The cytokine transforming growth factor-β (TGFβ) has strong antiproliferative activity in most normal cells but contributes to tumor progression in the later stages of oncogenesis. It is not fully understood which TGFβ target genes are causally involved in mediating its cytostatic activity. We report here that suppression of the TGFβ target gene encoding plasminogen activator inhibitor-1 (PAI-1) by RNA interference leads to escape from the cytostatic activity of TGFβ both in human keratinocytes (HaCaTs) and primary mouse embryo fibroblasts. Consistent with this, PAI-1 knock-out mouse embryo fibroblasts are also resistant to TGFβ growth arrest. Conversely, we show that ectopic expression of PAI-1 in proliferating HaCaT cells induces a growth arrest. PAI-1 knockdown does not interfere with canonical TGFβ signaling as judged by SMAD phosphorylation and induction of bona fide TGFβ target genes. Instead, knockdown of PAI-1 results in sustained activation of protein kinase B. Significantly, we find that constitutive protein kinase B activity leads to evasion of the growth-inhibitory action of TGFβ. Our data are consistent with a model in which induction of PAI-1 by TGFβ is critical for the induction of proliferation arrest.

Transferring growth factor-β (TGFβ) controls many processes, including cell proliferation, differentiation, and stress responses as well as the production of extracellular matrix-associated proteins and secretory growth factors (1, 2). TGFβ is a ubiquitous and potent growth-inhibitory cytokine in various cell types as, for example, keratinocytes and other epithelial cells, and acts through binding to a transmembrane receptor, which in turn phosphorylates SMAD2 and SMAD3 proteins. These activated SMADs then translocate to the nucleus with binding partner SMAD4, and this complex regulates a wide range of target genes, among which are both pro- and antiproliferative genes (3, 4). The cytostatic effect is mediated by the induction of cyclin-dependent kinase inhibitors p21cip1 and p15ink4b, and is dependent on retinoblastoma (pRb) function (1, 5–7). This way growth factor-dependent cyclin-dependent kinase activity is inhibited, and progression through the cell cycle is prevented. In addition, TGFβ induces genes implicated in invasion and cell motility, which includes secreted extracellular matrix-associated proteins as plasminogen activator inhibitor-1 (PAI-1) and multiple matrix metalloproteases (3, 8, 9).

The pleiotropic actions of TGFβ have important consequences in cancer. Abortion of the cytostatic response may provide cancer cells with a selective advantage including invasive behavior (10). Noticeably, resistance to the growth inhibitory activity of TGFβ is often found in cancer, for example by mutations that inactivate the TGFβ receptors or SMAD signal transducers (3, 8, 11). In addition, selective mutation of genes required for cytostatic TGFβ responses may also occur in the presence of apparently normal TGFβ receptor and SMAD function. Identifying these factors is essential to better understand how during tumor progression cells evade the growth-limiting response to TGFβ.

One of the target genes of SMAD activity that is potently induced by TGFβ in keratinocytes, fibroblasts, epithelial, and endothelial cells is PAI-1 (3, 12). Similar to TGFβ, PAI-1 is involved in extracellular matrix homeostasis and angiogenesis and is associated with cancer progression (13, 14). Recently we found that PAI-1 is a critical target of tumor-suppressor p53 in the induction of cellular senescence in fibroblasts (15). We, therefore, asked whether PAI-1 might also be causally involved in the cytostatic activity of TGFβ. Our present results reveal an unexpected role for PAI-1 in mediating the anti-proliferative effects of TGFβ.

**Experimental Procedures**

**Antibodies and Vectors**—Antibodies against PAI-1 (C9), p21cip1 (F5, C19), PKB/Akt1 (C20), PTEN (A2B1), and SMAD2 (H-2, S-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-PKB (Ser-473; #9271) and anti-phospho-PAI-1 (#3101L) were from Cell Signaling (Beverly, MA). α-Actin was from Sigma (Clone AC-74). Anti-BrdUrd antibody (M0744) was from Dako. Mouse and human cDNAs for PAI-1, urokinase-type plasminogen activator (uPA), PI3K, and PKB/AKT have been described (15). Small interfering RNAs in HaCaTs or mouse fibroblasts (MEFs) were produced using the pRETRO-SUPER vector (16). The 19-mer sequences for PAI-1, SMAD4, and p53 have been described (15, 17). For generation...
of the human TGFβRII and PTEN knockdown constructs, the following 19-mer sequences were used: 5’-GATTCAAGAAG-TATCTAC-3’ and 5’-GTAAGATGCAATCATGT-3’, respectively. Controls were retroviral nonfunctional short hairpin or red fluorescent protein (RFP) vectors.

Cell Culture, Transfection, and Retroviral Infection—Human keratinocyte HaCaT cells, mouse embryo fibroblasts, and Phoenix cells were cultured, transfected, infected, or selected as described previously (15). Human TGFβ (100-B) was from R&D Systems (Minneapolis, MN).

Colony Formation Assays—HaCaT cells were infected with shRNA or cDNA constructs and selected, and 20,000 cells were seeded onto 6-cm plates and stained or counted after 7 or 10 days of treatment with 200 pm TGFβ. TGFβ unresponsive and normal HaCaT cells were infected with control vector (RFP) or mouse PAI-1 cDNA and after 72 h plated under low density (20,000 cells per 6-cm plate) and 1 week later stained or counted. 10,000 or 20,000 young wild-type and post-senescent PAI-1 and p53 knockdown and knock-out and uPA-overexpressing MEFs were seeded onto 6-well plates and stained after 1 week treatment with no, 10, or 100 pm TGFβ. TGFβ was refreshed every other day.

Growth Curves—HaCaT cells and MEFs were infected with retroviral shRNA constructs and selected, and 20,000 or 50,000 HaCaT cells or 150,000 MEFs were plated in a 6-cm dish (time = 0 days). Every 4 days cells were counted, and 20,000, 50,000, or 150,000 cells were replated. TGFβ was refreshed every other day. Total cell amounts were displayed as cumulative over time.

Quantitative Real Time PCR—From untreated or TGFβ-treated HaCaT cells or MEFs, RNA was isolated with TRI-Zol® (Invitrogen) according to manufacturer’s instructions. Quantitative real-time PCR (QRT-PCR) was performed on an ABI Prism 7700 with Assays-on-Demand™ (Applied Biosystems) for mouse PAI-1 and TBP as a control housekeeping gene or for human PAI-1, p21CIP1, and p15INK4b with β-actin as a control housekeeping gene.

Cell Cycle Analysis by Fluorescence-activated Cell Sorter—MEFs were cultured overnight in the absence or presence of 100 pm TGFβ and subsequently incubated with BrdUrd for 45 min. Cells were fixed, permeabilized, and labeled with anti-BrdUrd and propidium iodide.

Cell Culture Images—Phase contrast images were obtained using a Zeiss Axiovert 25 microscope with A-Plan 10× or LD A-plan 20× objectives on a Canon Powershot G3 14× zoom camera.

Western Blotting—Lysates were from scraped subconfluent plates and complemented with protease inhibitor (Complete, Roche Applied Science) and phosphatase inhibitor (P2850, Sigma) according to manufacturer’s instructions. Lysis and analysis was performed as described (15). In all experiments representative examples of at least three independent experiments are shown.

RESULTS

Cytostatic TGFβ Activity in Human Keratinocytes Requires PAI-1—Human HaCaT cells are arrested in the G1 phase of the cell cycle in response to TGFβ while exhibiting strongly induced PAI-1 levels (3, 5, 12). We asked whether the induction of PAI-1 might be causally involved in the TGFβ-induced proliferation arrest. TGFβ receptor type II (TGFβRII) and SMAD4 knockdown vectors were used as positive controls (3). We stably infected HaCaT cells with retroviral constructs expressing two independent PAI-1 shRNAs (to reduce the chance of scoring for an off-target effect), a nonfunctional shRNA (used as a negative control), or the TGFβRII or SMAD4 shRNAs. We also transduced HaCaT cells with a vector encoding a cDNA for uPA as this secreted protease and growth factor acts downstream of its antagonist PAI-1. Consequently, enhanced uPA expression may override the PAI-1 induction by TGFβ. Fig. 1a shows that colony formation of parental HaCaT cells exposed to 200 pm TGFβ was severely inhibited but that knockdown of PAI-1 by two independent shRNA vectors or expression of uPA resulted in escape from growth arrest which was at least as potent as that caused by knockdown of TGFβRII or SMAD4 (Fig. 1a, supplemental Fig. S1, a).

A similar result was seen when growth curves were made of HaCaT cells having these knockdown vectors (Fig. 1b). There was no significant growth difference between the various cell lines in the absence of TGFβ (data not shown). To assess the knockdown efficiency in TGFβ-resistant cells, we determined the mRNA levels of PAI-1, TGFβRII, and SMAD4 in cells expressing the respective knockdown constructs by QRT-PCR. After stimulation with 200 pm TGFβ for 1 day, PAI-1 mRNA levels were highly induced in normal HaCaT cells, which was greatly suppressed in PAI-1kd cells. This became more exacerbated when cells were grown in TGFβ medium for 10 days (Fig. 1c). There was reduction of TGFβRII and SMAD4 in TGFβRIIkd and SMAD4kd cells, respectively, and progressive reduction over time when grown in the presence of TGFβ (Fig. 1c).

Next we asked whether overexpression of a mouse cDNA for PAI-1, which is insensitive to knockdown by the human PAI-1 shRNAs (15), could mimic TGFβ activity and induce a cell cycle arrest. We analyzed in a colony formation assay whether the growth of TGFβ-resistant cells expressing the various knockdown constructs was influenced by ectopic expression of murine PAI-1, as compared with infection with a RFP control. In all cases the cells were growth-arrested (Fig. 1d), and the inhibition of proliferation by PAI-1 expression was approximately comparable between the various cell lines (supplemental Fig. S1, b). We conclude that loss of PAI-1 expression results in bypass of the cytostatic activity of TGFβ and that ectopic PAI-1 expression can be sufficient for induction of a growth arrest in human keratinocytes.

Cytostatic TGFβ Activity in Mouse Fibroblasts Requires PAI-1—MEFs grown under low density are also sensitive to the cytostatic effect of TGFβ, which is retinoblastoma (pRb)-dependent (6). Therefore, we analyzed the consequences of both PAI-1 knockdown and knock-out in MEFs on the antiproliferative effect of TGFβ. Young rapidly growing primary MEFs were used as a TGFβ-sensitive control, whereas MEFs ablated for p53 (both knockdown and knock-out) served as positive controls for TGFβ growth arrest bypass (18). The wild-type MEFs or MEFs knockdown or knock-out for PAI-1 or over-expressing uPA exhibited normal p53 function (data not shown). When analyzing their proliferation in a colony formation assay in the
PAI-1 Knockdown Does Not Interfere with Canonical TGFβ Signaling—We next investigated to what extent reduction of PAI-1 levels in HaCaTs and MEFs would influence canonical TGFβ signaling, which is dependent on receptor-mediated SMAD2 activation by phosphorylation and specific cyclin-dependent kinase inhibitor target gene activation (4). After stimulation with 200 pm TGFβ for 10 days, HaCaT cells having knockdown of PAI-1 or SMAD4 or overexpression of uPA showed unaltered SMAD2 phosphorylation when compared with the control (Fig. 3a), whereas TGFβRIIkd cells had reduced SMAD2 phosphorylation (Fig. 3a). We subsequently determined whether target gene activation was influenced in TGFβ-resistant PAI-1kd cells. We analyzed the expression of the target genes PAI-1, p21cip1, and p15ink4b in various TGFβ-resistant cell lines by QRT-PCR. We found that, compared with control cells, there was a reduction of the TGFβ target genes PAI-1,
p21CIP1, and p15INK4b in TGFβRIIkd and SMAD4kd cells (Fig. 3b). In PAI-1kd cells PAI-1 levels were strongly reduced, whereas the induction of cyclin-dependent kinase inhibitors p21CIP1 and p15INK4b was unaltered compared with control TGFβ-treated cells (Fig. 3b). uPA overexpressing cells showed comparable PAI-1, p21CIP1, and p15INK4b levels to the control (Fig. 3b), consistent with the notion that uPA is downstream of PAI-1 and does not interfere with TGFβ target gene activation. The expression of protein levels of the TGFβ targets PAI-1 and p21CIP1 in the various knockdown cells mirrored the mRNA expression by the cells (supplemental Fig. S3). We conclude that loss of PAI-1 in HaCaT cells results in a bypass of the growth inhibitory effect of TGFβ in the presence of a functional SMAD signaling pathway and normal TGFβ target gene activation.

We further analyzed SMAD2 activation in the various TGFβ-resistant MEF cell lines. Ablation of PAI-1 expression or uPA overexpression did not seem to interfere with SMAD2 phosphorylation in cells treated with TGFβ for 10 days when compared with TGFβ-arrested controls (Fig. 3c), again suggesting that PAI-1 knockdown leaves SMAD activation by TGFβ intact. Using QRT-PCR, we measured complete loss and strong reduction of PAI-1 expression in the PAI-1/H11002/H11002 and PAI-1kd MEFs, respectively (Fig. 3c). The high expression of PAI-1 in uPA-expressing MEFs is again consistent with the notion that uPA is downstream of PAI-1 in TGFβ signaling (Fig. 3d). We conclude that loss of PAI-1 in MEFs results in a bypass of the growth inhibitory effect of TGFβ in the presence of a functional SMAD signaling pathway and cytostatic target gene activation.

Constitutive PI3K-PKB Signaling Induces Bypass of a TGFβ-induced Arrest—Next we sought to determine the downstream mechanism involved in bypass of the TGFβ arrest by PAI-1 knockdown.
Recently we found that loss of PAI-1 causes persistent PI3K-PKB growth factor signaling in aging fibroblasts (15). Because PKB (AKT) signaling is involved in evasion of the cytostatic activity of TGF\(\beta\), we asked whether in this system loss of PAI-1 expression might lead to alteration of PKB activation. Indeed, when PAI-1kd HaCaT cells cultured in the presence of 200 pM TGF\(\beta\) for 10 days were assayed for protein levels of active (phospho)-PKB we found that, compared with control-infected cells that arrest with strong reduction in phospho-PKB, both PAI-1kd and uPA-overexpressing cells retain activation of PKB as determined by phosphorylation of residue 473 (Fig. 4a). To address whether activated PI3K-PKB growth factor signaling might also be sufficient to induce a TGF\(\beta\)-bypass, we stably infected HaCaT cells with retroviral constructs of constitutively active PI3K or PKB, a PTEN knockdown, or control RFP and tested in a colony formation assay for evasion of the cytostatic activity of TGF\(\beta\). PTEN is a potent inhibitor of PKB activation, and loss of this tumor suppressor has been found in many cancers (23). We found both constitutively active PI3K and PKB activity as well as PTENkd to be sufficient for bypass of TGF\(\beta\) arrest (Fig. 4b). Moreover, activation of PKB was retained when the cells were treated with TGF\(\beta\) for 10 consecutive days (Fig. 4c). We suggest that loss of PAI-1 expression results in maintenance of PI3K-PKB signaling and that constitutive activation of PI3K-PKB growth factor signaling is sufficient for evasion of the cytostatic TGF\(\beta\) response in HaCaT cells.

**DISCUSSION**

PAI-1 is induced by TGF\(\beta\) in a variety of cell types and is considered a classical target of TGF\(\beta\) signaling. So far, however, it has mainly been recognized as being involved in extracellular matrix remodeling and tumor progression (13, 14). We show here that PAI-1 is directly required for the cytostatic activity of TGF\(\beta\) in human keratinocytes and mouse fibroblasts. Loss of PAI-1 is as efficient as reduction of canonical TGF\(\beta\) signaling or SMAD4 levels in bypassing a TGF\(\beta\) arrest. Apparently, in addition to the effect PAI-1 has on cell motility and invasion, it is also critically involved in TGF\(\beta\)-dependent proliferation. Our data imply that when the PAI-1-uPA balance shifts toward excess uPA, a cell no longer responds to the growth inhibitory effects of TGF\(\beta\). We propose that the PAI-1-uPA balance in a cell determines whether the response to TGF\(\beta\) is antiproliferative or stimulates tumor progression. Interestingly, in breast cancer, the uPA:PAI-1 ratio is a powerful predictor of disease outcome (24). PAI-1 may, therefore, be instrumental for the effects of TGF\(\beta\) on cancer progression.

We also provide evidence that knockdown of PAI-1 or overexpression of its target uPA enhances PI3K-PKB growth factor signaling in the presence of TGF\(\beta\) and that this itself is sufficient for bypass of a TGF\(\beta\) arrest. Our data support in vivo studies showing that PKB activation makes breast cancer cells...
PAI-1 Is a Critical Target of TGFβ

In summary, we suggest that PAI-1 is a critical TGFβ-target required for induction of a cytostatic response in human keratinocytes and mouse embryo fibroblasts, possibly by attenuating PI3K-PKB growth factor signaling.

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