

Original Article

PAI-1 secretion of endometrial and endometriotic cells is Smad2/3- and ERK1/2-dependent and influences cell adhesion

Cong Sui^{1,2}, Ezekiel Mecha^{1,3}, Charles OA Omwandho^{3,4}, Anna Starzinski-Powitz⁵, Angelika Stammeler¹, Hans-Rudolf Tinneberg¹, Lutz Konrad¹

¹Center of Gynecology and Obstetrics, Faculty of Medicine, Justus Liebig University Giessen, Germany; ²Reproductive Medicine Center, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; ³Department of Biochemistry, University of Nairobi, Nairobi, Kenya; ⁴Kirinyaga University College, Kerugoya, Kenya; ⁵Molecular Cell Biology and Human Genetics, Johann-Wolfgang-Goethe-University of Frankfurt, Germany

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Abstract: In the endometrium transforming growth factor-betas (TGF- β s) are involved mainly in menstruation and endometriosis. After binding of the ligands to the high-affinity receptors, TGF- β receptors (TBR1 and TBR2), TGF- β s activate Smad signaling to modulate gene expression and cellular functions. However, recently also Smad-independent pathways have been studied in more details. To evaluate both pathways, we have analyzed TGF- β signaling in human endometrial and endometriotic cells. Although endometrial and endometriotic cells secrete TGF- β 1, secretion by stromal cells was higher compared to epithelial cells. In contrast, secretion of TGF- β 2 was higher in endometriotic stromal and endometriotic epithelial cells compared to normal endometrial cells. Treatment of endometrial and endometriotic stromal and epithelial cells with TGF- β 1 or TGF- β 2 increased Smad-dependent secretion of plasminogen activator inhibitor-1 (PAI-1) dramatically in all three cell lines. Of note, endometriotic cells secreted clearly higher levels of PAI-1 compared to endometrial cells. Whereas a TBR1 kinase inhibitor completely blocked the TGF- β 1 or TGF- β 2-induced PAI-1 secretion, an ERK1/2 inhibitor only partially reduced PAI-1 secretion. This inhibition was not dependent on epidermal growth factor receptor (EGFR) activation by phosphorylation but on kinase activity of the TBR1. Finally, treatment of endometrial and endometriotic cell lines with recombinant PAI-1 showed reduced cell adhesion, especially of the endometrial cells. In summary, our results demonstrate that both Smad-dependent and TBR1-dependent ERK1/2 pathways are necessary for TGF- β -dependent high level secretion of PAI-1, which might increase cellular deadhesion.

Keywords: PAI-1, ERK, SMAD, TGF-betas, endometriosis

Introduction

TGF- β s are one of the most essential growth factors involved in cell differentiation, proliferation, motility, cancer and apoptosis in various cell types [1]. The Smad-dependent pathway is the main pathway of TGF- β signaling and is activated by phosphorylation of TBR1 which in turn phosphorylates Smad2/3 [2]. Additionally also Smad-independent pathways modulate signal transduction of TGF- β s (e.g. [3, 4]).

In the human endometrium TGF- β s are differentially and stage-specifically expressed [5]. The highest expression of TGF- β s was detected during menses [6] and TGF- β 1 is also elevated in serum and peritoneal fluid of women with

endometriosis [7, 8]. Furthermore, TGF- β 1 is also differentially expressed in ovarian endometriosis and might play a role as an activator [9].

Endometriosis affects 10% of the female population in their reproductive ages [10] and is characterized by endometrial-like tissue growing outside the uterine cavity, mostly in the ovary and the peritoneum [11]. Besides animal models to study endometriosis (e.g. [12]), endometriotic cell lines with characteristics comparable to primary endometriotic cells have been established [13, 14]. Of note, these cell lines offer the possibility to study epithelial cells, which are otherwise rarely analyzed in the research of endometriosis *in vitro*.

In endometrial cells, TGF- β s showed different effects on cell proliferation. TGF- β 1 stimulated proliferation of low epithelial cell numbers, but inhibited it at high cell numbers in women with and without endometriosis [15]. However, all three TGF- β isoforms inhibited proliferation of endometrial stromal cells *in vitro* [16].

In a nude mouse model, preincubation of endometrial tissue with TGF- β 1 together with progesterone before xenografting suppressed endometriosis-like lesion formation [17]. Possibly, TGF- β 1 restored the ability of progesterone to suppress matrix metalloproteinases (MMPs) and thus prevented the establishment of endometriosis. However, TGF- β 1 knockout mice on a background of SCID showed reduced lesion development of xenotransplanted human endometriotic tissue [18].

Remarkably, TGF- β s, especially TBR1, are also involved in myometrial development [19, 20]. Furthermore, TGF- β s induced contraction of endometrial stromal cells *in vitro* [16], which might contribute to abnormal myometrial contractions found in women with endometriosis [21, 22] probably resulting in increased dissemination of endometrial fragments.

In this study we aimed to investigate the TGF- β signaling pathways in endometrial and endometriotic cells to identify possible targets which might be involved in the pathology of endometriosis.

Materials and methods

Cell lines

The stromal T-HESC cells ([23] ATCC CRL-4003) have been isolated from normal endometrium and demonstrate typical endometrial characteristics [24]. The stromal cells 22B and epithelial cells 12Z have been isolated from active peritoneal endometriotic lesions ([14] generously provided by Dr. Starzinski-Powitz, Frankfurt, Germany). The cell lines show characteristics of the active phase of endometriosis and thus are suitable for studying cellular and molecular behaviour of endometriosis [24].

Cell culture

3×10^5 cells were seeded into 6-well plates (TPP, Switzerland) in DMEM high glucose or DMEM/F12 media (+ 10% FCS). After culturing over-

night (37°C, 5% CO₂), cells were starved in fresh medium (+ 1% FCS) for 6 hours. After removal of the old medium, fresh medium containing 10 ng/ml recombinant human (rh)-TGF- β 1 or rh-TGF- β 2 (Promokine, Germany), respectively was added. In the untreated controls only 1x PBS was added to the medium. Cells were cultured (37°C, 5% CO₂) for up to three days.

To investigate the pathways that might be involved in TGF- β signaling with respect to PAI-1 secretion, several inhibitors targeting different pathways were used: the TGF- β receptor type I kinase inhibitor: 5 μ M LY364947 (Sigma-Aldrich, USA [25]) and 5 μ M of the ERK inhibitor II (Merck, Germany [26]) dissolved in DMSO. The optimal dose was determined in prerun experiments. Inhibitors for other pathways like p38 MAPK (SB203580), PI3K (LY 294002), pKA (H-89) or JNK (JNK inhibitor II) were less effective on PAI-1 secretion.

Fresh media (+ 1% FCS) in 6-well with or without inhibitor(s) was added. The untreated controls were done with DMSO as vehicle. After an incubation of 2 hours (37°C, 5% CO₂), cells were stimulated with 10 ng/ml TGF- β 1 or TGF- β 2 as described above.

Supernatants were collected and mixed with a Protease Inhibitor cocktail (Sigma-Aldrich, USA). After centrifugation (5000x g, 10 min, 4°C) the supernatants were aliquoted and stored at -20°C until use in the ELISAs. Then cell numbers were determined as described below.

Cell numbers

After removal of the medium, cells were washed two times with 1x Dulbecco's PBS with Ca²⁺ and Mg²⁺. Then accutase was added at 37°C until all cells were detached. After adding fresh medium, 10 μ l of the cell suspension was transferred to a CASY tube with 10 ml CASY ton solution and mixed thoroughly. Then the cell numbers and cell viability were measured with a CASY-counter (Schaerfe System, Germany).

Cell adhesion assay

1×10^6 cells/ml were seeded in 6-well plates and treated with active recombinant human-PAI-1 (final concentrations of 40 nM, 20 nM, 10

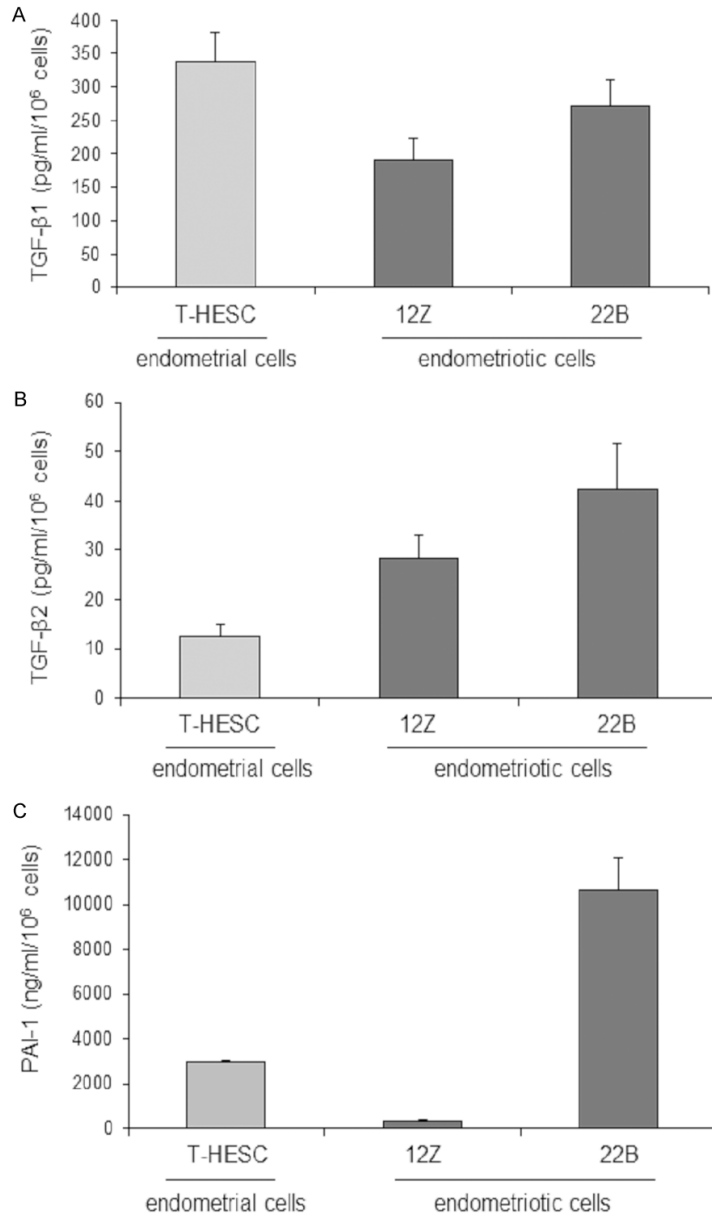


Figure 1. Quantification of TGF-β1, TGF-β2 and PAI-1 secretion by all cell lines. Stromal cell lines T-HESC and 22B secreted higher levels of TGF-β1 compared to epithelial cells 12Z (A). Endometriotic cell lines 22B and 12Z secreted more TGF-β2 compared to endometrial cells T-HESC (B). Endometriotic stromal cells 22B secreted 4-fold higher levels compared to endometrial stromal cells T-HESC. Endometriotic epithelial cells 12Z secreted modest amounts of PAI-1 (C). Each experiment was repeated three times in duplicates (A+B); each experiment was repeated nine times in duplicates (C).

nM or 5 nM). The buffer (50 mM NaH₂PO₄, 150 mM NaCl, 1 mM EDTA, pH 6.6) used to dissolve PAI-1 was used for the untreated control. After 4 hours (37°C, 5% CO₂) the cell numbers were quantitated with the CASY Counter.

ELISAs

Quantitation of protein secretion was performed with the following ELISAs: PAI-1 Antigen ELISA Kit (Technoclone), and TGF-β1 and TGF-β2 DuoSets (both from R&D Systems). Latent TGF-β isoforms were activated with HCl followed by neutralization with NaOH as indicated by the supplier. Each ELISA was performed according to the manufacturer's instructions and quantitated with the Benchmark Reader infinite M20-00 (Tecan). Cell numbers were used for standardization.

Statistical analysis

Each experiment was repeated independently at least three times in duplicate. Values from all experiments were used to calculate the means and the respective errors of the mean (SEM). Comparison between groups was done with the ANOVA followed by the post-hoc tests of Tukey and Dunnett by using GraphPad InStat 3 (GraphPad). *p* values less than 0.05 were considered statistically significant.

Results

To study endometrial and endometriotic cells *in vitro*, three cell lines from the human eutopic and ectopic endometrium were used. All cell lines secreted TGF-β1 (Figure 1A) as well as TGF-β2 (Figure 1B). The stromal cell lines (T-HESC and 22B) secreted higher levels compared to the epithelial cell line 12Z (Figure 1A). Interestingly, the endometriotic cell lines 22B and 12Z secreted more TGF-β2 than the normal endometrial cells (Figure 1B). All cell lines only secreted very low levels of TGF-β3 (data not shown). In the next step, we investigated secretion of the TGF-β-regulated protein PAI-1. In contrast to the epithelial cell line 12Z the stro-

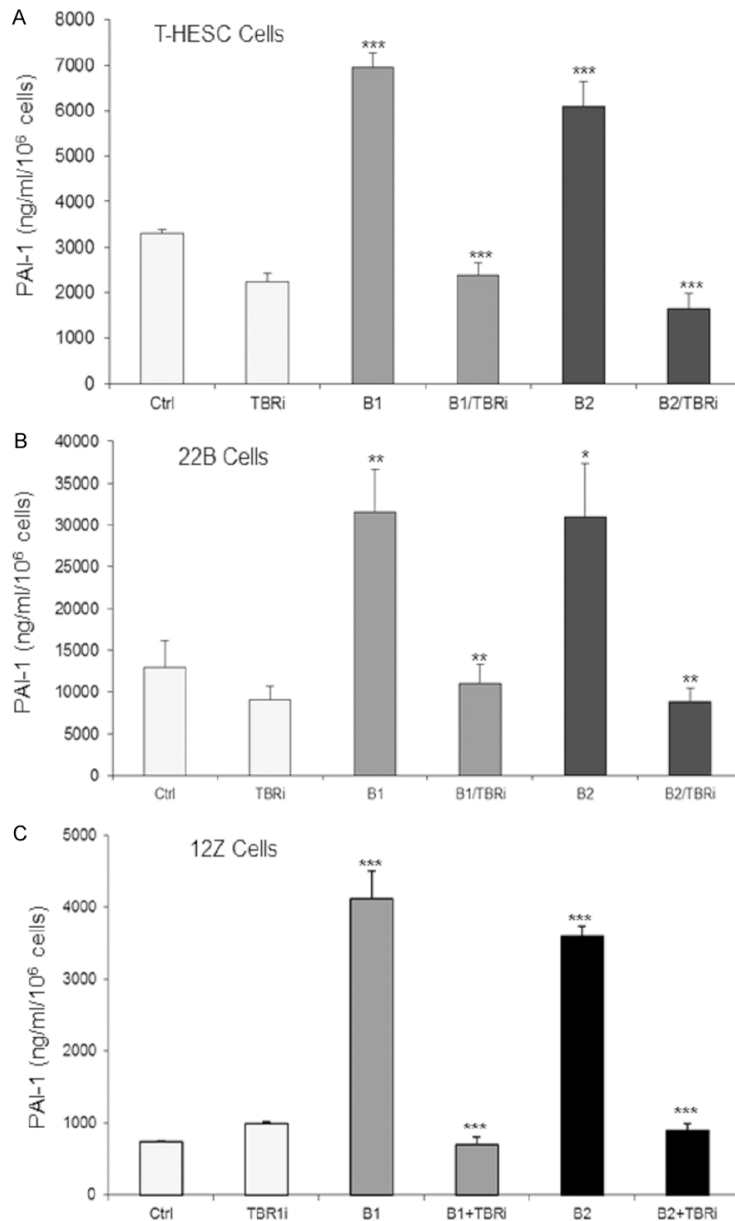


Figure 2. Treatment of cells with TGF- β 1 or TGF- β 2 (10 ng/ml), respectively, induced PAI-1 secretion in endometrial stromal T-HESC cells (A), endometriotic stromal 22B cells (B) and endometriotic epithelial 12Z cells (C). The TBR1 inhibitor (LY364947) blocked TGF- β -induced PAI-1 secretion of all cell lines studied completely to control levels (*** P <0.001; ** P <0.01; * P <0.05). Each experiment was repeated three times in duplicates. Ctrl, control; TBRi, TBR1 inhibitor; B1, TGF- β 1; B2, TGF- β 2.

mal cell lines produced high amounts of PAI-1 (Figure 1C). The endometriotic stromal cells 22B secreted approx. 4-fold higher levels compared to the endometrial stromal T-HESC. In contrast the endometriotic epithelial 12Z cells secreted only modest amounts of PAI-1. Taken together, PAI-1 secretion of the stromal cells is

much higher compared to the epithelial cells, whereas endometriotic cells secrete higher amounts of TGF- β s than the normal endometrial cells.

In order to elucidate the signaling pathways involved in secretion of PAI-1, we analyzed Smad-dependent and Smad-independent pathways with pharmacological inhibitors. Our results showed that both TGF- β 1 and TGF- β 2 strongly increased secretion of PAI-1 in all cell lines studied (Figure 2). The inhibitor LY364947 was described to inhibit phosphorylation of Smad2 and Smad3 by blocking the kinase activity of TBR1 [27]. Addition of LY364947 to the cells together with TGF- β 1 or TGF- β 2 strongly decreased PAI-1 secretion to levels comparable to the untreated controls (Figure 2), demonstrating that mainly the Smad pathway is responsible for TGF- β -induced PAI-1 secretion in endometrial cells.

Analysis of several Smad-independent pathway inhibitors as indicated in the Materials and Methods section revealed only for the ERK pathway a significant contribution to secretion of PAI-1 (Figure 3). Our results showed that the TGF- β 1-induced or TGF- β 2-induced PAI-1 secretion was reduced by a highly selective ERK1/2 inhibitor by 35-50% in the cell lines studied. Furthermore, our results showed that stimulation with TGF- β 1 or TGF- β 2 strongly enhanced phosphorylation of ERK1/2 in all three cell types

studied. However, we found that the EGF receptor is not phosphorylated by the TGF- β s (data not shown).

Besides effects of PAI-1 on activation of plasmin, it was also reported that PAI-1 inhibited cell attachment to the extracellular matrix (ECM

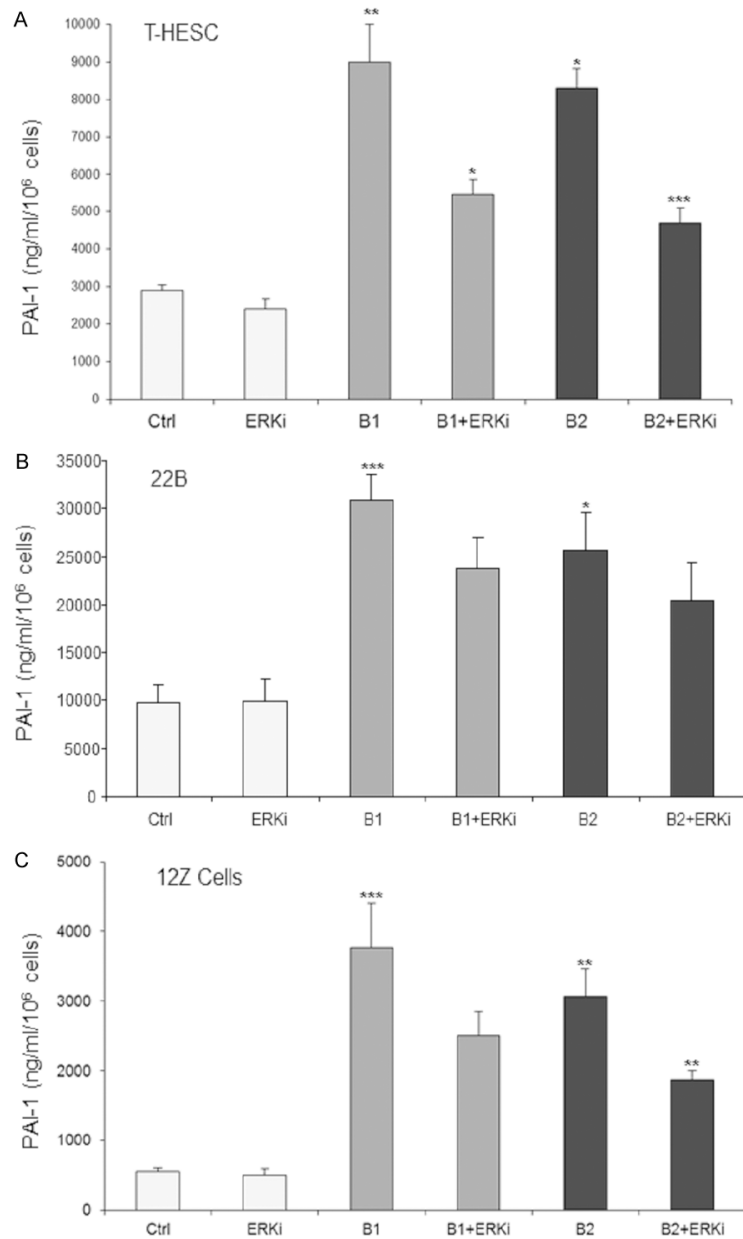


Figure 3. TGF- β 1 or TGF- β 2 induced PAI-1 secretion was moderately but significantly inhibited by the ERK inhibitor (ERKi) in endometrial stromal T-HESC cells (A), endometriotic stromal 22B cells (B), and endometriotic epithelial 12Z cells (C) by 50%, 30%, and 40%, respectively (** $P < 0.001$, ** $P < 0.01$; * $P < 0.05$). Each experiment was repeated three times in duplicates. Ctrl, control; B1, TGF- β 1; B2, TGF- β 2.

[28]). In order to test this hypothesis, cells were stimulated with or without recombinant PAI-1 for four hours and then the cell numbers were counted. PAI-1 decreased the number of adherent cells in a dose-dependent manner (Figure 4). Interestingly PAI-1 demonstrated stronger inhibitory effects on the endometrial cell line

T-HESC compared to the endometriotic cell lines 22B and 12Z. In addition to cell numbers, the CASY counter also measures cell vitality, which however was similar in all cell lines studies. Thus, we could exclude reduced cell adhesion by cell death.

Discussion

In our study, we quantified secretion of TGF- β 1 and TGF- β 2 by endometrial and endometriotic cell lines and demonstrated that TGF- β 1 secretion was higher in stromal cells in comparison to the epithelial cells. Of note, endometriotic cell lines secreted considerably higher levels of TGF- β 2 compared to normal endometrial cell lines. Our results concur with those of Pizzo *et al.* [7] who reported increased TGF- β 2 secretion by endometrial cells at advanced stages of endometriosis. Similarly, Chegini *et al.* [29] observed increased TGF- β 2 secretion in surgically induced endometriosis.

Besides the main function of PAI-1 to inhibit the tissue/urokinase plasminogen activator (tPA/uPA) induced fibrinolysis, PAI-1 is important also in adhesion, migration, signal transduction and anti-apoptosis [30, 31]. In endometrial and endometriotic tissues, Bruse *et al.* [32, 33] showed that expression of PAI-1 and uPA is much higher in endometriotic and endometrial tissue of women with endometriosis, compared to women without endometriosis. Also PAI-1 levels were higher in ectopic tissues than in eutopic tissues of the same woman [33].

Although there are many genes targeted by TGF- β s, PAI-1 is one of the most highly upregulated [34]. Accordingly we found a Smad-

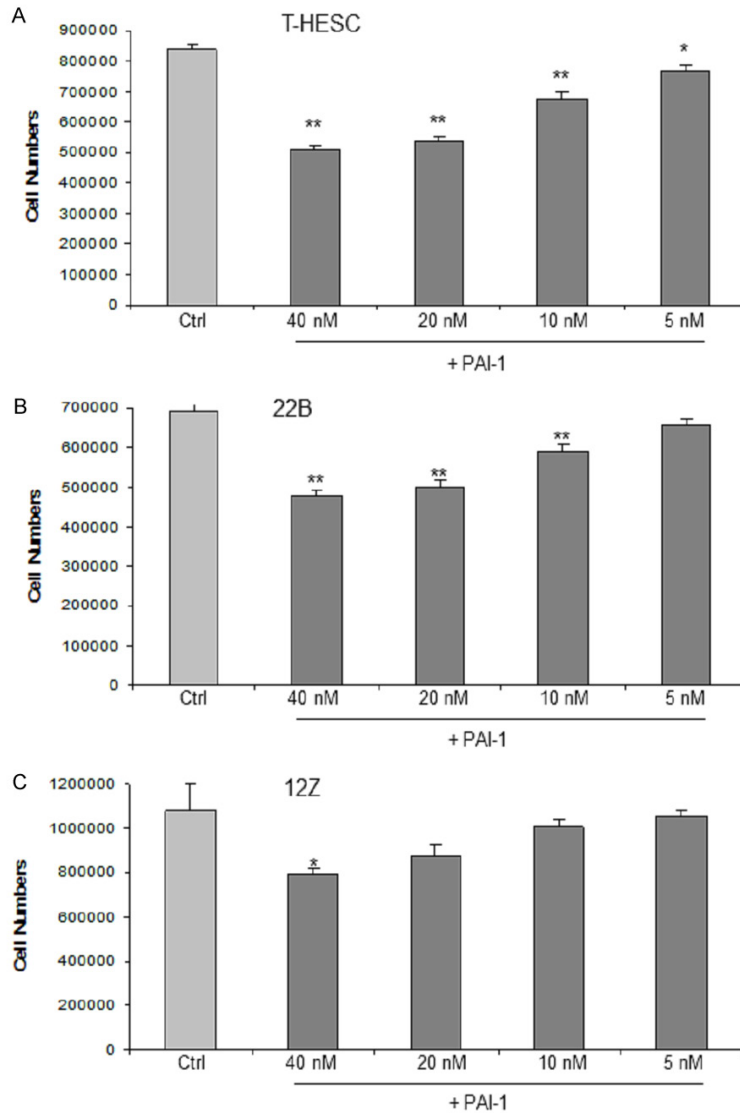


Figure 4. PAI-1 dose dependently inhibited cell adhesion of endometrial stromal T-HESC cells (A), endometriotic stromal 22B cells (B), and endometriotic epithelial 12Z cells (C). The numbers of attached cells decreased in a range of 2-40% after treatment for four hours with various concentrations of recombinant PAI-1 (** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$). Each experiment was repeated three times in triplicates.

dependent increased secretion of PAI-1 in endometrial and endometriotic cells upon stimulation with TGF- β 1 or TGF- β 2 *in vitro*. Our results showed clearly that endometriotic cells secreted more PAI-1 than the endometrial cells, and stromal cells (endometrial and endometriotic) secreted considerably more PAI-1 compared to epithelial cells. Our results concur with those of Bruse *et al.* [33] who showed that PAI-1 is mainly produced by endometrial endothelial and stromal cells.

To investigate possible Smad-independent pathways, we used several inhibitors, but found only for ERK a moderate but significant downregulation in TGF- β 1- and TGF- β 2-induced secretion of PAI-1 in all cell lines. Thus, besides Smad signaling, the ERK pathway contributes to high level secretion of PAI-1 in endometrial and endometriotic cells. Interestingly, there are several connections between the TGF- β -induced Smad pathway and the ERK pathway [35], for example the small GTPase Ras and the ERKs are implicated in TGF- β signaling [3]. Similarly, TGF- β phosphorylated ERK Ras-dependently and through activation of p21 activated kinase-2 [36]. Furthermore activated TBR1 directly phosphorylated ShcA (Src homology 2 domain containing) inducing association with GRB2 (EGF receptor-bound protein-2) and SOS (son of sevenless), which then leads to a sequential activation of Ras, Raf, MEK1/2 and ERK1/2 [37]. These data suggest that ERKs can be activated directly by the TBR1 without involvement of the Smad pathway or EGF receptor phosphorylation, the latter is normally required for phosphorylation of ERKs [38]. In this study, we did not observe phosphorylation of EGF receptors after TGF- β stimulation of

endometrial and endometriotic cells (data not shown).

Czekay *et al.* [39] showed that PAI-1 lowered attachment of cells to the ECM through inhibition of uPAR-vitronectin interaction, suggesting that PAI-1 negatively affects the cell-to-ECM connection. We found that recombinant PAI-1 reduced cell attachment in all cell lines studied. This effect is not due to increased apoptosis, because PAI-1 is nearly exclusively described

as an anti-apoptotic protein [31]. Of note, in our study PAI-1 demonstrated stronger inhibitory effects on endometrial compared to endometriotic cells. Thus, we suggest that PAI-1 might play a role in endometriosis by facilitating detachment of endometrial cells.

In conclusion, we showed in this study that mainly stromal cells secrete TGF- β s and PAI-1, which in the case of PAI-1 is regulated Smad-dependently and Smad-independently by ERKs. However, we could also show that epithelial cells contribute to secretion of TGF- β s and PAI-1 albeit only moderately. The connection of the ERK pathway to TGF- β signaling as suggested in this study seems to depend only on the kinase activity of the TBR1. Moreover, it is striking that we found no apparent differences in cell signaling between endometrial and endometriotic cells. However, we hypothesize that increased TGF- β and PAI-1 levels as observed in women with endometriosis might possibly contribute to endometriosis via enhanced cell shedding of endometrial cells by increased PAI-1 levels, a hypothesis which warrants further investigations.

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Disclosure of conflict of interest

None.

Address correspondence to: Lutz Konrad, Center of Gynecology and Obstetrics, Faculty of Medicine, Justus Liebig University Giessen, Feulgenstr, 10-12, D-35392 Giessen, Germany. Tel: +49 641 985 45282; Fax: +49 641 985 45258; E-mail: lutz.konrad@gyn.med.uni-giessen.de

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