USF binding

(77). This contrasts with the highly cooperative constraints for E-box recognition by other bHLH-LZ proteins (e.g. TFE3, MAX) that utilize accessory factors (e.g. SMADs) for optimal residence on the PAI-1 promoter (72, 107). An additional restraint on motif recognition and protein function resides at the level of protein phosphorylation. USF and TFE3 are phosphorylated at consensus MAP kinase target residues (108, 109) which initiates a conformational switch that exposes the DNA-binding domain (110). DNA binding and transcriptional activity requires USF phosphorylation (79, 108, 110, 111). At least one phosphorylation site (T153) is juxtaposed to a potential MAP kinase “docking” sequence (108). The recent identification of USF/ERK1/2 complexes and the requirement for MEK signaling in TGF- β 1-dependent ERK1/2 activation and PAI-1 transcription suggests a possible functional interaction between USF and one or more MAP kinases (32). DNA-anchored USF-1 could also complex with translocated MAP kinases (via kinase docking sites located within or closely juxtaposed to the USR) (e.g. [32, 78, 79, 109]) resulting in the hyper-phosphorylation of USF-1 (at secondary residues) potentially releasing E box-bound USF-1. USF activity may be further modified by either a recruited co-activator at the E-box site (112, 113) or, potentially, at the HRE (85) (e.g. USF-2, CREB) or direct replacement of USF-1 with USF-2 homodimers. By analogy, the HPV-16 oncoprotein E6 activates telomerase reverse transcriptase (TERT) transcription by c-MYC induction and release of USF-dependent repression at the -34 to -29 E-box site (114). Collectively, these findings suggest that the transcriptional effects of USF family members are context-dependent (112, 115, 116). USF-1 may function as a “basal repressor” of PAI-1 (or TERT) expression occupying E box sites to inhibit access of strong transcriptional activators that recognize the CACGTG motif (i.e. MYC, USF-2).

Conclusions

PAI-1 has emerged as an important causative factor and biomarker of cardiovascular disease. The continued definition of specific controls on PAI-1 transcription is important to realizing the potential of PAI-1 expression disruption (at both the transcriptional and intracellular signaling pathway levels) in the design of targeted, clinically-relevant, options for treatment of vascular pathologies (occlusive disease, neointima expansion, perivascular fibrosis) associated with TGF- β 1-induced PAI-1 expression. Recent data suggest that specific defined PAI-1 promoter regions, including PE1/2((E5/E4) and the HRE, may function as molecular “switches” that modulate PAI-1 transcription during cell “activation” by fibrogenic factors. These results, coupled with the success of small-molecule inhibitors of PAI-1 bioactivity, genetic-based approaches to attenuate PAI-1 expression at the mRNA transcript level or by interference with the involved signaling pathways, encourage speculation that PAI-1 disruption at various levels may have some promise in the manipulation of specific aspects of the atherogenic response. Indeed, suppression of PAI-1 activity has proven effective, in certain settings, in slowing disease progression (9, 117). The recent realization that complex cooperative EGFR→MEK/ERK and Rho signaling is an essential aspect of TGF- β 1-stimulated PAI-1 transcriptional control is not only novel but underscores the potential diversity of

new molecular targets (including members of the USF family of bHLH-LZ transcription factors) that can be exploited to disrupt or regulate PAI-1 expression levels for therapeutic benefit.

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Abbreviations

ALK5, activin receptor-like kinase 5; bHLH-LZ, basic helix-loop-helix/leucine zipper; ChIP, chromatin immunoprecipitation; DN, dominant-negative; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinases; HRE, hypoxia response element; MEK, mitogen-activated protein kinase/ERK kinase; Rho, Rho GTPase; ROCK, Rho kinase; NF- κ B, nuclear factor-kappa B; SERPIN, serine protease inhibitor; SMAD, Sma/Mad homologues; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; USF, upstream stimulatory factor; VSMC, vascular smooth muscle cells.

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