Identification of a cis-Acting Sequence in the Human Plasminogen Activator Inhibitor Type-1 Gene That Mediates Transforming Growth Factor-β1 Responsiveness in Endothelium in Vivo*

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The mechanism of regulation of the plasminogen activator inhibitor type-1 (PAI-1) gene by transforming growth factor-β1 (TGF-β1) was studied in vitro and in vivo in endothelial cells. We constructed adenovirus vectors containing PAI-1 5′-flanking sequences driving expression of a β-galactosidase (β-gal) reporter gene. Cultured bovine endothelial cells were transduced with the vectors and treated with TGF-β1. β-Gal expression was up-regulated 10–20-fold by TGF-β1 when vectors contained 799-base pair (bp) of 5′-flanking sequence, but only minimally (2–3-fold) from a vector containing only 82-bp of 5′ PAI-1 flanking sequence. TGF-β1 up-regulated β-gal expression at the mRNA level, congruently with TGF-β1 up-regulation of expression of the endogenous PAI-1 gene. The constructs were transduced into intact rat carotid endothelium, and TGF-β1 was injected systemically. In vivo, TGF-β1 up-regulated endothelium-specific expression of β-gal 3-fold (p < 0.03) from a vector containing the 799-bp sequence, but did not alter expression from a vector containing the 82-bp sequence. The sequence between −799 and −82 mediates up-regulation of reporter gene expression by TGF-β1 in endothelial cells in vitro and in vivo. This general method permits the elucidation of mechanisms of gene regulation by physiologic stimuli delivered to the endothelium of intact animals.

Plasminogen activator inhibitor type-1 (PAI-1), the major physiological inhibitor of tissue-type plasminogen activator and urokinase plasminogen activator (1, 2), is thought to participate in the regulation of several plasmin-dependent processes including fibrinolysis, thromboplast implantation, cell migration, ovulation, angiogenesis, and wound repair (3). Among these potential biological roles of PAI-1, much attention has been devoted to its role in the arterial wall. In human tissues, PAI-1 is expressed by both vascular smooth muscle and endothelial cells (EC) (4). PAI-1 is up-regulated in vivo in association with atherosclerosis (5), endotoxemia (6), thrombosis (7), and vascular injury (8). Definition of the molecular mechanisms by which PAI-1 expression is up-regulated in these pathological states may lead to a better understanding of vascular pathobiology and could also lead to the development of novel approaches to the prevention of thrombosis and atherosclerosis.

Regulation of PAI-1 expression is thought to occur primarily at the level of gene transcription (3). Specific agents that up-regulate PAI-1 expression in experimental systems include endotoxin (9, 10), thrombin (11), glucocorticoids (12), tumor necrosis factor-α (13), angiotensin II (14), and transforming growth factor-β1 (TGF-β1) (15, 16). Among these agents, the mechanism of regulation of PAI-1 expression in response to TGF-β1 has been studied most extensively (17–21). Transforming growth factor-β1 is a major constituent of platelet α-granules (22), from which it may be released during vascular injury or thrombosis. Locally increased expression of PAI-1, up-regulated in response to platelet TGF-β1 (7, 23), may enhance the resistance of platelet-rich thrombi to plasminogen activator-mediated fibrinolysis and may also result in increased fibrin deposition leading to the progression of atherosclerosis (5, 24). The mechanism by which TGF-β1 regulates PAI-1 expression in endothelium is therefore of pathophysiological significance.

Despite the likely importance of PAI-1 regulation by TGF-β1 in EC in vivo, virtually all of the data on the mechanisms of regulation of PAI-1 gene expression have been produced in transformed hepatocyte and fibroblast lines in vitro (17–21, 25). While these studies are informative, the extent to which one may extrapolate the results of gene regulation experiments across cell types and from in vitro to in vivo is unclear. For example, the mechanisms of regulation of vascular cell adhesion molecule-1 in cultured skeletal muscle and epithelial cells differ from those operative in cultured EC (26, 27). Insulin and proinsulin up-regulate PAI-1 production in cultured HepG2 cells but not in cultured EC (28), and the effects of TGF-β1 on PAI-1 mRNA half-life also differ between HepG2 cells and EC (18). Moreover, even if gene regulation studies were performed solely with cultured EC, patterns of gene expression and regulation in EC (both baseline transcript levels (29) as well as regulation of transcription in response to added cytokines (30)) are quite variable in vitro, leading to the generation of conflicting experimental results in different laboratories. Finally, PAI-1 protein is nearly undetectable in normal unstimulated endothelium in vivo (31); yet when placed in vitro, EC synthesize and secrete large amounts of PAI-1 (32). For all of these reasons, to understand the physiological mechanisms of regulation of PAI-1 expression in EC, it is most appropriate to perform experiments in vivo in endothelium. Until recently,
however, this has not been feasible.

We recently reported an animal model of in vivo endotheli-um-specific gene transfer (33) and speculated on the utility of this model to permit the definition of molecular mechanisms of in vivo EC gene regulation. Here we report the use of this animal model to define a functional cis-acting sequence in the human PAI-1 promoter that mediates up-regulation of EC gene expression in response to TGF-β1 administration in an intact animal.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—We obtained the following plasmids: pSP72 (Promega, Madison, WI); pAdRSV4 and pAdBglII (34) (Dr. Blake Roessler and Dr. Beverly Davidson, University of Michigan); pLZ11 (35) (Dr. Joshua Sanses, Washington University); p3P, a derivative of pUC13 containing a fragment of the human PAI-1 promoter extending from 3.0 kb 5'- to the transcription start site at the EcoRI site in the 5'-untranslated sequence (12) (Dr. Thomas Quertermous, Vanderbilt University); and pPK9A (36) (Dr. Anita Roberts, National Cancer Institute). To generate recombinant replication-defective adenovirus (Ad) vectors (Fig. 1), five plasmids (pAdPAI800-β-Gal, pAdIAP800-β-Gal, pAdPAI82β-Gal, pAdRSVnLacZ, and pAdRSVTFG-β1) were constructed. Isolated restriction fragments contained the elements of the 5' end (0–1 and 9.2–16.1 map units) of the Ad 5 genome (37), with individual expression cassettes inserted at the site of the E1 deletion. pAd-PAI800-β-Gal, pAdIAP800-β-Gal, and pAdPAI82β-Gal were constructed as follows. A BgIII fragment containing the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter and SV40 polyadenylation sequences (termed "SVPA") was excised from pAdRSV4 and ligated into BamHI-digested pSP72. The resulting plasmid, designated pSP72BglII, was digested with SmaI and BamHI then ligated to a blunt-ended, BglII-XbaI fragment of pLZ11. This BgII-XbaI fragment of pLZ11 contained a nuclear-targeted Escherichia coli β-gal gene (nLacZ). The plasmid product of the ligation of the BgII-XbaI fragment to pSP72BglII, termed pSP72β-Gal, contained nLacZ fused to SVPA, with cloning sites upstream for insertion of promoter fragments. To obtain fragments of the human PAI-1 promoter, we first digested p3P with HindIII and EcoRI, releasing an 874-bp (all base pair enumeration includes unpaired overhangs) sequence of the human PAI-1 gene, including 799 bp upstream of the transcription start site and 75 bp of the first (untranslated) exon. This 874-bp fragment was blunt-ended and ligated into PvuII-cut pSP72, yielding pSP72P2P, and restoring the EcoRI site. PAI-1 promoter fragments were removed from pSP72P2P either as: 1) a 887-bp XhoI-EcoRI fragment containing the original 874-bp promoter + exon 1 fragment along with 13 bp of 5'-flanking polylinker sequence ("PAI100"); containing 799 bp of PAI-1 sequence 5' to the PAI-1 transcription start site ("PAI800"), or 2) a 157-bp BsrI-EcoRI fragment, truncated by 717 bp of 5' PAI-1 promoter sequence, but identical to the longer XhoI-EcoRI promoter fragment at the 3' end ("PAI282," containing only 82 bp of PAI-1 sequence 5' to the PAI-1 transcription start). PAI800 and PAI822 were blunt-ended, and ligated into the StuI site of pSP72β-Gal. The resulting plasmids, pPA800β-Gal and pPA82β-Gal, contained expression cassettes consisting either of PAI800 or PAI822 sequences driving expression of nLacZ-SVPA. pPA800β-Gal and pPA82β-Gal were then digested with BglII and XhoI, sites that flanked the PAI-1-SVPA expression cassette. These BgII-XhoI fragments were blunt-ended and ligated into a BgII-digested blunt-ended pAdBglIII. Both orientations of the PAI800 expression cassette as well as the forward (with respect to the Ad genome sequences) orientation of the PAI822 expression cassette were propagated as pAd-PAI800-β-Gal, pAdIAP800-β-Gal, and pAdPAI82β-Gal, respectively. Both pAdPAI800-β-Gal and pAdIAP800-β-Gal were used to construct vectors because use of an exogenous expression cassette that was transcribed toward the Ad E1A enhancer resulted in elimination of baseline expression of the cassette. Our in vitro experiments, however, demonstrated similar baseline levels of expression for both orientations of the PAI822 expression cassette (data not shown), therefore pAdIAP800-β-Gal-derived vector was not used in in vivo experiments.

The plasmid pAdRSVTFG-β1 was constructed by digestion of pPK9A with ClaI releasing a 1.35-kb fragment containing 5'-flanking region of porcine TGF-β1. This BgIII fragment was ligated into the BamHI site of pAdRSV4, creating an expression cassette consisting of the RSV LTR promoter driving nLacZ-SVPA, oriented 5' → 3' with respect to the Ad5 sequences of pAdRSV4. The fidelity of all plasmid constructions was confirmed by restriction mapping.

Recombinant Ad Production—Adenoviral vectors were generated by cotransfection of linearized plasmids pAdPAI800-β-Gal, pAdIAP800-β-Gal, pAdPAI82β-Gal, pAdRSVnLacZ, or pAdRSVTFG-β1 with the 35-kb ClaI fragment of Ad-d327 (an E3-deleted Ad (38)). The resulting replication-defective Ad vectors are shown in Fig. 1. To screen for recombinant Ad vectors AdRSV5-β-Gal, AdIAP800-β-Gal, AdIAP800-β-Gal, and AdPAI82β-Gal, freeze-thaw lysates made from plaques were tested for ability to transfer recombinant expression to 293 cells. For screening putative AdRSVTFG-β1 recombinants, lysates were tested for the ability to transfer the capacity to synthesize and secrete TGF-β1 to 293 cells. TGF-β1 was detected by assaying conditioned media with the Predica TGF-β1 ELISA kit (Genzyme, Cambridge, MA), without prior acid activation. Virus harvested from recombinant plaques was propagated and titered on 293 cells. An additional recombinant Ad, AdHV1.1, was used as a control in certain experiments. AdHV1.1 has a structure similar to the other vectors, containing a hirudin cDNA fused to SVPA, driven by the RSV LTR promoter (39). Titers of purified stocks of all viruses ranged from 5 × 10^9 to 2 × 10^11 plaque-forming units (pfu/ml).

In Vitro Gene Transfer into EC—Calf pulmonary artery EC (CPAEC, passage 15; CCL 209) were obtained from the American Type Culture Collection (Rockville, MD). CPAEC were maintained in growth medium consisting of Dulbecco's minimum essential medium (Biofluids, Rockville, MD) with 20% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Biofluids). CPAEC were grown at 37°C under 5% CO2 and passed upon confluency at a 1:4 ratio. CPAEC (passage 17–23) were transduced by incubation with Ad vector stocks diluted in Opti-MEM (Life Technologies, Inc.) for 90 min followed by replacement with growth medium. In vitro gene transfer was performed at multiplicities of infection (m.o.i.) ranging from 25 to 500.

For experiments testing the effect of exogenous TGF-β1 on recombinant gene expression, infected EC were incubated in medium containing either purified human TGF-β1 (Signa) dissolved in 10 μg/ml bovine serum albumin or vehicle only. Twenty-four hours after addition of TGF-β1, EC were lysed in buffer (100 μM potassium phosphate, pH 7.2, 0.2% Triton X-100, 1 mM diethiothreitol). Extracts were assayed for β-gal activity by chemiluminescence assay (Tropix, Inc., Bedford, MA), using E. coli β-gal (Bohringer Mannheim) as a standard. To visualize the expression of recombinant gene products, parallel wells of transduced EC were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 37°C for 1 h (40).

For experiments analyzing the time course of recombinant gene expression, CPAEC were infected with Ad vectors, then were incubated with growth medium containing 10 ng/ml TGF-β1 or vehicle only. Endothelial cells were harvested either immediately (t = 0) or at various time points after infection. Cell extracts were assayed for β-gal activity, as above.

In certain experiments, CPAEC transduced with one of the four β-gal expressing vectors were subsequently infected with a second virus, either AdRSVTFG-β1 or AdHV1.1. Immediately following infection with β-gal vector, EC were rinsed once with OPTI-MEM, incubated with AdRSVTFG-β1 or AdHV1.1 for 90 min, then fed with growth medium. At defined time points, the EC were harvested and assayed for β-gal activity and protein. The conditioned media were also collected and assayed for TGF-β1 antigen by ELISA. As a specificity control, in certain experiments a rabbit polyclonal neutralizing antibody to TGF-β1 (100 μg/ml; Promega, Madison, WI) was added at the time of growth medium addition.

The primary purpose of this study was to identify cis-acting elements in the PAI-1 promoter that are TGF-β1-responsive in vivo, not to perform promoter deletion or reconstitution analyses to identify and quantify cis-acting elements that are functional under baseline conditions. For this reason, in both in vitro and in vivo gene transfer studies, reporter gene expression is normalized to cell or tissue protein, and comparisons are generally made between expression levels in cells receiving the same vector in the presence or absence of TGF-β1 stimulation. We do not normalize data based on expression of a second reporter gene, as is usually done with plasmid-mediated transfection. There are two reasons for this. First, the in vitro transduction efficiencies are essentially 100%, and therefore do not require controls specif-
which encodes rat PAI-1 (41); saline citrate and 0.1% SDS, and autoradiographed at room temperature followed by 30 min wash at 60°C in 0.1 mM Tris, pH 7.4, 0.1% SDS, and autoradiographed at ~70°C. cDNA probes were: PAI-1, a 2.4-kb HindIII fragment of plasmid pSKPAI53, which encodes rat PAI-1 (41); β-gal, a 3.0-kb Stul-XbaI fragment of plasmid pLZ11 (35); GAPDH, a 1.4-kb BamHI-HindIII fragment of GAPDH (a gift from Dr. Stephen Karlinos, NINDS). All probes were 32P-labeled with the random primer method to specific activity of approximately 1 × 10^9 cpm/μg. An initial blot was subjected to autoradiography to obtain a figure for presentation. The experiment was then repeated six times, followed each time by Northern analysis and visualization of transcripts on blots with a FUJIX Bas1000 Bioimaging Analyzer. The radioactivity present in each band was quantitated with an analyzer (Fig. 1). A two-tailed t-test, \( t \) = 0.05. Statistical analyses were performed on a microcomputer with the aid of the SigmaStat program (Jandel Scientific, San Rafael, CA).

RESULTS

Regulation of Human PAI-1 Promoter by TGF-β1 in EC in Vitro—Infection of CPAEC with each of the β-gal vectors at 4 × 10^8 pfu/ml resulted in gene transfer efficiencies of essentially 100% (data not shown), thus eliminating concerns that results were obtained only from a small, potentially unrepresentative population of cells. Endothelial cells were infected with AdPAI800-β-Gal, AdIAP800-β-Gal, AdPAISβ-Gal, or AdRSVβ-Gal at 4 × 10^8 pfu/ml followed by addition of TGF-β1 protein or vehicle (Fig. 2). β-Galactosidase expression was up-regulated by TGF-β1 in a dose-related manner in EC transduced with AdPAI800-β-Gal and AdIAP800-β-Gal, reaching maximal levels at 10 ng/ml TGF-β1 up-regulated β-gal expression by a mean of 15-fold for 6.5–13-fold; \( n = 6 \) in EC transduced with AdPAI800-β-Gal and by a mean of 21-fold in EC transduced with AdIAP800-β-Gal (range 10–35-fold; \( n = 6 \). Higher peak levels of expression from the AdIAP800-β-Gal vector versus the AdPAI800-β-Gal vector were found consistently; this may result from a relatively greater interference with reporter gene transcription by factors bound to the adenovirus E1A enhancer in AdPAI800-β-Gal, in which PAI-1 5′-flanking sequences are adjacent to the E1A enhancer sequences (Fig. 1). Expression of β-gal was up-regulated by TGF-β1 in EC transduced with AdPAISβ-Gal or AdRSVβ-Gal to a relatively small extent (mean 2.5-fold, range 1–3-fold; \( n = 4 \); mean 2.7-fold, range 2–3-fold; \( n = 3 \), respectively). Of note, in individual experiments, baseline (i.e. no TGF-β1) β-gal activity assays might be inappropriate due to endogenous activity in rat tissue. The ELISA (5–3’ Inc., Boulder, CO), while less sensitive than the activity by 30 min was specific for the E. coli versus mammalian enzyme (data not shown).

Protein Determinations—Protein concentrations of cell and tissue extracts were determined with the BCA assay (Pierce), using bovine serum albumin as a standard.
Fig. 1. Structure of Ad vectors. Vectors were made by homologous recombination between shuttle vector plasmids (see "Experimental Procedures") and the large ClaI fragment of adenovirus 5 di327 mutant. This recombination results in insertion of each of the five illustrated expression cassettes at the site of deletion of the adenovirus E1 gene. Structural features of the vectors and expression cassettes are indicated: ITR, inverted terminal repeat; SV40 poly A, polyadenylation signal from simian virus-40; n LacZ, nuclear targeted E. coli lacZ gene; IAP800, 800-bp promoter fragment of human PAI-1 gene expressed 3' to 5' relative to adenovirus genome; PAI82, 82-bp promoter fragment of human PAI-1 gene; PAI800, 800-bp promoter fragment of human PAI-1 gene expressed 5' to 3' relative to adenovirus genome. Directions of transcription within the expression cassettes are indicated by arrows. The location of the TATA box and of consensus binding sequences for transcription factors AP-1 and NF-1 are shown.

Fig. 2. Up-regulation by TGF-β1 of expression from the 800-bp PAI-1 promoter fragment in cultured CPAEC. Endothelial cells infected with the listed Ad vectors for 90 min were exposed to the indicated concentrations of human TGF-β1 for 24–36 h. β-Galactosidase activity in cell lysates is expressed as fold induction above activity present in EC transduced with the same vector and receiving vehicle only. Data are expressed as means ± S.D. of results from 3–6 independent experiments.

We considered that the observed differences in TGF-β1 inducibility among the constructs might be kinetic rather than absolute. To explore this possibility, transduced TGF-β1-stimulated EC were assayed for β-gal activity at later time points (Fig. 4). β-Galactosidase expression was again inducible in EC transduced with AdPAI800β-Gal and AdIAP800β-Gal (peak inducibility mean 16-fold, range 9–28-fold (n = 4); mean 29-fold, range 24–35-fold (n = 5), respectively). β-Galactosidase expression was only mildly inducible in CPAEC transduced with AdPAI82β-Gal and AdRSVβ-Gal (peak up-regulation mean 3-fold, range 2–4-fold (n = 2); mean 4-fold, range 2–8-fold (n = 3), respectively). Thus, the lower TGF-β1-inducibility of the PAI82 and RSV promoters was absolute, not kinetic.

Two central premises underlie experiments in which fragments of genomic DNA are fused to reporter genes for the purpose of drawing inferences regarding the regulation of expression from intact genomic DNA: 1) increased reporter protein levels are reflective of the abundance of corresponding mRNA and 2) the behavior of the largest promoter fragment (into which deletions and mutations are introduced) fused to the reporter gene accurately models the behavior of the endogenous promoter fused to the endogenous gene, in this case PAI-1. To verify these two premises for the PAI-1 promoter-β-gal constructs used in our study, we performed Northern analysis of CPAEC that had been infected with AdPAI800β-Gal, AdIAP800β-Gal, AdPAI82β-Gal, or AdRSVβ-Gal and treated with 0, 1, or 10 ng/ml TGF-β1 (Fig. 5). Endogenous PAI-1 mRNA was up-regulated by TGF-β1 (at 10 ng/ml) in all cells to a similar extent (2–5-fold; p = 0.35). Specific mRNA encoding β-gal was up-regulated by TGF-β1 (at 10 ng/ml) in parallel with endogenous PAI-1 mRNA when expression of β-gal was driven by the 799-bp PAI-1 promoter fragment in either orientation, but not when expression of β-gal was driven by the 82-bp PAI-1 promoter (2.4-fold for the IAP800 promoter; 2.0-fold for PAI800 promoter versus 1.3-fold for PAI82 promoter; p = 0.011 and 0.024 for IAP800 and PAI800 versus the 82-bp fragment, respectively). These mRNA data are in qualitative agreement with the protein data obtained with the constructs (Fig. 2). Use of the PAI800β-Gal Ad vector and deletion mutants thereof, with measurement of β-gal activity, appears to represent a valid approach with which to study mechanisms of regulation of the endogenous PAI-1 gene.

Co-infection of CPAEC with a TGF-β1-expressing Ad Vector—As a prelude to in vito studies examining the ability of TGF-β1 to up-regulate expression from PAI-1 promoter fragments transduced into intact endothelium, we tested whether co-infection of CPAEC with a TGF-β1-expressing Ad vector could serve as a source of TGF-β1 protein to up-regulate PAI-1 promoter activity in vitro. The CPAEC infected with AdPAI800β-Gal, AdIAP800β-Gal, AdPAI82β-Gal, or AdRSVβ-Gal were exposed to AdRSVβGIF1 at m.o.i. of 25 or 250. As a control for nonspecific effects of infection with AdRSVβGIF1, parallel wells of transduced CPAEC were infected with the hirudin-expressing vector AdHV1.1, also at m.o.i. of 25 or 250. Delivery of TGF-β1 by infection with AdRSVβGIF1 up-regulated β-gal expression in a manner both qualitatively and quantitatively similar to that found with addition of TGF-β1 protein (Fig. 6A; compare to Fig. 2). A time course study of
up-regulation of β-gal expression by AdRSVTGF-β1 also produced results similar to those obtained by addition of recombinant TGF-β1 protein (Fig. 6B; compare to Fig. 4). The slower kinetics of induction of β-gal expression in these co-infection experiments is likely due to the gradual accumulation of TGF-β1 in the medium (Fig. 6B, inset), compared to the immediately high levels of TGF-β1 that are obtained following addition of recombinant protein to the culture dish.

Regulation of the Human PAI-1 Promoter in Endothelium in Vivo—To test the ability of fragments of the human PAI-1 promoter to respond to TGF-β1 stimulation in vivo, we introduced AdPAI800β-Gal, AdPAI82β-Gal, or AdRSVβ-Gal into the endothelium of rat common carotid arteries. In initial studies we attempted to deliver TGF-β1 to these rats by means of in vivo expression from the AdRSVTGF-β1 virus. First we attempted co-local delivery: AdPAI800β-Gal was infused for 15 min followed by infusion of either AdRSVTGF-β1 or a control adenovirus, AdHV1.1 (3 × 10¹⁰ pfu/ml of either virus, also for 15 min). No up-regulation of β-gal expression was observed, although the interpretation of this negative result is clouded by our previous observations on potential interference between co-infecting viruses (see “Experimental Procedures”). We next attempted to achieve systemically elevated levels of TGF-β1 protein by intravenous injection of a bolus of up to 8 × 10⁹ pfu of AdRSVTGF-β1 (injection of higher amounts of AdRSV-β1 was fatal). Although injection of 5 × 10⁹ pfu of AdRSVβ-Gal yielded the expected evidence of significant recombinant gene expression in hepatocytes (numerous blue hepatocyte nuclei; not shown), in no rat injected with up to 8 × 10⁹ pfu/ml AdRSVTGF-β1 did we detect an elevated plasma TGF-β1 level. We therefore attempted up-regulation of PAI-1 promoter activity by systemic injection of recombinant TGF-β1 protein, a technique shown to up-regulate endogenous PAI-1 expression in mice (43).

In preliminary time course experiments, carotid arteries were transduced with AdPAI800β-Gal. Three days later, rats were injected intravenously with 100 or 200 μg/kg TGF-β1, or vehicle only. Arteries were harvested 24, 48, or 72 h later. β-Galactosidase expression was up-regulated, as determined by X-gal staining followed both by en face viewing and by counting transduced EC in histologic sections (data not shown). Up-regulation appeared maximal 48 h after TGF-β1 injection and was not further increased either at a dose of 200 μg/kg TGF-β1 or in arteries harvested at 24 and 72 h. Thus, we carried out a series of definitive in vivo experiments with the optimized protocol of injection of 100 μg/kg TGF-β1 and vessel harvest 48 h later.

In rats with carotid endothelium transduced with AdPAI800β-Gal (n = 6), systemic injection of TGF-β1 resulted in a 3-fold up-regulation of β-gal activity in carotid artery ex-
treatments: 2.0 ± 1.7 milliunits/mg versus 6.1 ± 3.6; p < 0.03 (Fig. 7A). In contrast, TGF-β1 injection did not alter β-gal activity in rat carotid endothelium transduced with either AdPAI82β-Gal (n = 5), 4.1 ± 1.3 (control) versus 3.4 ± 1.0 milliunits/mg with TGF-β1 (p = 0.57); or AdRSVβ-Gal (n = 3), 21 ± 9.6 (control) versus 16 ± 6.1 milliunits/mg with TGF-β1 (p = 0.50). Nearly identical results were obtained by assaying the same vessel extracts for β-gal antigen (Fig. 7B). β-Gal antigen in arteries transduced with AdPAI800β-Gal was significantly increased from 0.65 ± 0.49 to 2.4 ± 1.5 ng/mg (p < 0.03) following systemic injection of TGF-β1. Again, systemic TGF-β1 injection did not alter β-gal expression in rat carotid endothelium transduced with either AdPAI82β-Gal, 1.7 ± 1.6 (control) versus 1.1 ± 0.37 ng/mg with TGF-β1 (p = 0.49), or AdRSVβ-Gal, 10 ± 9.1 (control) to 6.0 ± 3.0 ng/mg with TGF-β1; (p = 0.49). Of note, similar to the in vitro data, the baseline levels of β-gal expression (both activity and antigen) were increased (2-fold) in PAI82β-Gal versus PAI800β-Gal-transduced arteries.

Additional carotid arteries transduced with AdPAI800β-Gal, AdPAI82β-Gal, or AdRSVβ-Gal and taken from rats exposed to systemic injection of either TGF-β1 or vehicle were harvested 48 h later and stained with X-gal. Arteries transduced with AdPAI800β-Gal were viewed both en face and by histologic sectioning (Fig. 8). There was a visible increase in β-gal expression in the endothelium of TGF-β1-exposed arteries (Fig. 8, B and D) when compared to that present in arteries taken from animals injected with vehicle only (Fig. 8, A and C). No increase in β-gal expression in response to TGF-β1 was seen following X-gal staining of arteries transduced with either AdPAI82β-Gal or AdRSVβ-Gal (not shown). Thus, an in situ assay of β-gal expression confirmed the results of tissue extract assays and provided additional data that localized the up-regulation of β-gal expression specifically to the endothelium. Taken together, the in vivo data identify a TGF-β1-responsive element of the human PAI-1 promoter within a defined sequence of 717 bp (between HindIII and BsrI sites), and indicate that in the endothelium of an intact animal there are no DNA elements strongly responsive to TGF-β1 present in the 3′ 82 bp of the promoter sequence.

**DISCUSSION**

We used Ad-mediated gene transfer to identify a sequence in the human PAI-1 gene that is responsive to TGF-β1 in endothelium in vivo. Our major findings were: 1) 799 bp of the human PAI-1 promoter, along with 75 bp of 5′-untranslated sequence, confer TGF-β1-responsiveness on a reporter gene in arterial EC in vitro and carotid endothelium in vivo; the presence of E1A enhancer sequences adjacent to the promoter does not prevent TGF-β1-responsiveness. 2) Transcripts from the 799-bp PAI-1 promoter fragment are up-regulated in parallel with transcripts of the PAI-1 gene in cultured EC, validating the use of these Ad-encoded constructs to study the regulation of PAI-1. 3) Recombinant TGF-β1, delivered by co-infection with a TGF-β1-expressing Ad vector in vitro, duplicates the up-regulation of PAI-1 promoter activity achieved with purified TGF-β1 protein. 4) The proximal 82 bp of the human PAI-1 promoter are minimally responsive to TGF-β1 in EC in vitro, and are unresponsive to TGF-β1 in rat endothelium in vivo.

Two previous studies examined the regulation of PAI-1 expression in endothelium. Sawdey et al. reported that lipopolysaccharide, tumor necrosis factor-α, or TGF-β1 up-regulated PAI-1 transcription in cultured bovine EC; however, no functional analysis of the PAI-1 promoter was performed (44). Van Zonneveld et al. (12), using plasmid transfection into both cultured rat FTO2B hepatoma cells and bovine EC, analyzed the human PAI-1 promoter. In FTO2B cells, dexamethasone-responsive sequences between −800 and +75 mediated a 40-fold induction of reporter gene expression. In bovine EC, PAI-1 promoter fragments of 187 bp and 1.5 kb were both functional, but no data were presented on their responsiveness to dexamethasone. Of note, we treated CPAEC transduced with AdPAI800β-Gal with dexamethasone and (in contrast both to our results with TGF-β1 and to those of van Zonneveld in FTO2B cells) found no up-regulation of β-gal expression. Thus, glucocorticoid regulation of the PAI-1 promoter may be cell type-specific, as reported elsewhere for PAI-1 regulation by insulin (28). These results underscore the importance of performing
promoter analysis in the cell type of interest, in this case EC. To our knowledge, the present study is the first to provide a mechanistic analysis of the regulation of the PAI-1 promoter in EC either in vitro or in vivo.

We determined that elements present between −799 and −82 in the human PAI-1 promoter mediate TGF-β1-responsiveness in endothelium. Westerhausen et al. (18), working with HepG2 cells, localized TGF-β1-responsive elements between −791 and −328 (5-fold) and −328 to −187 (2-fold). No TGF-β1-responsive elements were found from −187 to +72.

Keeton et al. (17), working with Hep3B cells, found TGF-β1-responsive elements from −800 to −636 (50-fold) and from −87 to −49 (11-fold; 7-fold in a construct beginning at −82). Riccio et al. (19), also working with Hep3B cells, localized a TGF-β1-responsive sequence from −598 to −532 (5-fold); sequences from −115 to +72 were less responsive (2-fold) and further deletions were not studied. Thus, our results are consistent with those produced with cultured hepatoma cells in identifying bp −799 to −82 as mediating TGF-β1 responsiveness. In contrast to Keeton et al. (17) and potentially to Riccio et al. (19) as well, in experiments performed in EC, we found only minimal TGF-β1 responsiveness in vitro and no responsiveness in vivo within the −82 to +75 sequence.

The 82-bp PAI-1 promoter fragment had a consistently higher constitutive activity (mean 6-fold) than the 799-bp fragment in the in vitro transduction assays. In the in vivo experiments, this increase was still present, but was smaller (2-fold). We are cautious in our interpretation of these data because the experiments were not designed prospectively to test the relative strengths of promoter sequences. Nevertheless, this was a consistent finding and is similar to the 2-fold increase in activity of a 187-bp versus a 1.5-kb PAI-1 promoter fragment found in cultured bovine EC (12). A negative regulatory element that is functional in EC may be present upstream of −82 bp. Further prospective quantitative studies are required to address this conclusively.

In addition to defining TGF-β1-responsive sequences in the PAI-1 gene that function in endothelium, one of our primary goals was to establish a quantitative system for studying gene regulation in vivo in endothelium. For these initial studies, we used constructs containing sequences that mediate TGF-β1 responsiveness in vitro in other cell types (17–21). Accordingly, the finding that TGF-β1 up-regulated expression from AdPAI800β-Gal was to a certain extent anticipated. Nevertheless, our results are novel in that they elucidate an in vivo mechanism of PAI-1 regulation in endothelium, the existence of which was previously only hypothetical. The correlation of our in vitro
and in vivo results is useful: the in vivo results prove biological relevance while the in vitro system permits a more robust experimental approach. In vitro strategies will be required, for example, for the eventual identification and cloning of transcription factors involved in gene regulation by TGF-β1. In vivo approaches will be required for a confirmation that such factors not merely in vitro phenomena but are present and functional in intact animals.

The method described herein permits the definition of pathways of EC gene regulation by physiologic stimuli in intact animals. While exposure of EC to TGF-β1 may be accomplished both in vitro and in vivo, other important physiologic stimuli that are delivered to the endothelium, such as alterations in blood pressure or flow (45), cannot be adequately modeled (46) (and therefore are not conclusively studied) in vitro. We anticipate that the Ad vector-reporter gene system described herein will permit further in vivo definition of cis-acting sequences that modulate EC gene expression in response to all types of physiologic stimuli, including flow and pressure. Our ability to measure significant up-regulation of gene expression with only 5–6 animals per group (Fig. 7) also demonstrates the utility of this somatic cell gene transfer approach in comparison to analogous experiments that might be performed by germ-line targeting of genetic material to the endothelium (47).

Our experimental system also has shortcomings. Preparation of the large amounts of purified agonists such as TGF-β1 that must be injected systemically to produce biological effects at a distance can be time consuming and costly. Moreover, side effects of agonist delivery into the systemic circulation could potentially confound experimental results. For example, we cannot be certain that TGF-β1 is the proximal effector up-regulating PAI-1 expression in the transduced carotid endothelium. It is possible that other downstream agonists, induced in vivo by TGF-β1, participate in the induction of PAI-1. Nevertheless, the consistency of our in vivo results with those obtained in the far more simple in vitro system supports a direct role for TGF-β1 in vivo. A more conclusive demonstration of a proximal role for TGF-β1 in vivo might be obtained by incorporating a dominant negative TGF-β1 receptor into the Ad vector used to transduce endothelium; the consequent elimination of PAI-1 promoter up-regulation would support a role for TGF-β1 at the surface of transduced EC. In addition, incorporation of promoter elements that are highly active in hepatocytes (48) may permit systemic delivery of agonists by intravenous administration of Ad vectors, eliminating the need for large amounts of purified recombinant proteins.

Side effects specific to the Ad delivery system are another potential difficulty inherent in our in vivo experimental system. While we have excluded certain significant alterations in the vascular phenotype consequent to Ad infusion in this rat system (33), we cannot eliminate the possibility that the artery wall is somehow altered by exposure to Ad, such that it is no longer representative of a “normal” artery. In a rabbit model, we recently reported marked alterations in vascular phenotype following exposure to Ad (49); similar findings have not been reported by us or by others in the rat. Nevertheless, no somatic gene transfer system is completely free of such considerations. The potential occurrence of confounding local biological phenomena must be considered in somatic gene transfer experiments, just as the potential for developmental activation of compensatory gene expression must be considered in germline transgenic and knockout experiments.

In summary, we defined a functional cis-acting sequence in the PAI-1 promoter in endothelium in vivo. Future experiments will include transduction of additional deleted and mutated promoter sequences as well as the co-transduction of dominant negative and constitutively active components of signal transduction pathways that participate in PAI-1 regulation (50). This general method permits definition of pathways that mediate gene regulation in endothelium in vivo.

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REFERENCES
1. van Meijer, M., and Pannekoek, H. (1995) Fibrinolysis 9, 263–276
2. Sprengers, E. D., and Kluft, C. (1987) Blood 69, 381–387
3. Vassalli, J.-D., Sappino, A.-P., and Belin, D. (1991) J. Clin. Invest. 88, 1067–1072
4. Simpson, A. J., Booth, N. A., Moore, N. R., and Bennett, B. (1991) J. Clin. Pathol. 44, 139–143
5. Schneiderman, J., Sawday, M. S., Keeton, M. R., Bordin, G. M., Bernstein, E. F., Dilley, R. B., and Loskutoff, D. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6998–7002
6. Pralong, G., Calandra, T., Glauer, M.-P., Schellekens, J., Verhoef, J., Bachmann, F., and Kruthoff, E. K. O. (1989) Thromb. Haemostasis 61, 459–462
7. Fuji, S., Sawa, H., Safita, J. E., Lucore, C. L., and Seibel, B. E. (1992) Circulation 86, 2000–2010
8. Hasenstab, F., Frenouh, R., and Cloves, A. (1994) FASEB J. 8, A319 (abstr.)
9. Coluci, M., Paramo, J. A., and Cullen, D. (1985) J. Clin. Invest. 75, 818–824
10. Emeis, J. J., and Kooistra, T. (1986) J. Exp. Med. 163, 1260–1266
11. Duche, D., and Quertermous, T. (1989) Blood 74, 222–228
12. van Zonneveld, A.-J., Curriden, S. A., and Loskutoff, D. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 85, 5525–5529
13. Schlee, R. R., Bevilacqua, M. P., Sawday, M., Gimbrone, M. A., Jr., and...
Loskutoff, D. J. (1988) J. Biol. Chem. 263, 5797–5803
14. van Leeuwen, R. T. J., Kol, A., Andreotti, F., Kluft, C., Maseri, A., and Sperti, G. (1994) Circulation 90, 362–368
15. Saksela, O., Moscatelli, D., and Rifkin, D. B. (1987) J. Cell Biol. 106, 857–963
16. Mimuro, J., and Loskutoff, D. J. (1987) Thromb. Haemostasis 58, 447
17. Keeton, M. R., Curriden, S. A., van Zonneveld, A. J., and Loskutoff, D. J. (1991) J. Biol. Chem. 266, 23048–23052
18. Westerhausen, D. R., Jr., Hopkins, W. E., and Billadello, J. J. (1991) J. Biol. Chem. 266, 1092–1100
19. Riccio, A., Pedone, P. V., Lund, L. R., Olesen, T., Steen Olsen, H., and Andreasen, P. A. (1992) Mol. Cell. Biol. 12, 1846–1855
20. Sandler, M. A., Zhang, J.-N., Westerhausen, D. R., Jr., and Billadello, J. J. (1994) J. Biol. Chem. 269, 21500–21504
21. Chang, E., and Goldberg, H. (1995) J. Biol. Chem. 270, 4473–4477
22. Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M., and Sporn, M. B. (1983) J. Biol. Chem. 258, 7155–7160
23. Slivka, S. R., and Loskutoff, D. J. (1991) Blood 77, 1013–1019
24. Juhan-Vague, I., and Alessi, M. C. (1993) Gene (Amst.) 73, 459–468
25. Resnick, N., Collins, T., Atkinson, W., Bonthron, D. T., Dewey, C. F., Jr., and Gimbrone, M. A., Jr. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 4591–4595
26. Dewey, C. F., Jr. (1979) in Dynamics of Arterial Flow (Wolf, S., and Werthessen, N. T., eds) pp. 55–103, Plenum Press, New York
27. Aird, W. C., Jahroudi, N., Weiler-Guettler, H., Rayburn, H. B., and Rosenberg, R. D. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 4567–4571
28. Aird, W. C., Jahroudi, N., Weiler-Guettler, H., Rayburn, H. B., and Rosenberg, R. D. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 4567–4571
29. Kozarsky, K. F., McKinley, D. R., Austin, L. L., Raper, S. E., Stratford-Perricaudet, L. D., and Wilson, J. M. (1994) J. Biol. Chem. 269, 13695–13702
30. BRAND, T., MACLELLAN, W. R., and SCHNEIDER, M. D. (1993) J. Biol. Chem. 268, 11500–11503