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TGF- β 1-induced plasminogen activator inhibitor-1 expression in vascular smooth muscle cells requires pp60^{c-src}/EGFR^{Y845} and Rho/ROCK signaling

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Abstract

TGF- β 1 and its target gene encoding plasminogen activator inhibitor-1 (PAI-1) are major causative factors in the pathology of tissue fibrosis and vascular disease. The increasing complexity of TGF- β 1 action in the cardiovascular system requires analysis of specific TGF- β 1-initiated signaling events that impact PAI-1 transcriptional regulation in a physiologically-relevant cell system. TGF- β 1-induced PAI-1 expression in both primary cultures and in an established line (R22) of vascular smooth muscle cells (VSMC) was completely blocked by inhibition of epidermal growth factor receptor (EGFR) activity or adenoviral delivery of a kinase-dead EGFR^{K721A} construct. TGF- β 1-stimulated PAI-1 expression, moreover, was preceded by EGFR phosphorylation on Y845 (a *src* kinase target residue) and required pp60^{c-src} activity. Infection of VSMC with an adenovirus encoding the EGFR^{Y845F} mutant or transfection with a dominant-negative pp60^{c-src} (DN-Src) expression vector effectively decreased TGF- β 1-stimulated, but not PDGF-induced, PAI-1 expression implicating the pp60^{c-src} phosphorylation site EGFR^{Y845} in the inductive response. Consistent with these findings, TGF- β 1 failed to induce PAI-1 synthesis in *src* kinase-deficient (SYF^{-/-}) fibroblasts and reexpression of a wild-type pp60^{c-src} construct in SYF^{-/-} cells rescued the PAI-1 response to TGF- β 1. TGF- β 1-induced EGFR activation, but not SMAD2 activation, moreover, was virtually undetectable in SYK^{-/-} fibroblasts in comparison to wild type (SYK^{+/+}) counterparts, confirming an upstream signaling role of *src* family kinases in EGFR^{Y845} phosphorylation. Genetic EGFR deficiency or infection of VSMCs with EGFR^{K721A} virtually ablated TGF- β 1-stimulated ERK1/2 activation as well as PAI-1 expression but not SMAD2 phosphorylation. Transient transfection of a dominant-negative RhoA (DN-RhoA) expression construct or pretreatment of VSMC with C3 transferase (a Rho inhibitor) or Y-27632 (an inhibitor of p160ROCK, a downstream effector of Rho) also dramatically attenuated the TGF- β 1-initiated PAI-1 inductive response. In contrast to EGFR pathway blockade, interference with Rho/ROCK signaling effectively inhibited TGF- β 1-mediated SMAD2 phosphorylation and nuclear accumulation. TGF- β 1-stimulated SMAD2 activation, moreover, was not sufficient to induce PAI-1 expression in the absence of EGFR signaling both in VSMC and mouse embryonic fibroblasts. Thus, two distinct pathways involving the EGFR/pp60^{c-src}/MEK-ERK pathway and Rho/ROCK-dependent SMAD2 activation are required for TGF- β 1-induced PAI-1 expression in VSMC. The identification of such novel interactions between two TGF- β 1-activated signaling networks that specifically impact PAI-1 transcription in VSMC may provide therapeutically-relevant targets to manage the pathophysiology of PAI-1-associated cardiovascular/fibrotic diseases.

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Keywords

Plasminogen activator inhibitor-1; PAI-1; TGF- β ; Vascular smooth muscle cells; Rho/ROCK signaling; EGFR; SMAD

1. Introduction

TGF- β family members activate multiple signaling intermediates (e.g., SMAD, MAPK, Rho/ROCK) that control the transcription of genes involved in tissue fibrosis, cell migration, tumor invasion, and angiogenesis [1–7]. One such TGF- β 1 target gene encodes plasminogen activator inhibitor-type 1 (PAI-1; SERPINE1), the major physiologic regulator of the plasmin-based pericellular proteolytic cascade [8,9]. PAI-1 is a prominent causative factor in several progressive and chronic fibrotic disorders, particularly in the context of elevated tissue TGF- β 1 levels, as well as an important contributor to the pathophysiology of vascular sclerosis [10–20]. Indeed, the Framingham Heart Study identified PAI-1 as a significant biomarker and notable predictor of cardiovascular disease-related death [20]. In vivo studies in PAI-1^{-/-} mice, moreover, confirmed the role of PAI-1 in the development of arteriosclerosis and perivascular fibrosis [14,15,21]. Clarifying the signaling network underlying TGF- β 1-induced PAI-1 expression, therefore, may well provide novel, therapeutically useful, targets to manage TGF- β 1/PAI-1-dependent cardiovascular disorders.

SMAD-dependent as well as SMAD-independent (e.g., Rho/ROCK) pathways contribute significantly to both PAI-1 gene control and progression of cardiovascular disease [5,22–28]. Neointima formation, VSMC migration and proliferation, for example, are suppressed by inhibitors of Rho/ROCK signaling [24]. Angiotensin II-induced perivascular fibrosis is markedly reduced in ROCK^{+/-} mice compared to wild-type littermates [25] while PAI-1 induction by specific profibrotic stimuli (e.g., angiotensin II, C-reactive protein, hyperglycemia) requires Rho/ROCK signaling [26–28]. Pharmacologic blockade and use of dominant-negative expression constructs, moreover, also implicated MAP kinases (MEK/ERK), pp60^{c-src} and the epidermal growth factor receptor (EGFR) in TGF- β 1-initiated PAI-1 transcription [1,29–33]. Whether cross-talk or pathway integration exists among the various effectors of TGF- β 1 signaling (pp60^{c-src}, EGFR/ERK, Rho/ROCK, SMADs) in the response of the PAI-1 gene to TGF- β 1 is addressed in the current study. TGF- β 1-stimulated PAI-1 expression in VSMC was found to require both EGFR and Rho/ROCK signaling impacting MEK/ERK activation and SMAD2/3 phosphorylation, respectively. The most novel findings, however, are that the duration, but not initiation, of SMAD2/3 activation was regulated by the Rho/ROCK pathway and that SMAD phosphorylation in the absence of EGFR signaling is not sufficient to initiate PAI-1 expression. TGF- β 1-treatment, moreover, resulted in phosphorylation of the EGFR at the Y845 *src*-target residue and either mutation of this residue (EGFR^{Y845F}) or transfection of a dominant-negative (DN) pp60^{c-src} construct completely blocked PAI-1 induction in TGF- β 1-stimulated cells. The continued definition of signaling events underlying TGF- β 1-initiated PAI-1 transcription will ultimately lead to the clinical utility of PAI-1 network targeting in the treatment of PAI-1-associated vascular fibrotic disease.

2. Materials and methods

2.1. Cell culture

R22 rat VSMC (gift of Dr. Peter A. Jones, USC/Norris Comprehensive Cancer Center) were grown to near confluence in low glucose (1 g/l) DMEM supplemented with 10% FBS then maintained in serum-free medium for 2–3 days prior to stimulation with TGF- β 1 (1 ng/ml) [31,34]. EGFR^{+/+} and EGFR^{-/-} mouse embryonic fibroblasts (MEFs) (kindly provided by Dr.

Jennifer R. Grandis, University of Pittsburgh Medical Center) and triple *src* (*c-src*, *c-yes*, *c-fyn*)-deficient MEFs (*SYF^{-/-}*) as well as *SYF^{-/-}* cells engineered to re-express pp60^{*c-src*} (gifts of Dr. Harold Singer, Albany Medical College) were serum-deprived for 24–48 h before addition of TGF- β 1. Primary cultures of rat aortic SMC (also the gift of Dr. H. Singer) were propagated in DMEM/F-12 (1:1) medium containing 10% FBS then maintained in DMEM/F-12/0.1% FBS for 2 days prior to growth factor stimulation. Pretreatment with AG1478 (EGFR), C3 transferase (Rho), SU6656 (*src* family kinases), Y-27632 (p160ROCK) and the MEK inhibitors U0126 and PD98059 is described in the text.

2.2. Western blotting

R22 cells were lysed at 4 degrees C in 0.5% deoxycholate, 0.1% SDS, 50 mM HEPES, pH 7.5, 1% Triton X-100, 1% NP-40, 150 mM NaCl, 50 mM NaF, 1 mM vanadate, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin and 1 mM PMSF and extracts clarified at 14,000 $\times$ g for 15 min. MEFs were disrupted in 4% SDS/PBS for 10 min, lysates vortexed briefly, boiled for 5 min then centrifuged at 14,000 rpm for 15 min. Aliquots (30 μ g cellular protein) were electrophoretically-separated, transferred to nitrocellulose, membranes blocked in 5% milk in 0.05% Triton-X 100/PBS, incubated overnight with specific antibodies (to rat PAI-1 [American Diagnostica; polyclonal, 1:1000], EGFR [Cell Signaling; polyclonal, 1:1000], pERK1/2 [Santa Cruz Biotechnology; monoclonal, 1:2000], ERK2 [Santa Cruz Biotechnology; polyclonal, 1:2000], pSMAD2^{Ser465/467} [Cell Signaling; polyclonal, 1:1000], pSMAD3^{Ser423/425} [Cell Signaling; polyclonal, 1:1000], SMAD2/3 [Cell Signaling; polyclonal, 1:1000], Rho [Santa Cruz Biotechnology; polyclonal, 1:1000], EGFR^{Y845} [Cell Signaling; polyclonal, 1:750], pp60^{*c-src*}-pY416 [Cell Signaling; polyclonal, 1:1000], phosphotyrosine (4G10 monoclonal, Upstate Biotechnology; 1:2000), TGF- β RI [Santa Cruz Biotechnology; polyclonal, 1:1000]) in blocking buffer and washed three times in 0.05% Triton X-100/PBS prior to incubation with secondary antibodies. Immunoreactive proteins were visualized with ECL reagent and quantitated by densitometry. Stripped membranes were reprobed with antibodies to actin, EGFR or ERK2 and, in certain instances, to pp60^{*c-src*}, SMAD2 and SMAD2/3 to confirm protein loading levels. The results obtained with all normalization antibody probes were equivalent. Statistical analyses of data utilized the t test to derive p values.

2.3. Fluorescence microscopy

Cells were fixed in 3.7% paraformaldehyde, permeabilized in 0.1% Triton X-100/PBS for 10 min, blocked in 2% BSA for 20 min then incubated with Texas Red X-phalloidin (Molecular Probes) for 45 min for visualization of actin microfilament organization. For immunocytochemical localization of Rho, activated (pY845) EGFR and pSMAD2, formalin-fixed cells were washed 3 times with Ca²⁺/Mg²⁺-free PBS, permeabilized in 0.1% Triton X-100 in PBS for 10 min followed by 3 PBS washes and a BSA block (as above). Cells were incubated with antibodies to Rho (1:50), pY845 EGFR (1:50) or pSMAD2 (1:50) for 1 h at room temperature, washed 3 times, and incubated with appropriate secondary antibodies (1:250; Molecular Probes) for 45 min. Following a final series of PBS rinses, coverslips were mounted with Vectashield reagent containing DAPI (to visualize nuclei) (Vector Laboratories).

2.4. Rho GTPase assay

PBS-washed cells were extracted (in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol containing leupeptin and 1 mM sodium orthovanadate) by constant agitation for 15 min at 4 degrees C. Clarified lysates (600 μ g protein) were incubated with Rhotekin RBD-agarose beads for 45 min at 4 C. Active (i.e., Rhotekin-bound) Rho and total Rho levels (GTP-Rho + GDP-Rho) were determined by Western blotting with Rho antibodies.

2.5. Delivery of adenoviral and dominant-negative (DN) constructs

R22 cells were infected (MOI=50–100) with the GFP-tagged kinase-dead EGFR1 mutant EGFR^{K721A}, the *src* kinase phosphorylation site mutant EGFR^{Y845F} (gifts of Ds. Paula J. McKeown-Longo, Albany Medical College and Sarah Parsons, University of Virginia) or control-GFP adenoviruses in low-serum medium for 48 h. All mutations were confirmed by sequencing. Stimulation with TGF- β 1 (1 ng/ml) or PDGF (25 ng/ml) was for 4 h prior to cell extraction. Subconfluent 35-mm cultures of R22 cells were transfected with 2.5 μ g of DN-pp60^{c-src} (gift of Dr. Giulio Superti-Furga, EMBL), DN-RhoA^{N17} (gift of Dr. Andrew Aplin, Albany Medical College) or vector DNA (without insert) as described previously [31]. Following incubation with lipid/DNA complexes for 4 h, the medium was changed to DMEM/10% FBS overnight and cells serum-deprived for 2 days prior to TGF- β 1 stimulation. Transfection efficiency was 50–70% while adenovirus infectivity was >90% (assessed by GFP fluorescence microscopy).

3. Results

3.1. Role of EGFR signaling in TGF- β 1-induced PAI-1 expression

TGF- β 1 mobilizes both SMAD-dependent and -independent signaling [5–7] although the impact of such cross-pathway events on PAI-1 induction is not well understood. Pharmacologic blockade previously implicated EGFR involvement in response to TGF- β 1 [31]. A more specific molecular approach was utilized, therefore, to identify the involved signaling intermediates in this pathway. TGF- β 1-stimulated PAI-1 expression was effectively suppressed by the EGFR inhibitor AG1478 in primary and R22 VSMC (Fig. 1A,B), by adenoviral delivery of an EGFR^{K721A} kinase-dead (EGFR^{KD}) receptor construct (Fig. 1C,D) or by genetic ablation of EGFR1 (Fig. 1E,F). While TGF- β 1 (0.05 to 0.2 ng/ml) effectively up-regulated PAI-1 levels in wild-type (EGFR^{+/+}) MEFs, this SERPIN was not inducible at any tested TGF- β 1 concentration (0.05 to 10 ng/ml) in EGFR^{-/-} fibroblasts (Fig. 1E,F). TGF- β 1 stimulated ERK1/2 phosphorylation, moreover, in EGFR^{+/+} but not EGFR^{-/-} MEFs (Fig. 1E) consistent with prior observations that ERK1/2 activation is downstream of EGFR signaling in the TGF- β 1-stimulated pathway [31,32]. EGFR^{-/-} MEFs, however, are fully capable of responding to TGF- β 1 as SMAD2 was effectively activated (i.e., phosphorylated) in both wild-type and EGFR^{-/-} cells (Fig. 1E).

pp60^{c-src} kinase activity, as assessed in the IgG immune complex assay, increases within 30 min after TGF- β 1 stimulation of quiescent R22 cells [31]. pp60^{c-src} phosphorylation at the Y416 residue in the activation loop of the kinase domain was evident, in fact, within 15 min and remained elevated for 1 h after TGF- β 1 addition (Fig. 2A). Transient transfection of a dominant-negative pp60^{c-src} (DN-Src) construct, moreover, effectively blocked PAI-1 induction in response to TGF- β 1 (Fig. 2B) and TGF- β 1 failed to stimulate PAI-1 expression in MEFs deficient in *src* family kinases (i.e., *c-src*-, *c-yes*-, *c-fyn*-null fibroblasts; SYF^{-/-/-}) compared to identically stimulated wild-type SYF^{+/+/+} cells (Fig. 2C,D). Importantly, PAI-1 induction was restored in SYF^{-/-/-} MEFs engineered to re-express a wild-type pp60^{c-src} construct (Fig. 2E). Since these findings confirmed the participation of pp60^{c-src} in TGF- β 1-initiated PAI-1 transcription, it was necessary to determine if specific *src* kinase sites (e.g., Y845) in the EGFR were specifically phosphorylated in response to TGF- β 1 and to evaluate their potential involvement in PAI-1 induction. Increased EGFR phosphorylation at the pp60^{c-src} target Y845 residue occurred rapidly after addition of TGF- β 1 to both wild-type (EGFR^{+/+}) MEFs (Fig. 3A,B) and R22 VSMC (Fig. 3C). EGFR^{Y845} phosphorylation in R22 cells increased significantly above basal levels at 15 min after exposure to TGF- β 1 (Fig. 3B) correlating well with the time course of pp60^{c-src} activation (Fig. 2A). Infection with the EGFR^{Y845F}-GFP mutant adenovirus significantly attenuated (by approximately 70%) TGF- β 1-dependent, but not PDGF-induced, PAI-1 expression (Fig. 3D,E). Enhanced EGFR

phosphorylation (particularly at the Y845 site) in response to TGF- β 1, as was the case for PAI-1 induction, required the activity of *src* family kinases. Pretreatment of VSMC with the specific *src* family kinase inhibitor SU6656 effectively blocked TGF- β 1-initiated increases in both pp60^{c-src} and EGFR phosphorylation (as detected with the phospho-tyrosine specific antibody 4G10) (Fig. 4A,B) and pp60^{c-src} and EGFR activation specifically at the Y416 and Y845 residues, respectively (Fig. 4C). pEGFR^{Y845} phosphorylation in response to TGF- β 1 was evident in wild type but not SYF^{-/-} MEFs (Fig. 4D). The time course of TGF- β 1-initiated SMAD2 activation, in contrast, was similar in both WT and SYF^{-/-} MEFs (Fig. 4D) confirming that in the context of either EGFR or *src* family kinase deficiency (Figs. 1E, 2C–E, 4D) SMAD2 activation still occurs but is not sufficient for PAI-1 induction.

3.2. TGF- β 1-induced PAI-1 expression and SMAD phosphorylation requires Rho/ROCK signaling

SMAD2 was rapidly phosphorylated (within 5 to 15 min) of TGF- β 1 addition to quiescent R22 cells and sustained for at least 4 h (Fig. 5A). Although AG1478 pretreatment completely inhibited TGF- β 1-stimulated ERK1/2 phosphorylation (Fig. 5B) and PAI-1 induction (Fig. 1A,B), TGF- β 1-initiated SMAD2 phosphorylation is not affected by AG1478 (Fig. 5B). Similarly, while the MEK inhibitors U0126 and PD98059 completely blocked TGF- β 1-induced PAI-1 expression in both R22 and primary VSMC (Fig. 5C,D) as well as ERK1/2 phosphorylation (Fig. 5E), as anticipated from previous findings [1,31], SMAD2 activation is not affected by U0126 (Fig. 5E) suggesting that TGF- β 1-directed SMAD2 phosphorylation is EGFR/MEK-independent. Indeed, SMAD2 was efficiently phosphorylated in response to TGF- β 1 in both EGFR^{+/+} and EGFR^{-/-} MEFs (Fig. 5F) consistent with results in the R22 VSMC system (Fig. 5A,B) and confirming, importantly, over comparable concentrations and time courses that TGF- β 1→SMAD signaling is intact in EGFR^{-/-} as well as SYF^{-/-} cells (Figs. 1E, 4D, 5F). In contrast, increased ERK1/2 phosphorylation in TGF- β 1-stimulated cells was clearly EGFR-dependent (Fig. 5G). These data collectively suggest that while the EGFR/*src*/MEK-ERK axis is required for TGF- β 1-mediated PAI-1 expression, this pathway does not regulate SMAD2 phosphorylation in either VSMC or MEFs.

TGF- β 1 also activates the Rho GTPase, a regulator of actin microfilament organization in various cell types and an important intermediate in the induced expression of CTGF as well as the smooth muscle differentiation genes SMA and SM22 [4,35,36]. TGF- β 1 increased Rho GTP loading within 2–4 h after addition to serum-deprived VSMC returning to basal levels by 24 h (Fig. 6A). The kinetics of Rho activation in response to TGF- β 1, moreover, precedes optimal PAI-1 induction, suggesting a potential role in PAI-1 gene control. To evaluate this possibility, VSMC were incubated with increasing concentrations (1.5–5.0 μ g/ml) of cell-permeable C3 transferase. The microfilament network was clearly altered by C3 transferase confirming the functionality of this Rho GTPase inhibitor, at the concentrations used, in R22 cells (Fig. 6B). Pretreatment with C3 transferase effectively suppressed TGF- β 1-stimulated PAI-1 expression in both R22 cells (Fig. 6C) and primary VSMC cultures (Fig. 6D). Transient transfection of a dominant-negative RhoA (DN-RhoA^{N17}) construct, moreover, markedly attenuated the PAI-1 response to TGF- β 1 compared to control (vector without insert) transfectants (Fig. 6E) confirming the involvement of RhoA signaling in PAI-1 induction. Quantitative analysis of multiple individual Western blots (e.g., Fig. 6C) confirmed the significant dose-dependent inhibition of TGF- β 1-initiated PAI-1 induction at all C3 transferase concentrations tested (Fig. 6F).

A major downstream effector of RhoA is ROCK, a serine/threonine kinase which modulates cytoskeletal architecture and the activity of several signaling intermediates (e.g., MLCK, MAPK) [22]. Hyperglycemia- and angiotensin II-induced PAI-1 expression appears to be ROCK-dependent [26,28] highlighting the requirement for ROCK signaling in the control of

several genes (e.g., PAI-1, MCP-1) that mediate progression of vascular disease [23,37]. The specific involvement of ROCK in TGF- β 1-stimulated PAI-1 gene control, however, remained to be determined. Treatment with Y-27632 resulted in disruption of actin stress fiber organization with accompanying alterations in cell shape (Fig. 7A), confirming the functional status of this ROCK inhibitor (similar to C3 transferase) in R22 cells. Pre-incubation with Y-27632 (from 5–20 μ M) ablated PAI-1 induction in response to TGF- β 1 in primary VSMC (Fig. 7B) and R22 cells (Fig. 7C,D) at even the lowest concentration tested.

Activated SMADs are important effectors of TGF- β 1 signaling to specific target genes including PAI-1 [38–40]. Since the EGFR/*src*/MEK-ERK axis did not regulate SMAD2 phosphorylation in TGF- β 1-stimulated VSMC (Figs. 1E,4D,5B,E,F), it was necessary to evaluate the potential contribution of the Rho/ROCK pathway to SMAD activation and/or cellular trafficking. TGF- β 1-stimulated SMAD2/3 phosphorylation and nuclear accumulation (at 4 h) is completely inhibited by pretreatment of quiescent R22 cells with either C3 transferase (Fig. 8A) or Y-27632 (Fig. 8B–D). Interestingly, time course analysis revealed that while SMAD2 activation at early time points (i.e., within 2 h) of TGF- β 1 addition is minimally affected by Y-27632 pretreatment, SMAD2 phosphorylation at 4 h is virtually ablated by ROCK pathway inhibition (Fig. 8E). The effect of Y-27632 on pSMAD3 was less dramatic although there was a considerable reduction in TGF- β 1-induced pSMAD3 levels at the 4 h time point as well (Fig. 8B,E). This suggests that Rho/ROCK signaling events regulate the duration of SMAD2/3 activation (up to 4 h) but not the initiation of SMAD2/3 phosphorylation by TGF- β R complexes (Fig. 8E). Total cellular SMAD2/3, TGF- β RI or Rho levels were relatively unchanged regardless of treatment conditions. Since MEK/ERKs are major participants in TGF- β 1-stimulated PAI-1 expression (Fig. 5C,D) [1,2,31] and ROCK inhibition virtually ablates TGF- β 1-mediated PAI-1 induction (Fig. 7B–D, 8E), it was important to evaluate the potential role of the Rho/ROCK pathway in TGF- β 1-initiated ERK1/2 activation. Y-27632 pretreatment significantly reduced TGF- β 1-stimulated pERK1/2 levels as did AG1478 (Fig. 9A,B) suggesting that ROCK is also an upstream regulator of ERK activity. EGFR phosphorylation, however, is not affected by Y-27632 (Fig. 9C). TGF- β 1-initiated Rho/ROCK activation, therefore, may impact PAI-1 gene regulation by modulating both SMAD2/3 and ERK1/2 signaling.

4. Discussion

TGF- β 1 stimulates PAI-1 expression in VSMC through two distinct but cooperating pathways that involve EGFR/pp60^{c-src}→MEK/ERK signaling and EGFR-independent, but Rho/ROCK-modulated, TGF- β R-directed SMAD and ERK activation. Interference with any of the individual elements in this dual cascade (EGFR/pp60^{c-src}/MEK or Rho/p160ROCK) markedly reduced, and in some cases, completely inhibited PAI-1 expression. ERK phosphorylation and PAI-1 induction in response to TGF- β 1 are significantly attenuated by an EGFR pharmacologic inhibitor (AG1478), by molecular targeting of EGFR activity (i.e., by adenoviral delivery of EGFR^{Y721A} kinase-dead or EGFR^{Y845F} mutant constructs) and, more importantly, by genetic ablation of the EGFR or *src* family kinases in mouse embryonic fibroblasts. The TGF- β 1-dependent formation of EGFR/pp60^{c-src} complexes [31] and EGFR^{Y845} phosphorylation, the inhibition of TGF- β 1- (but not PDGF-) induced PAI-1 expression by the EGFR^{Y845F} mutant as well as a DN-Src construct collectively implicate EGFR/pp60^{c-src} interactions and, in particular, the EGFR^{Y845} pp60^{c-src} site in the kinase domain activation loop [41] in signal propagation.

Bifurcation of the signaling cascade downstream of the TGF- β R suggests the existence of differential levels of control on genes that regulate VSMC function. Indeed, TGF- β 1-induced expression of connective growth factor (CTGF) and the smooth muscle differentiation markers SM-22 and α -SMA required RhoA-mediated SMAD2 activation, highlighting the importance

of Rho/ROCK in vascular fibrosis and VSMC phenotypic switching [35,36,42]. While TGF- β 1 receptors phosphorylate SMADs downstream of growth factor engagement, it appears that the Rho/ROCK pathway modulates the duration of SMAD2/3 activation as interference with Rho/ROCK signaling inhibits SMAD2/3 phosphorylation and nuclear accumulation (at 4 h post-growth factor addition). How Rho/ROCK impact TGF- β 1-initiated SMAD2/3 activation and sub-cellular localization [35,36; this paper] is not known but this pathway may function to provide efficient SMAD2/3 activation for extended periods. Alternatively, Rho/ROCK signaling may be required to inhibit negative regulation of SMAD2/3 function by inactivation of SMAD phosphatases sustaining, thereby, SMAD2/3 transcriptional actions [e.g., 43]. These possibilities are currently under investigation.

SMAD2 phosphorylation in TGF- β 1-treated VSMC is not altered by EGFR blockade either pharmacologically (with AG1478), molecularly (by expression of EGFR^{KD} or EGFR^{Y845F}) or by the genetic absence of EGFR (EGFR^{-/-} cells). It is apparent, moreover, that while SMAD2 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 present data [9, 44–46] suggests that SMADs and specific MAP kinase-targeted bHLH-LZ factors (such as USF) occupy their separate binding motifs at the critical TGF- β 1-responsive PE2 region E box in the PAI-1 promoter (Fig. 10).

The actual mechanism underlying EGFR activation in response to TGF- β 1 is currently under investigation but may involve, among other possibilities, direct recruitment of *src* kinases to the EGFR or the processing and release of a membrane-anchored EGFR ligand (e.g., HB-EGF). PAI-1 expression upon TGF- β 1 addition is at least partly attenuated by the broad-spectrum MMP/ADAM-inhibitor GM6001 (in preparation) suggesting the requirement for MMP activity in signal propagation. Increased PAI-1 expression is a major causative factor in several progressive and chronic fibrotic disorders particularly in the context of systemic vascular disease, diabetes, obesity and the metabolic syndrome [e.g., 16,21,47–50]. Indeed, transgenic mice engineered to overexpress PAI-1 develop arterial thrombosis and perivascular fibrosis with age unlike wildtype littermates [16,21,51]. Suppression of PAI-1 activity is one proposed approach to therapy that, in certain settings, has proven effective in slowing disease progression [16]. The continued clarification of molecular controls on PAI-1 gene regulation and dissection of the associated upstream signaling pathways may well lead to the design of targeted therapeutics to manage the diverse in vivo pathophysiologic cardiovascular consequences of TGF- β 1-mediated PAI-1 overexpression.

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Fig. 1.

EGFR signaling is required for TGF-β1-stimulated PAI-1 expression. Serum-deprived primary VSMC (A) and R22 cells [52] (B) were maintained under quiescence conditions or stimulated with TGF-β1 (1 ng/ml; for 1–4 h) with or without pretreatment with the EGFR inhibitor AG1478. AG1478 decreased basal PAI-1 levels in primary VSMC in a dose-dependent manner and completely blocked PAI-1 induction in response to TGF-β1 in primary cells as well as in R22 VSMC at a final concentration of 2.5 μM (A,B). A molecular genetic approach was utilized to confirm involvement of the EGFR in TGF-β1-initiated signaling. TGF-β1-induced PAI-1 expression was effectively suppressed by adenoviral delivery of a kinase-dead (EGFR^{KD}) EGFR^{K721A}-GFP mutant receptor but not with a control-GFP viral construct (C). Use of an antibody specific for EGFR1 confirmed over-expression of the EGFR^{KD} in infected R22 cells which migrated above endogenous EGFR due to the presence of the GFP tag. A summary of three independent Western blot experiments (mean±SD) with statistical analysis appears in (D). Similarly, TGF-β1 (0.05 to 0.2 ng/ml) stimulated PAI-1 synthesis and ERK1/2 phosphorylation in EGFR^{+/+} MEFs but not in EGFR^{-/-} fibroblasts (E,F). Confluent EGFR^{+/+} and EGFR^{-/-} MEFs were serum-deprived for one day prior to addition of TGF-β1 (at concentrations indicated in E or at 0.1 ng/ml in F). After 4 h, cells were extracted, lysate proteins separated by electrophoresis and membrane transfers probed with antibodies to EGFR (to confirm EGFR status), PAI-1, ERK2, pERK1/2 and pSMAD2 (E). TGF-β1 effectively increased pSMAD2 levels in both EGFR^{+/+} and EGFR^{-/-} MEFs indicating that TGF-β1 signaling (at least to SMAD2) is intact in wild-type and mutant fibroblasts. Data plotted (in F) is the mean±SD of 3 independent experiments. Actin, EGFR and ERK2 provided loading controls.

Fig. 2.

TGF-β1-stimulated PAI-1 expression requires *src* kinase signaling. *src* phosphorylation at the Y416 residue in the activation loop of the kinase domain was evident 15 min after TGF-β1 stimulation (A). Transient transfection of a dominant-negative pp60^{c-*src*} (DN-Src) construct ablated TGF-β1-initiated PAI-1 expression (B). Comparison of wild-type (SYF^{+/+}) and triple (*c-src*, *c-yes*, *c-fyn*) *src* family kinase-deficient (SYF^{-/-}) mouse embryonic fibroblasts (MEFs) indicated that PAI-1 was inducible by TGF-β1 only in wild-type cells confirming *src* kinase involvement in the inductive response (C,D). Data plotted (D) is the mean±SD of triplicate independent assessments. Reexpression of pp60^{c-*src*} in SYF^{-/-} MEFs (SYF^{-/-} c-*src*⁺) restores PAI-1 inducibility in response to TGF-β1 (E). Total cellular pp60^{c-*src*} or ERK2 served as a loading controls for Western analyses.

Fig. 3. EGFR^{Y845} phosphorylation is required for TGF- β 1-induced PAI-1 expression. EGFR phosphorylation (at the Y845 site) was evident within 7 min (by immunocytochemistry; A) and 10 min (by Western blotting; B) after TGF- β 1 (0.1 ng/ml) addition to serum-deprived EGFR^{+/+} MEFs (A,B). VSMC exhibited similar EGFR activation kinetics. Serum-deprived R22 cells were incubated with TGF- β 1 (1 ng/ml) and EGFR^{Y845} phosphorylation determined by Western analysis with pY845-specific antibodies (C). Phosphorylation of the Y845 site in R22 cells occurred between 5 and 15 min post-TGF- β 1 stimulation. TGF- β 1-induced PAI-1 expression was effectively attenuated by adenoviral delivery of a EGFR^{Y845F}-GFP mutant receptor but not with a control-GFP viral construct (D). PDGF-dependent PAI-1 induction, in contrast, was not affected by expression of the EGFR^{Y845F} mutant. Visual examination of R22 cultures infected with the EGFR^{Y845}-GFP or GFP-only adenoviruses confirmed expression of the adenoviral constructs (top panels in D). The histogram (in E) is a summary of three independent Western blot experiments (mean $\pm$ SD), at a similar multiplicity of infection (MOI=50–100), for both the GFP and EGFR^{Y845F}-GFP viruses; insert is representative blot of the triplicate Western analysis. Stripped membranes were probed with antibodies to ERK2 or the EGFR to assess protein loading levels.

Fig. 4.

EGFR activation in response to TGF- β 1 stimulation requires *src* tyrosine kinase activity. TGF- β 1 induced tyrosine phosphorylation of the EGFR and the pp60^{c-src} non-receptor tyrosine kinase (as detected with the phospho-tyrosine-specific antibody 4G10) within 30 min of growth factor addition (1 ng/ml) (A). The identity of the phospho-proteins was determined by blotting with antibodies to pp60^{c-src} and the EGFR (B). Inhibition of *src* kinase activity with the specific *src* family inhibitor SU6656 (1 μ M) dramatically decreased both basal and TGF- β 1-induced Src Y416 phosphorylation (C) without altering total pp60^{c-src} levels (B). TGF- β 1-stimulated phosphorylation of the EGFR (A), particularly at the Y845 site (C), is completely blocked by pretreatment with SU6656 suggesting an upstream signaling role of *src* family members in EGFR activation. Consistent with these findings (in A–C), EGFR phosphorylation in response to TGF- β 1 was evident in SYF^{+/+/+} but not SYF^{-/-/-} MEFs whereas SMAD2 activation (pSMAD2) was similar in both cell types (D). Total SMAD2 levels provided a loading control.

Fig. 5.

SMAD2 phosphorylation in TGF- β 1-treated VSMC is independent of EGFR/MEK-ERK signaling. TGF- β 1-stimulates a sustained phosphorylation (for at least 4 h) of SMAD2 and ERK1/2 activation upon addition to quiescent R22 cells (A). Pretreatment with AG1478 for 30 min prior to a 2 h incubation with TGF- β 1 did not affect SMAD2 phosphorylation but effectively blocked both TGF- β 1-mediated PAI-1 induction (see Fig. 1A) and ERK1/2 phosphorylation (B). Similarly, the MEK inhibitors U0126 (10 μ M) and PD98059 (10–20 μ M) dramatically attenuated TGF- β 1-stimulated PAI-1 expression in both R22 (C) and primary (D) VSMC. U0126 blocked the TGF- β 1-stimulated increase in pERK1/2 levels but did not impact SMAD2 activation in R22 cells (E). Each experimental group is presented in triplicate (in B,E). As anticipated from data in (B,E), SMAD2 was rapidly phosphorylated in both EGFR^{-/-} and wild-type MEFs in response to TGF- β 1 (F). Increased ERK1/2 phosphorylation was evident only in EGFR^{+/+} fibroblasts, initially at 15 min (F) and more significantly at 2 h (G) after growth factor addition, confirming participation of the EGFR in ERK activation by TGF- β 1. The low basal pERK1/2 in EGFR^{-/-} cells suggests a role for EGFR signaling in ERK phosphorylation even in quiescent fibroblasts. ERK2 (A,C), the EGFR (B,E), actin (D), SMAD2/3 (F), ERK1/2 (G) provide loading controls.

Fig. 6.

RhoA signaling is required for TGF- β 1-mediated PAI-1 expression. TGF- β 1 stimulation of serum-deprived R22 cells significantly increased cellular levels of the GTP-bound form of Rho declining to the basal state by 24 h (A). Pretreatment with the Rho inhibitor C3 transferase (for 12 h) induced actin cytoskeletal modifications and cell shape changes, consistent with role of Rho proteins in actin remodeling and confirming the activity of C3 transferase in the VSMC cell system (B). TGF- β 1-induced PAI-1 expression, was effectively suppressed by pretreatment of R22 cells (C) and primary VSMC (D) with C3 transferase as well as by transient transfection of R22 VSMC with a dominant-negative RhoA^{N17} (DN-RhoA) construct (2.5 μ g) (E). Data plotted in (F) is a summary of three independent Western blot experiments (mean $\pm$ SD) using R22 cells. C3=C3 transferase; numbers in parentheses in (F) on the x-axis is the concentration (in μ g/ml) of C3 transferase. Total Rho (A), ERK2 (C,E) EGFR (D) levels provided loading controls.

Fig. 7.

Crucial role of ROCK in PAI-1 induction. To determine the potential involvement of ROCK as a downstream Rho effector of PAI-1 expression, quiescent VSMC were treated with the ROCK inhibitor Y-27632 (20 μ M). Visualization of Texas Red phalloidin-stained R22 cells clearly indicated that actin organization and cell shape were dramatically altered by addition of Y-27632 to R22 cells (A). Y-27632 incubation prior to a 4-h stimulation with TGF- β 1 (1 ng/ml) completely blocked PAI-1 induction even at the lowest concentration (5 μ M) in both primary VSMC (B) and R22 cells (C). Data plotted (D) is the mean+SD of three independent Western blot experiments for R22 VSMC. Y=Y-27632 in (D); numbers in parentheses indicate drug concentration (μ M). Actin, EGFR (B) and ERK2 (C,D) served as loading controls.

Fig. 8.

TGF-β1-induced SMAD2/3 activation and nuclear accumulation is suppressed by inhibition of Rho/ROCK signaling. Pretreatment of quiescent R22 cells with C3 transferase (C3) or Y-27632 effectively inhibited SMAD2 phosphorylation in response to TGF-β1 (A–C). Data plotted (C) is the mean±SD of three independent Western blot experiments for R22 VSMC. Y=Y-27632 in (D); numbers in parentheses indicate drug concentration (μM). TGF-β1-stimulated pSMAD2 (depicted in green) nuclear accumulation (D) is similarly suppressed by pretreatment with Y-27632 (at all concentrations between 5–20 μM, a range that effectively blocked PAI-1 induction [Figs. 7B–D]) indicating that interference with either member of the Rho pathway (Rho, ROCK) had similar consequences on both SMAD2 and SMAD3 activation. Nuclei were visualized by DAPI staining (D). Exposure to TGF-β1 was for 4 h in each case. Membranes were re probed with antibodies to Rho (A) and SMAD2/3 (B) to confirm loading levels. For the time course study, quiescent R22 cells were treated with TGF-β1 for times indicated in the text with or without Y-27632 (20 μM) pretreatment. Equal amounts of lysates from each time point were probed with pSMAD2, pSMAD3, total SMAD2/3, TGF-βRI, PAI-1 or Rho antibodies (E).

Fig. 9.

Inhibition of ROCK signaling with Y-27632 attenuates ERK1/2 but not EGFR^{Y845} phosphorylation in TGF- β 1-stimulated VSMC. To determine the effect of ROCK signaling in ERK1/2 activation, R22 cells were stimulated with TGF- β 1 for 2 h with or without pretreatment with Y-27632 (20 μ M) or AG1478 (2.5 μ M) which was used as a control since interference with EGFR signaling blocks TGF- β 1-induced ERK activation, Fig. 5B); lysates were probed for pERK1/2 and total ERK1/2 levels. Y-27632 effectively attenuated the increase in ERK1/2 phosphorylation in response to TGF- β 1 (A,B). Histogram in (B) illustrates the mean+SD of three independent Western blot experiments. Although inhibition of ROCK signaling significantly decreased TGF- β 1-stimulated pERK1/2 levels (A,B), Y-27632 did not affect TGF- β 1-induced EGFR phosphorylation (170 kDa pEGFR as detected with the 4G10

monoclonal antibody; e.g., Fig. 4A) (C). Blots were reprobed with antibodies to ERK2 (A,B) or total EGFR (C) for loading assessments.

Fig. 10.

Model for TGF-β1-induced PAI-1 expression in VSMC. The available data indicates that TGF-β1 activates two distinct signaling pathways that initiate transcription of PAI-1 in VSMC. Rho/ROCK are required for 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→USF-2) switch [e.g., 45,46] 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-βR occupancy. The actual mechanism underlying EGFR activation in response to TGF-β1 is currently under investigation 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-β1 stimulation of the RhoA/ROCK pathway are presently unclear.