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RAPID COMMUNICATION

Differential Regulation of PAI-1 Gene Expression in Human Fibroblasts Predisposed to a Fibrotic Phenotype

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Synthesis of the major negative physiologic regulator of plasmin activation [plasminogen activator inhibitor type-1 (PAI-1)] is elevated during progressive cellular senescence, in premature aging disorders (e.g., Werner's syndrome), and in conditions associated with tissue fibrosis and excessive fibrin accumulation (e.g., sclerosis, keloid formation). Dermal fibroblasts derived from Werner's patients as well as from keloid lesions markedly overexpress PAI-1 mRNA transcripts compared to normal skin fibroblasts. Such cell type-related differences in steady-state PAI-1 mRNA content, and variances in the relative abundance of the 3.0- compared to the 2.2-kb PAI-1 mRNA species, served to discriminate normal from Werner's and keloid fibroblasts. This disparity in PAI-1 mRNA levels paralleled transcriptional activities of the PAI-1 gene; *de novo* synthesis of PAI-1 protein among the three cell types, moreover, closely approximated the respective differences in total PAI-1 mRNA content. Despite the markedly elevated levels of PAI-1 mRNA and protein evident in newly confluent keloid fibroblasts, these cells effectively suppressed PAI-1 synthesis (as did normal dermal fibroblasts) upon culture in serum-free medium. Werner's syndrome skin fibroblasts, in contrast, continued to maintain high-level PAI-1 expression even after 3 days of growth arrest. Adhesion-mediated attenuation of serum-stimulated PAI-1 expression, a characteristic of normal cells involving sequences which mapped to the distal 5′ flanking region of the PAI-1 gene, was retained in keloid but not Werner's fibroblasts. Collectively, these data suggest that (1) specific controls on PAI-1 gene expression are fundamentally different between these two clinically significant high PAI-1-synthesizing cell types and (2) the localized keloid may define the emergence of a distinct profibrotic dermal fibroblastoid phenotype in genetically predisposed individuals.

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INTRODUCTION

Extracellular matrix organization in cultured cells is a function of the complement of structural proteins synthesized and subject to remodeling by a complex protease cascade dependent upon the initial activation of plasmin by serine proteases [1, 2]. Perturbation of this cascade has specific consequences particularly within the setting of trauma repair [3, 4]. Gene-targeting studies and transgenic analysis of defined injury systems have implicated plasminogen, urokinase plasminogen activator (uPA), and PA inhibitor type-1 (PAI-1) as critical components in normal wound healing [4], fibrin accumulation [5], and tissue remodeling and fibrosis [3, 5]. PAI-1, moreover, modulates cell migration by complex interactions with vitronectin, uPA, and the uPA receptor [6–11]. Constitutive PAI-1 overexpression, therefore, as occurs during *in vitro* cellular senescence as well as in genetically linked premature aging and fibrotic syndromes [3, 5, 12–15], has specific ramifications within the context of wound healing [16, 17]. Indeed, the program of matrix remodeling/injury repair [18–20] is fundamentally altered during normal and premature cellular aging [12–14, 17, 21] as well as in localized and diffuse sclerotic disorders resulting in deficits in fibrin degradation [22–25]. Atypical fibroblasts derived from keloid lesions (KF) or patients with Werner's syndrome (WSF), for example, synthesize elevated levels of extracellular matrix proteins and proteoglycans and maintain this metabolic profile *in vitro* [16, 21, 26–29]. Moreover, while normal skin fibroblasts characteristically have high uPA to PAI-1 ratios [25], PAI-1 mRNA abundance is significantly increased in KF and WSF [14, 25, 30] as well as during progressive senescence of human diploid fibro-

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blasts [12] resulting in marked deficits in pericellular proteolytic activity [e.g., 25]. Although PAI-1 expression is modulated as a function of growth state, cellular age, substrate adhesion, and susceptibility to inflammatory cytokines [3, 5, 12–15, 25, 27], constraints on PAI-1 gene control may be differentially operative in cells genetically predisposed to a profibrotic phenotype. The present study, therefore, was designed to assess the extent to which differences relating to cell type influence PAI-1 gene regulation with regard to growth state-associated mechanisms of expression control.

MATERIALS AND METHODS

Cell culture. KF (Wound Healing Center, Medical College of Virginia, Richmond, VA) [16, 27] and normal dermal fibroblasts and WSF (Coriell Institute, Camden, NJ) were used prior to entry into a replicative end state (Table 1). Normal rat kidney (NRK) cells were cultured as described [12]. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; 1000 mg/liter D-glucose) supplemented with 10% (v/v) fetal bovine serum (FBS; DMEM/10). In certain experiments, cells were grown to approximately 70% confluency in DMEM/10, washed twice with Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (CMF-HBSS), and maintained in serum-free medium (DMEM/0) for 3 days. For suspension experiments, growth-arrested cells were washed twice with CMF-HBSS, trypsinized, and collected by centrifugation at 500*g* for 5 min. Cells were resuspended in DMEM/0 and plated into 100-mm dishes previously coated with 15 ml of 0.9% agarose in DMEM/0. After 15 h, the cells were either stimulated (for 4 h) in suspension culture by direct addition of serum to a final concentration of 20% (v/v) or harvested and allowed to adhere to tissue culture plastic during exposure to 20% FBS-containing DMEM for 4 h. Pretreatment with puromycin (20 μg/ml final concentration) was for 15 min prior to and during serum stimulation.

Metabolic labeling and gel electrophoresis. Media were aspirated, the monolayers washed twice with HBSS containing 0.5 mM $MgCl_2 \cdot 6H_2O$ and 1.3 mM $CaCl_2$, and cultures incubated in DMEM/0 for 3 days to initiate growth arrest [12]. Cells were labeled (in 9-cm^2 dishes for normal dermal cells and KF or in 2-cm^2 wells for WSF) in either the absence (quiescence) or presence (stimulated growth) of 20% dialyzed FBS in methionine-free RPMI 1640 containing [^{35}S]methionine (sp act >1100 Ci/mmol, 50 μCi/ml; New England Nuclear, Boston, MA) [12, 31]. After 3 h, the medium containing [^{35}S]methionine-labeled secreted proteins was aspirated and clarified by centrifugation at 13,000*g*. Cell monolayers were washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (CMF-PBS) and extracted with 0.2% (w/v) saponin in CMF-PBS to isolate cell–substratum contact regions (i.e., focal contacts) and associated undersurface material [32–34]. This saponin-resistant protein fraction was solubilized in electrophoresis sample buffer [50 mM Tris–HCl, pH 6.8, 10% [SDS], 1% 2-mercaptoethanol], boiled, and separated by SDS/10% acrylamide gel electrophoresis [35, 36]. Secreted PAI-1 was quantified by combined immunoprecipitation/electrophoresis analysis [33, 34, 37, 38].

Northern blotting and transcriptional analysis. Cellular RNA was isolated using RNAzol B, 3 μg per lane separated on 1.2% agarose/formaldehyde gels, transferred to Nytran membranes using 10× SSPE [1.8 M NaCl, 100 mM $NaPO_4$, pH 7.4, 10 mM ethylenediaminetetraacetic acid (EDTA)], and uv crosslinked. Blots were prehybridized at 42°C for 2 h [12] prior to hybridization at 42°C for 16 h with ^{32}P-labeled cDNA probes to human PAI-1 [3] or glyceraldehyde phosphate dehydrogenase (GAPD) as described [37, 38]. Nuclear run-off transcriptional assessments of PAI-1 gene activity were as described previously with exposure compensation for the relatively low nuclear yield from WSF [13, 38].

TABLE 1

Human Dermal Fibroblast Isolates Used in This Study

Strain[a]	Strain designation	Race[b]	Donor age (years)/sex	Reference
WS_1	AG06300	C	37/M	Coriell Institute
WS_2	AG12797	O	36/M	Coriell Institute
WS_3	AG00780	C	60/M	Coriell Institute
KF_1	Keloid 2	B	22/F	16, 27–29
KF_2	Keloid 8	B	22/F	16, 27–29
KF_3	Keloid 75	B	21/M	16, 27–29
NF_1	A1F	C	NB[c]/M	50
NF_2	SF	C	31/M	51
NF_3	HF	B	47/F	

[a] WAS, Werner's syndrome; KF, keloid-derived; NF, normal.
[b] C, caucasian; O, oriental; B, black.
[c] Newborn.

Plasmid constructs. The reporter constructs rPAI-CAT(-2395) and rPAI-CAT(-1237) consisted of various identified lengths of the rat PAI-1 5′ flanking genomic sequence (from the indicated nucleotide to the transcription start site) ligated to a chloramphenicol acetyl-transferase (CAT) gene (gift of Dr. T. D. Gelehrter, University of Michigan Medical School). rPAI-CAT(-1912) was generated by exonuclease III (*Exo*III)/mung bean nuclease (MBN) digestion of the full-length construct. rPAI-CAT(-2395) was restricted with *Sph*I [in 0.1 M potassium acetate, 25 mM Tris–acetate (pH 7.6), 10 mM magnesium acetate, 0.5 mM β-mercaptoethanol, 10 μg/ml BSA] at 37°C for 16–20 h. *Sal*I was added for an additional 3 h digest at 37°C. DNA was phenol:chloroform extracted, precipitated, and suspended (at 0.25 μg/μl) in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0); 500 ng of *Sph*I/*Sal*I-digested DNA was time cleaved with 50 U *Exo*III [in 33 mM Tris–acetate (pH 7.8), 66 mM potassium acetate, 10 mM magnesium acetate, and 0.5 mM dithiothreitol] at 30°C for 15 min and then at 65°C. MBN [15 U in 33 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM ZnOAc, and 0.001% Triton X-100] was added for 30 min incubation at 37°C, DNA extracted with phenol:chloroform, precipitated with 0.3 M NaOAC, resuspended in TE buffer, self-ligated, and transformed into competent JM109 *Escherichia coli.* Colonies were screened by restriction analysis with *Hin*dIII; candidate deletion constructs were sequenced.

Transfection. NRK cells were transfected with LipofectAMINE DNA complexes; after 5 h, 7 ml of DMEM/10 was added; cells were allowed to recover overnight and maintained for 3 days in DMEM containing 0.5% FBS [38]. Quiescent cells were trypsinized, resuspended in DMEM/0, and plated into 100-mm dishes precoated with 15 ml of 0.9% agarose in DMEM. After 15 h, cells were stimulated with FBS to a final concentration of 20% either in suspension or after transfer to tissue culture plastic; cells were harvested 2 h later and divided into two aliquots, one for assessment of CAT activity and the other for measurement of CAT plasmid copy number.

CAT activity assay. Cells were resuspended in 0.25 M Tris–HCl buffer (pH 7.8) and subjected to three alternating dry ice:37°C freeze/thaw cycles, and lysates were heated at 65°C for 10 min to inactivate endogenous acetyl-transferase activity and clarified by centrifugation. Supernatants were incubated with 0.54 μg acetyl-CoA and 3.8 nCi [^{14}C]chloramphenicol in 0.25 M Tris–HCl buffer (pH 7.8) at 37° for 2 h, extracted with ethyl acetate, concentrated, and chromatographed on silica plates for 1 h in 19 parts chloroform:1 part methanol. The plates were exposed to X-OMAT film and autoradiographs

scanned. CAT activity was defined as the integrated optical density of the acetylated forms of chloramphenicol normalized to CAT plasmid hybridization signal for each cell population (see below). Statistical evaluations utilized the Student *t* test.

Southern quantitation of CAT plasmid DNA. The remaining half of the cell pellet was lysed in 0.6% SDS, 10 mM EDTA (pH 8.0) at 25°C for 20 min, NaCl was added to a final concentration of 1 M, and lysates were refrigerated at 4°C for at least 8 h and centrifuged at 13,000*g* for 30 min. Supernatants were digested with 10 μg RNase A for 30 min at 37°C; 20 μg proteinase K was added for an additional 1 h incubation at 56°C. Following phenol:chloroform extraction, the recovered DNA was denatured with 0.3 M NaOH for 45 min at 65°C, dot-blotted onto Nytran membranes, uv crosslinked, and prehybridized in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA at 67°C for 2 h. Hybridization utilized 5×10^6 cpm of a random prime-labeled *Eco*RI CAT-specific coding fragment in 6× SSC, 0.5% SDS, 100 μg/ml salmon sperm DNA at 67°C for 16 h. Blots were washed twice in 2× SSC/1% SDS for 15 min and twice in 0.2× SSC/1% SDS for 15 min prior to autoradiography. Signal intensity of excised dots was quantified by scintillation counting.

RESULTS

PAI-1 mRNA and protein levels in near-confluent WSF and KF were significantly elevated compared to normal dermal fibroblasts cultured at an equivalent cell density, although there was considerable variation in PAI-1 transcript abundance and protein synthesis among the three independent isolates of WSF (Fig. 1). The relative abundance of PAI-1 mRNA expressed by WSF and KF (Fig. 1), moreover, closely paralleled PAI-1 transcriptional activity (11- and 4-fold that of normal dermal fibroblasts, respectively) as assessed by nuclear run-off analysis (Fig. 2). Despite this considerable cell type-associated difference in transcriptional rate and steady-state mRNA levels, the stability of both the 3.0- and 2.2-kb PAI-1 mRNA species among normal dermal, WSF, and KF was similar for each cell type (not shown). Similarly, differential mRNA stability was not a factor in age-associated PAI-1 overexpression in IMR-90 fibroblasts [13]. Calculations derived from such stability data suggest a mean half-life for both transcripts (in dermal fibroblasts) of approximately 6–12 h. These observations are consistent with previous assessments of dermal fibroblast PAI-1 mRNA half-life [39] and indicate that the decay rate for PAI-1 transcripts expressed by skin compared to lung fibroblasts is different [cf., 13, 39].

PAI-1 expression is growth state regulated in normal human lung fibroblasts and these kinetics are altered in progressively senescing cells [12, 13]. It was necessary to assess, therefore, whether WSF (which have a significantly curtailed *in vitro* life span [40]) and KF (which, like WSF, express elevated levels of PAI-1 under confluent culture conditions in contrast to their normal dermal counterparts; Fig. 1) exhibited dysregulation of the growth state-related pattern of PAI-1 gene

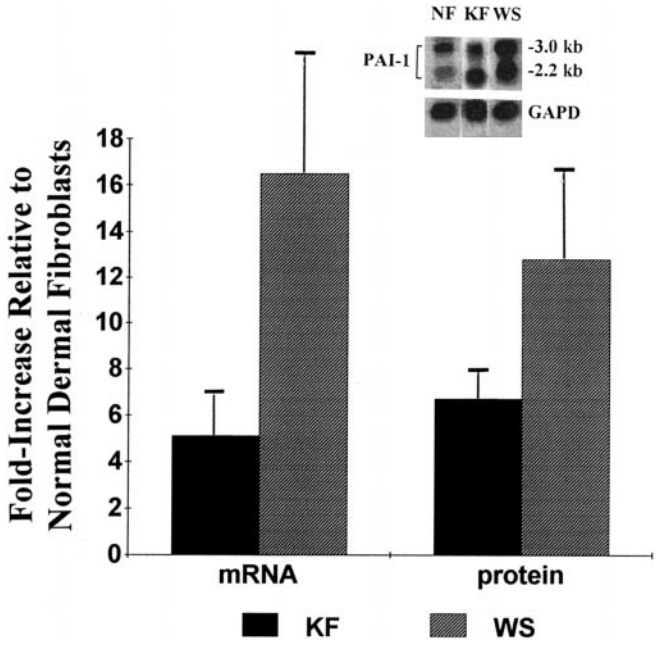

FIG. 1. Expression of PAI-1 mRNA and protein in cultured human fibroblasts upon attainment of confluence. Compared to normal dermal fibroblasts, keloid and WSF cells expressed approximately 5- and 16-fold more PAI-1 mRNA, respectively (total of both the 3.0- and 2.2-kb transcript species). Inset illustrates a typical Northern blot of RNA isolated from normal dermal (NF), keloid-derived (KF), and Werner's syndrome (WS) fibroblasts (isolate group #1; Table 1) hybridized with a ^{32}P-labeled cDNA probe to human PAI-1 and GAPD. For assessment of relative levels of extracellular accumulated [^{35}S]methionine-labeled PAI-1 protein, immunoprecipitated protein was separated on SDS–10% acrylamide gels, bands were visualized by fluorography, and PAI-1 levels were determined by scanning densitometry. Relative to normal dermal fibroblasts, keloid-derived and Werner's fibroblasts accumulated approximately 6- and 13-fold more PAI-1 protein during the period of metabolic labeling. Data represent means ± standard deviation of evaluations on three separate isolates of each cell type (Table 1).

expression [i.e., 12, 38]. Since PAI-1 gene expression in early-passage normal cells is repressed upon growth factor deprivation [12, 13] and serum-induced PAI-1 transcription in quiescent cells is modulated by substrate attachment [38], both parameters were evaluated. WSF continued to synthesize significant levels of PAI-1 protein when cultured for 3 days in serum-deprived medium (Fig. 3) and, in this respect, were indistinguishable from fully senescent IMR-90 cells [13]. KF, while maintaining a high PAI-1-expressing phenotype under conditions of near-confluent growth (Fig. 1), in contrast, effectively repressed PAI-1 protein synthesis upon serum-free culture as is typical of normal dermal fibroblasts (Fig. 3). Saponin fraction PAI-1 protein deposition, indicative of matrix-associated PAI-1 accumulation [12, 33–37], was significantly enhanced (5- to 12-fold) upon addition of serum (to a concentration of 20%) to quiescent cultures of both normal and

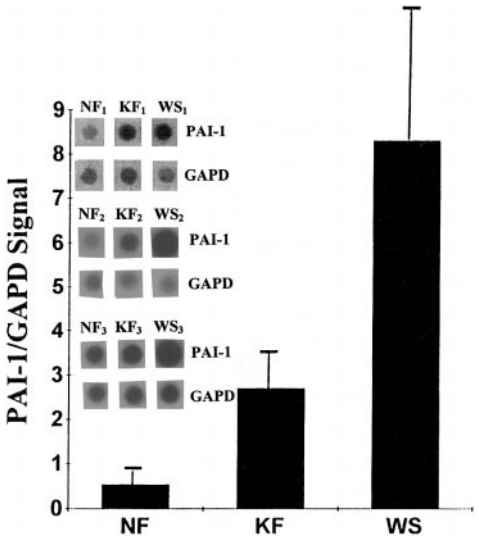

FIG. 2. Assessment of PAI-1 transcriptional activity. Nuclei were isolated from normal dermal (NF), keloid-derived (KF), and Werner's syndrome (WS) fibroblasts, and nascent RNA transcripts were elongated in the presence of [^{32}P]UTP, RNA extracted, and hybridized to nitrocellulose-immobilized human PAI-1 and GAPD cDNA probes (inset). Hybridization signal was quantified by computerized densitometry and plotted as the ratio of the PAI-1 to GAPD signal; data represent means ± standard deviation of run-off assays for three separate isolates of each cell type (Table 1).

keloid fibroblasts. This rather typical [for early passage fibroblasts; e.g., 12, 13, 21] response of the PAI-1 gene to serum deprivation and subsequent stimulation in KF contrasted with the elevated expression of PAI-1 mRNA and protein in near-confluent KF cultures (Fig. 1). A similar differential response to adhesion-mediated PAI-1 attenuation was evident among the high PAI-1-expressing phenotypes. PAI-1 induction was attenuated in normal dermal fibroblasts, early-passage NRK cells, and KF as a consequence of concomitant serum stimulation and substrate adhesion but not in WSF (Fig. 4). Since adhesion influences the amplitude as well as kinetics of serum-induced PAI-1 expression in NRK cells [38] and a similar process modulates PAI-1 induction in early-passage but not senescent human fibroblasts [13], it was important to determine the mechanism(s) involved in adhesion-mediated attenuation. Early-passage NRK cells were selected for this analysis since they are easily transfected (relative to the several human cell strains compared here) and readily express the various CAT reporter plasmids constructed (see Materials and Methods). Attachment of quiescent NRK cells to culture plastic in the presence of serum attenuated levels of induced PAI-1 transcripts by >50% compared to the amplitude of expression for cells stimulated with serum in suspension (Figs. 4 and 5). Induced PAI-1 expression under both suspension and adhesive culture conditions was resistant to puromycin (Fig. 5), consistent with the immediate-early mode of serum-stimulated PAI-1 synthesis defined previously [38]. The 2-fold differential in PAI-1 mRNA abundance (and levels of induction relative to non-puromycin-treated populations) between suspension and adhesive cultures (at the 2-h postserum addition time point) was retained in such inhibitor-treated cells suggesting that the basis for this response was likely transcriptional (Fig. 5). Puromycin effectively reduced PAI-1 expression at 4 and 8 h postserum addition in adhering cells, moreover indicating that an adhesion-dependent requirement for maintenance of serum-induced PAI-1 transcripts is operative and regulated by a 2° (i.e., protein synthesis-dependent) mechanism (compare Adhesion in Fig. 5). To assess whether this muted, anchorage-associated induction by serum involved adhesive response elements in the PAI-1 gene, promoter–reporter assays (using constructs containing varying lengths of PAI-1 genomic 5′ flanking

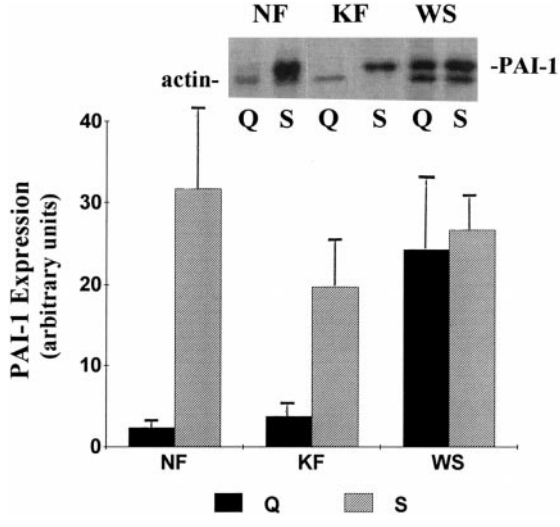

FIG. 3. Growth state-dependent accumulation of PAI-1 protein in the saponin-resistant fraction of human fibroblasts. Normal dermal (NF) and keloid-derived (KF) fibroblasts effectively suppress PAI-1 synthesis under conditions of prolonged serum deprivation (3 days) resulting in creation of a G_0 or quiescent (Q) growth state. Upon addition of serum to a final concentration of 20% (stimulated phenotype; S) there is a rapid accumulation of [^{35}S]methionine-labeled PAI-1 protein in the SAP fraction of both cell types (inset). Werner's syndrome (WS) cells, in contrast, failed to repress PAI-1 synthesis after 3 days in serum-free medium, accumulating approximately comparable levels of PAI-1 protein under both quiescent and stimulated culture conditions. Electrophoresis of 25,000 cpm of SAP fraction protein derived from NF, KF, and WSF cells after 3 days in serum-free medium (Q) or 6 h after serum stimulation (S) [inset; isolate group #1 (Table 1)]; actin and PAI-1 bands are indicated. The PAI-1 band for each cell type under both culture conditions was quantified by densitometry. Data plotted represent means ± standard deviation of densitometric assessments made on SAP fraction electrophoretic separations from two different isolates (#1 and #2; Table 1) from each group.

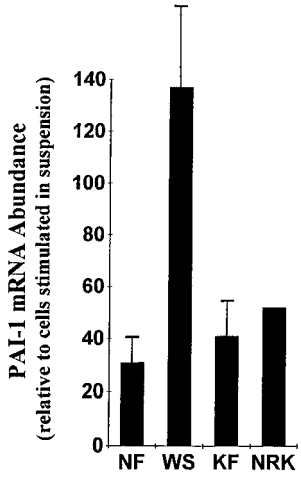

FIG. 4. Expression of PAI-1 transcripts in cells maintained in agarose suspension culture or upon substrate attachment. Normal dermal fibroblasts (NF), WSF, KF, and NRK cells were maintained in suspension culture (see Materials and Methods) and RNA was isolated from cells stimulated with serum for 4 h in suspension or cells stimulated with serum during adhesion onto plastic surfaces. Northern blots were hybridized with ^{32}P-labeled cDNA probes to PAI-1 and GAPD; PAI-1 mRNA abundance (normalized to GAPD signal) for cells stimulated with serum during reattachment to plastic surfaces was plotted as the percentage expression compared to cells serum stimulated in suspension. Data represent means ± standard deviation for analysis of two different isolates (#2 and #3; Table 1) for each cell type.

sequence; Fig. 6) were designed within the real time of stimulated expression. CAT activity driven by 2395 bp of PAI-1 5′ flanking DNA in response to serum addition under adhesive culture conditions was <50% that of cells stimulated in suspension culture (Fig. 6). Such adhesion-dependent attenuation of the serum-mediated CAT inductive response closely approximates the difference in steady-state PAI-1 mRNA levels between suspension and adherent NRK cell populations (Figs. 4 and 5). Normalized CAT activity derived from the rPAI-CAT(-1237) construct, in contrast, was not statistically different under the two culture conditions. To identify more specifically the topographic position of PAI-1 adhesion-dependent response elements, *Exo*III/MBN digestion was used to directionally delete portions of the PAI-1 distal genomic DNA. rPAI-CAT-(-2395) was restricted with *Sph*I and *Sal*I to specifically create an *Exo*III substrate 5′ of the PAI-1 sequence that is not present adjacent to the plasmid vector. Digestions with *Exo*III were timed specifically to remove PAI-1 sequences but not the plasmid backbone. Subsequent MBN treatment created blunt ends suitable for self-ligation of the digested plasmids. Transformants were screened by restriction analysis with *Hin*dIII (Fig. 7). Using this strategy, a clone was isolated lacking the 400-bp *Hin*dIII fragment retaining approximately 1.8 kb of PAI-1 proximal sequence as determined by electrophoresis of *Bam*HI/*Xho*I digests (Fig. 7). PAI-1-specific sequences within this construct were confirmed as beginning at nt -1912 relative to the PAI-1 transcriptional start site (Fig. 7). Transfection analysis indicated that this deletion abrogated adhesion-dependent transcriptional attenuation (Fig. 6). Similar to the rPAI-CAT(-1237) vector, there was no significant difference in reporter activity expressed by rPAI-CAT(-1912) transfectants following serum stimulation under adhesive compared to suspension culture conditions (Fig. 6) suggesting that adhesion-dependent response elements may localize within the distal 483 bp of the PAI-1 5′ gene (extending from nt -2395 to -1912).

DISCUSSION

Enhanced synthesis of both collagen and fibronectin is maintained upon culture of atypical KF and WSF *in vitro* [e.g., 40–42] and changes in matrix protein composition accompany senescence of cultured human fibroblasts [e.g., 12, 13]. Fibronectin transcription is significantly elevated (by approximately threefold) in KF and senescent fibroblasts [13, 29]; in KF, such reprogramming appears due to response differences (compared to normal dermal fibroblasts) to specific wound-associated growth factors (i.e., TGF-β) or glucocorticoids [16, 29, 44, 45]. While the enhanced synthesis of structural proteins contributes significantly to the net accumulation of matrix in keloids and in WS, the overall pericellular proteolytic balance in these two cell types clearly differs from that of normal dermal fibroblasts [e.g., 25]. The data presented here indicate that one basis for this proteolytic deficit may reside at the level of PAI-1 transcriptional differences between the two cell types which is reflected in increases in both steady-state PAI-1 mRNA abundance and PAI-1 protein biosynthesis. Analysis of the relative abundance of the 3.0- and 2.2-kb PAI-1 transcripts indicated that the 2.2-kb species was the predominant PAI-1 mRNA expressed by KF. WSF and fully senescent IMR-90 lung fibroblasts also express appreciable levels of the 2.2-kb mRNA (relative to normal dermal fibroblasts) [12, 46]. Despite this similarity, KF were not at a stage of replicative senescence and, moreover, failed to maintain PAI-1 expression and matrix accumulation under conditions of serum deprivation which is a hallmark of the senescent phenotype [13]. Whether the elaboration of a subset of senescent traits by KF suggests emergence of a distinct profibrotic phenotype in genetically predisposed individuals or indicates commitment to an end-stage growth program remains to be determined.

Serum-induced PAI-1 mRNA synthesis was puromycin resistant in cells cultured under either suspension

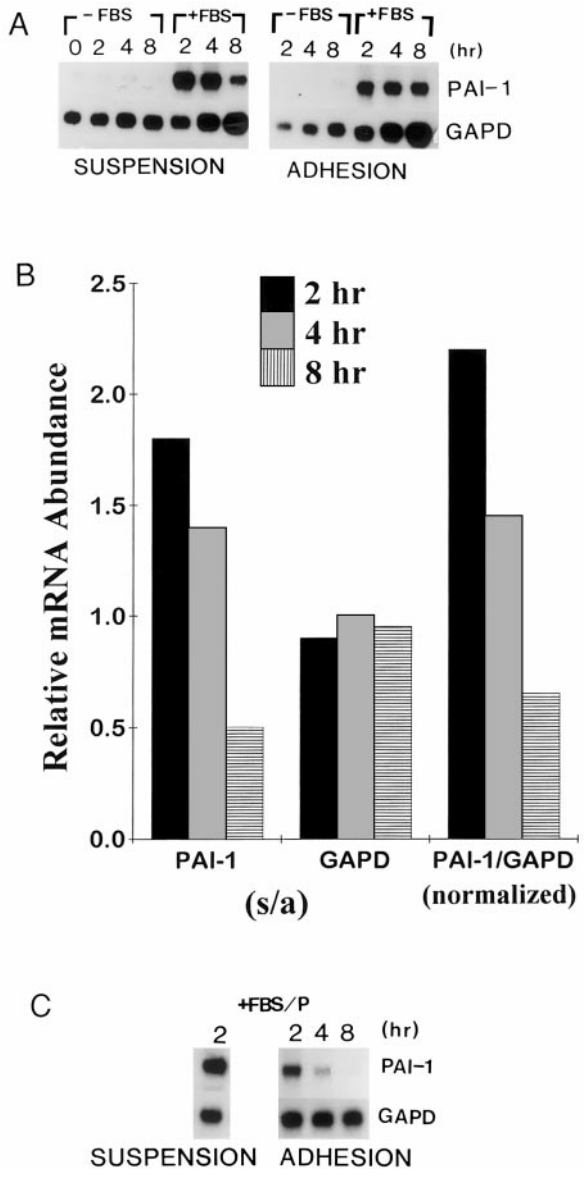

FIG. 5. (A) Northern analysis of serum-induced PAI-1 expression. NRK cells were growth arrested by serum deprivation for 3 days, trypsinized, and cultured over agarose in serum-free DMEM for an additional 15 h. Cells were maintained in serum-free medium (−FBS) or stimulated with serum to a final concentration of 20% (+FBS) either in suspension or upon subsequent adhesion to tissue culture plastic. At the indicated times (in hours), RNA was isolated and Northern blots were hybridized with ^{32}P-labeled cDNA probes to PAI-1 and GAPD. (B) Scanning densitometry served to quantitatively assess the relationship between serum-induced PAI-1 mRNA levels in cells stimulated in suspension (s) compared to cells exposed to serum during substrate attachment (a). The integrated optical density of the PAI-1 and GAPD mRNA signals for cells in suspension was divided by the corresponding value for attaching cells (s/a); relative PAI-1 mRNA abundance at each time point was calculated after normalization to GAPD signal density (normalized). (C) Puromycin (P) pretreatment did not alter either the levels of PAI-1 mRNA induced or the relative PAI-1 mRNA abundance differential evident between cells stimulated with serum (+FBS) in suspension or during adhesion to culture plastic. The adhesion-associated maintenance of PAI-1 transcript abundance at 4 and 8 h postserum addition, however, was dependent on ongoing protein synthesis (compare Adhesion in A and C).

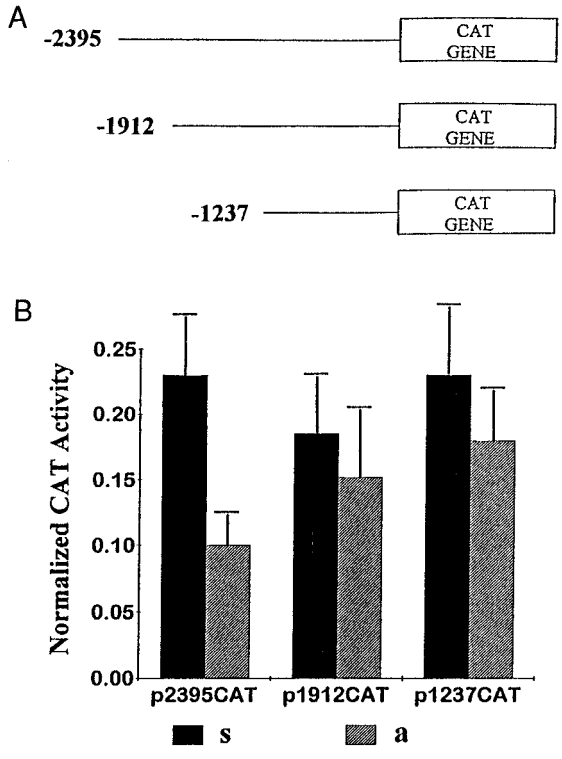

FIG. 6. (A) Schematic representation of PAI-1 "promoter"–CAT constructs. Insets representing various lengths of PAI-1 5′ genomic flanking sequences, from nt -2395, -1912, or -1237 and extending to +23 relative to the transcriptional start site, were positioned immediately upstream of a CAT reporter gene (see Materials and Methods). (B) Cells were transfected with each construct, growth arrested by serum deprivation for 3 days, cultured over agarose for 15 h, and then stimulated by addition of serum to 20% in suspension (s) or in conjunction with transfer to tissue culture plastic (a). CAT activity (transfection efficiencies normalized to CAT DNA abundance, as assessed by Southern analysis) represents the mean ± standard error of six to eight independent measurements. The difference in suspension vs adhesion CAT levels was statistically significant only for the p2395CAT transfectants.

or adherent conditions, consistent with the anchorage-independent immediate-early mode of transcriptional activation established previously [38]. Induction was also attenuated by concomitant substrate adhesion in all cell types except WSF. CAT activity expressed by cells stimulated under adhesive conditions was <50% that of cells stimulated in suspension, which approximates the reduced steady-state PAI-1 mRNA levels evident upon Northern analysis of newly attaching NRK cells. Sequences involved in such muted induc-

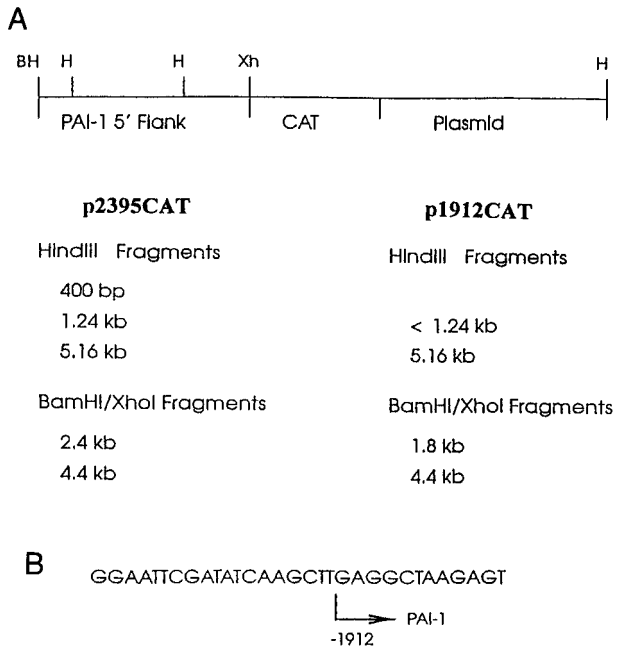

FIG. 7. (A) Diagram illustrating the relative position of the PAI-1 5′ flanking region, CAT reporter gene, and plasmid vector backbone in the 2395-bp PAI-1 construct. Digestion of p2395CAT with *Hin*dIII (H) generates three fragments of 400 bp, 1.24 kb, and 5.16 kb. Similar digestion of p1912CAT results in two fragments, one less than 1.24 kb and the other 5.16 kb. The 400-bp *Hin*dIII fragment of p2395CAT, which represents the distal-most sequence of the PAI-1 5′ flanking region (relative to the start site of transcription), is eliminated in the p1912CAT construct. *Bam*HI/*Xho*I double digestion, which separates PAI-1 sequences from the remaining plasmid, yields a 2.4-kb PAI-1-specific fragment of p2395CAT and a 1.8-kb PAI-1-specific fragment of p1912CAT. (B) Sequence analysis of p1912CAT indicates that PAI-1 sequences (above and to the right of the arrow) begin with nt -1912. Plasmid backbone sequences are located to the left of the arrow.

tion appeared to reside in a region of the PAI-1 gene between nt -2395 and -1912. The distal 483-bp putative attenuating sequence alone, when placed upstream of an exogenous (i.e., SV40) or a truncated PAI-1-specific (i.e., proximal 266 nt) promoter segment, however, functions as a repressor regardless of the culture conditions (i.e., suspension or adhesion) and defines the first functional attributes to PAI-1 flanking sequences located upstream of nt -1800 [46]. Additional transcriptional repressor elements occur within the rat PAI-1 gene between nt -764 to -628 and nt -266 to -188 [47, 48]. In exponentially growing cells, these elements interact with similar protein complexes as assessed by competitive mobility shift analysis [47], suggesting potential interactions between these sequence sets in regulating basal PAI-1 expression. The role of PAI-1 sequences from nt -764 to -628 and -266 to -188, if any, in mediating adhesion-dependent PAI-1 repression (either alone or by interaction with repressor elements located within the distal 483-bp region extending from nt -2395 to -1912) remains unknown. PAI-1 transcript levels, moreover, were not maintained (over an 8-h period) in suspended or adherent cells exposed to puromycin. Continued PAI-1 expression (or transcript stability), thus, is regulated in the post-immediate-early response phase in an adhesion-dependent puromycin-sensitive fashion. Since substrate attachment imparts 2° (i.e., protein synthesis-dependent) transcriptional level controls on the PAI-1 gene [38; Kutz and Higgins, in preparation], a complex model of PAI-1 gene regulation in response to serum can be constructed which incorporates both immediate-early (1°) and 2° inductive influences [38]. This model, and previous findings, indicate that regulated expression of the PAI-1 gene is closely linked to cell cycle progression [12, 13, 38, 43] and modulated as a function of substrate adhesion [38]. Cycle-associated events that dictate the kinetics as well as amplitude of induced PAI-1 synthesis are just emerging [e.g., 43] and transcriptional elements which function in the elevated expression characteristic of KF and senescent (including WSF) cells are as yet undefined. Clearly, both cell cycle transit and regulation of growth state-dependent genes are aberrant in WSF and senescent IMR-90 fibroblasts [12, 13]. While the latter may also underlie the creation of an activated or "synthetic" phenotype by KF [25, 41, 42, 46], as reflected in increased PAI-1 synthesis [25], it is apparent that controls on PAI-1 gene expression between KF and WSF are fundamentally different.

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