

# Transforming growth factor- $\beta$ 1 enhances the antifibrinolytic and prothrombotic state of growing endothelial cells in a cell cycle-specific manner

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## SPECIFIC AIMS

In this study, we investigated how TGF- $\beta$ 1 and cell cycle-specific events can regulate prothrombotic and antithrombotic protein expression and modulate the hemostatic microenvironment in endothelial cells. Our findings demonstrate that the expression of a subset of hemostatically relevant proteins is regulated during endothelial cell cycle and that TGF- $\beta$ 1 can differentially modulate cell cycle-controlled protein expression.

## PRINCIPAL FINDINGS

### 1. Prothrombotic and antithrombotic molecule expression during the cell cycle

To investigate cell cycle-regulated protein expression, we characterized human umbilical vein endothelial cells (HUVEC) in the G<sub>1</sub> and in the S/G<sub>2</sub> phase by DNA staining with propidium iodide. A secondary immunostaining with specific antibodies revealed that thrombomodulin (**Fig. 1**), both plasminogen activators (u-PA and t-PA), and plasminogen activator inhibitor 1 (PAI-1) were predominantly expressed in the S/G<sub>2</sub> phase compared to the G<sub>1</sub> phase. As assessed by flow cytometric analyses, the expression of thrombomodulin, t-PA and u-PA increased by 31%, 41%, and 41%, respectively, in replicating tetraploid cells, compared to G<sub>1</sub>-arrested diploid cells. The cell cycle-dependent expression of PAI-1 increased even by up to 72% in the S/G<sub>2</sub> phase compared to cells in the G<sub>1</sub>-phase, whereas the expression of von Willebrand factor (vWF) remained unaltered during progression of the cell cycle.

### 2. Prothrombotic and antithrombotic molecule expression on TGF- $\beta$ 1 treatment

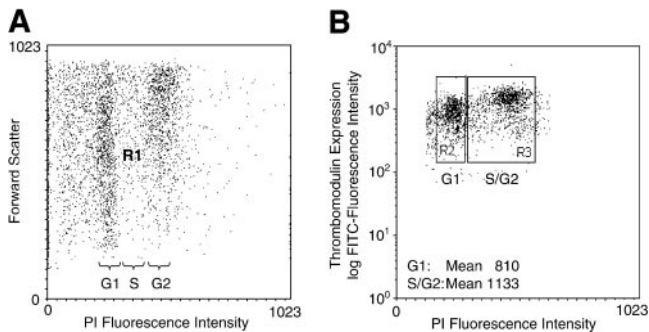
TGF- $\beta$ 1 arrests endothelial cells mainly in the G<sub>1</sub> phase. When cells have passed the G<sub>1</sub>/S transition, they are

not longer responsive to TGF- $\beta$ 1-dependent cell cycle control. Thus, after incubation with TGF- $\beta$ 1, the expression of hemostatically relevant molecules in the G<sub>1</sub> and S/G<sub>2</sub> phase is comparable. On treatment with TGF- $\beta$ 1, the cell cycle-dependent expression of thrombomodulin was elevated by ~34% in the S/G<sub>2</sub> phase as compared to the G<sub>1</sub> phase. Expression of t-PA and u-PA showed an increase of 66% and 49%, respectively, when replicating tetraploid cells were treated with 1 ng/ml TGF- $\beta$ 1 in comparison to the equally treated diploid cells. When the combined effects of TGF- $\beta$ 1 and cell cycle-dependent protein expression were studied, an increase of PAI-1 expression by 165% was observed. In contrast, no change in the expression concentration of vWF was observed under the same experimental conditions.

### 3. Specific induction of PAI-1 expression by TGF- $\beta$ 1 in the S/G<sub>2</sub> phase

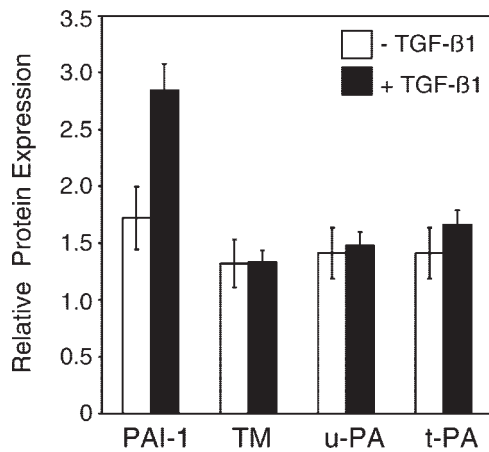
To investigate the potential physiological relevance of the S/G<sub>2</sub> phase and the role of TGF- $\beta$ 1 in modulating the expression of prothrombotic and antithrombotic molecules in growing HUVEC, we compared their expression during the cell cycle in the presence and absence of TGF- $\beta$ 1. The ratio of cells expressing prothrombotic and antithrombotic molecules in the S/G<sub>2</sub> phase of TGF- $\beta$ 1-treated and untreated HUVEC represents the hemostatic capacity during endothelial growth. **Figure 2** demonstrates that cells in the S/G<sub>2</sub> phase augment PAI-1 expression on treatment with 1 ng/ml of TGF- $\beta$ 1. The protein expression ratio (G<sub>2</sub>/G<sub>1</sub>) increased from 1.7 to 2.9. In contrast, this ratio was

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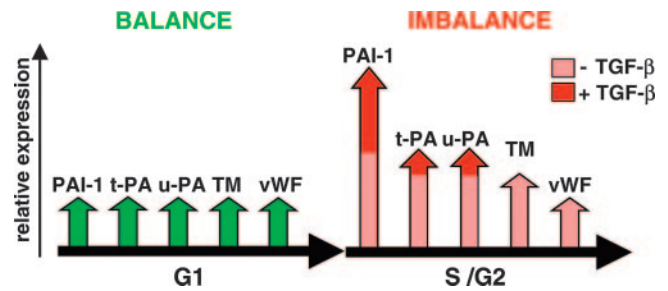


**Figure 1.** Differential expression of thrombomodulin during human umbilical vein endothelial cell (HUVEC) cycling. *A*) Principle of cell cycle analysis.  $1 \times 10^5$  propidium iodide (PI)-stained cells were live-gated using sideward and forward scatter characteristics. Cells were analyzed using forward scatter and red fluorescence to detect the cells in the G<sub>1</sub>, S, and G<sub>2</sub> phase according their DNA amount. These cells were gated in R1. *B*) The R1-gated cells were further gated in R2 and R3 related to their cell cycle phase to determine thrombomodulin expression detected by a specific antibody (Ab) and corrected by nonspecific Ab binding. R2 contains cells in the G<sub>1</sub> phase and R3 contains cells in the S and the G<sub>2</sub> phase of the cell cycle. The mean FITC-fluorescence in gate R2 (G<sub>1</sub> phase) was 810, and in gate R3 (S/G<sub>2</sub> phase) 1133 arbitrary units. A representative example with a 40% increase of thrombomodulin expression in the S/G<sub>2</sub> phase is shown.

not affected by TGF- $\beta$ 1 with regard to the expression of thrombomodulin, t-PA, and u-PA. This finding demonstrates that TGF- $\beta$ 1 specifically modulates cell cycle-controlled expression of PAI-1.



**Figure 2.** Effect of tumor growth factor (TGF)- $\beta$ 1 on G<sub>2</sub> phase-specific protein expression. Exponentially growing HUVEC were treated for 24 h with 1 ng/ml TGF- $\beta$ 1, and stained with propidium iodide and specific antibodies against thrombomodulin (TM), tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA), and plasminogen activator inhibitor-1 (PAI-1). TM, t-PA, u-PA and PAI-1 expression was measured in relation to the cell cycle phases G<sub>1</sub> and G<sub>2</sub>. The ordinate indicates the ratio of the corresponding protein expression in the G<sub>2</sub> and G<sub>1</sub> phase. The results are shown as means  $\pm$  SD of mean fluorescence intensity from 4 independent experiments.



**Figure 3.** Schematic diagram of the cell cycle- and TGF- $\beta$ 1-dependent modulation of hemostasis in growing endothelial cells. *Left*) Unperturbed blood vessels with resting endothelial cells are balanced due to the expression of both prothrombotic and antithrombotic proteins (green). *Right*) In contrast, during the S/G<sub>2</sub> phase the expression of certain proteins is enhanced (pink). Moreover, TGF- $\beta$ 1 potentiates the expression in a synergistic manner (red), resulting in an imbalanced hemostasis of endothelial cells. Prothrombotic molecules: plasminogen activator inhibitor-1 (PAI-1), von Willebrand Factor (vWF). Antithrombotic molecules: tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA), thrombomodulin (TM).

### CONCLUSIONS AND SIGNIFICANCE

It is well established that TGF- $\beta$ 1 has the ability to arrest cells in the G<sub>1</sub> phase and to regulate the expression of several proteins involved in endothelial homeostasis. However, whether and how both endothelial responses to TGF- $\beta$ 1 are linked to each other, is unknown. Our findings now constitute the first report demonstrating that growing endothelial cells modulate their hemostatic microenvironment in a cell cycle-dependent manner under the specific control of TGF- $\beta$ 1.

Resting endothelial cells are hemostatically balanced (Fig. 3). In contrast, growing endothelium in the S/G<sub>2</sub> phase increases the expression of antithrombotic molecules, including thrombomodulin, u-PA, and t-PA, and the prothrombotic molecule PAI-1. Interestingly, vWF is constantly expressed throughout the cell cycle. Thus, not all hemostatically relevant proteins are under cell cycle control, and the potential to influence the hemostatic properties of the endothelium in a cell cycle-dependent manner is restricted to a specific subset of proteins.

The expression of thrombomodulin, t-PA, and u-PA increase in the S/G<sub>2</sub> phase compared to the G<sub>1</sub> phase of TGF- $\beta$ 1-treated cells. On treatment with TGF- $\beta$ 1, expression of PAI-1 show an extensive net increase in the S/G<sub>2</sub> phase. Thus, growing endothelial cells produce an antifibrinolytic and prothrombotic microenvironment in a cell cycle-dependent manner and under specific control of TGF- $\beta$ 1 (Fig. 3).

In summary, the enhanced expression of PAI-1 that is triggered by TGF- $\beta$ 1 selectively in the S/G<sub>2</sub> phase might constitute a new aspect of hemostatic functions of endothelial cells, which could be relevant for different conditions, including angiogenesis and wound healing. EJ

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**ABSTRACT** Cell cycle-dependent modulation of protein expression may influence the balance of antithrombotic and prothrombotic properties of endothelial cells. In the present study, we examined the regulation of prothrombotic and antithrombotic molecules by transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) during distinct phases of the cell cycle in human umbilical vein endothelial cells. In the absence of TGF- $\beta$ 1, the expression of thrombomodulin, the plasminogen activators u-PA and t-PA, and their inhibitor PAI-1 was significantly increased in the S/G2 compared to the G<sub>1</sub> phase. Treatment of endothelial cells with TGF- $\beta$ 1, however, resulted in elevated expression of PAI-1 specifically in the S/G2 phase, while t-PA and u-PA increased to the same extent in both the G<sub>1</sub> and S/G2 phase. These findings demonstrate that the expression of a subset of hemostatically relevant proteins is regulated during endothelial cell cycle and that TGF- $\beta$ 1 can differentially modulate cell cycle-controlled protein expression.—Stoldt, V. R., Schnorr, O., Schulze-Osthoff, K., Scharf, R. E. Transforming growth factor- $\beta$ 1 enhances the antifibrinolytic and prothrombotic state of growing endothelial cells in a cell cycle-specific manner. *FASEB J.* 20, E55–E60 (2006)

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ENDOTHELIAL CELLS ARE key players in the regulation of the hemostatic balance by modulating the expression of prothrombotic and antithrombotic molecules (1). Resting diploid endothelial cells facilitate blood flow by providing an antithrombotic surface that inhibits platelet adhesion and coagulation (2). When endothelium is perturbed, the cells undergo programmed biochemical changes that can lead to the expression of a prothrombotic surface (1). Vascular lesions and exposed subendothelial matrix promote platelet adhesion, activation and, in turn, platelet aggregation. Platelet activation mediates the release of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) (3), which plays an important role in tissue repair (4). In this context, TGF- $\beta$ 1 is able to block endothelial cells from entering S phase (5). In a variety of different cell types, TGF- $\beta$ 1 inhibits cell proliferation by its ability to down-regulate the

proto-oncogene *c-myc* and to induce the transcription of cyclin-dependent kinase inhibitors such as p15<sup>INK4b</sup>, p27<sup>Kip1</sup>, and/or p21<sup>Cip1</sup> (6).

TGF- $\beta$ 1-postreceptor signaling leads to Smad-dependent gene expression (7,8). Transcription factors of the Smad family additionally interact with diverse DNA-binding cofactors that direct the resulting complex to specific target genes (9). The prothrombotic molecule plasminogen activator inhibitor 1 (PAI-1) is a well-characterized example for TGF- $\beta$ 1-regulated gene expression. In human mesothelial cells, *PAI-1* is transcriptionally induced by TGF- $\beta$ 1 (10). In this case, TGF- $\beta$ 1 induces a Smad-containing nuclear complex to bind to a specific promoter sequence in the *PAI-1* gene (10,11).

TGF- $\beta$ 1-modulated endothelium repair is a complex network of regulatory mechanisms that culminate in the hemostatic balance. This balance could be established by either a Smad-related transcriptional response and/or by cell cycle-driven regulatory mechanisms. We hypothesized that TGF- $\beta$ 1 modulates expression of hemostatically relevant proteins differentially throughout the G<sub>1</sub> phase and the S/G2 phase, thus providing a microenvironment that supports wound healing. Therefore, we examined the regulation of the prothrombotic molecules PAI-1 and von Willebrand factor (vWF), as well as the antithrombotic molecules thrombomodulin and the plasminogen activators t-PA and u-PA during the different cell cycle phases. We now demonstrate that TGF- $\beta$ 1 modifies the prothrombotic and antithrombotic state of human umbilical vein endothelial cells in a cell cycle-specific manner.

## MATERIALS AND METHODS

### Reagents

Platelet-derived transforming growth factor- $\beta$ 1 was from R&D Systems (Wiesbaden, Germany). Propidium iodide from

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Sigma (Deisenhofen, Germany) was used for DNA labeling. The propidium iodide stock solution contained 1 mg/ml in 0.1% (wt/vol) sodium citrate and was used at a final concentration of 1  $\mu$ g/ml. The following polyclonal or monoclonal antibodies were used: sheep anti-human PAI-1, goat anti-human u-PA and anti-human t-PA were from Chemicon (Hofheim, Germany); mouse monoclonal antibody (mAb) against human thrombomodulin was from Biozol (Eching, Germany) and anti-human vWF mAb from DAKO (Hamburg, Germany). Antibody (Ab) binding was detected using FITC-conjugated F(ab')<sub>2</sub>-specific antibodies: rabbit antimouse-F(ab')<sub>2</sub> Ab from DAKO; mouse anti-goat-F(ab')<sub>2</sub> and anti-sheep-F(ab')<sub>2</sub> antibodies from Chemicon. RNA digest was performed with 10  $\mu$ g/ml RNase from Roche Diagnostics (Mannheim, Germany).

### Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Bioproducts (Heidelberg, Germany) and grown in endothelial growth medium EGM-2 (Bioproducts) in a 5% CO<sub>2</sub> atmosphere at 37°C. The medium was supplemented with recombinant human epithelial growth factor, basic fibroblast growth factor, vascular endothelial growth factor insulin-like growth factor-1, ascorbic acid, hydrocortisone, heparin, FBS, gentamicin and amphotericin at the recommended concentrations (12). The cultures were routinely used between passage 3 and 5. Trypsin (500  $\mu$ g/ml) and EDTA (200  $\mu$ g/ml) from Sigma were used for cell harvest. Exponentially growing cells were treated with concentrations of TGF- $\beta$ 1 ranging from 0.01 to 10 ng/ml for 24 h in order to analyze effects on cell growth arrest and induction of protein expression.

### Immunolabeling and FACS analysis

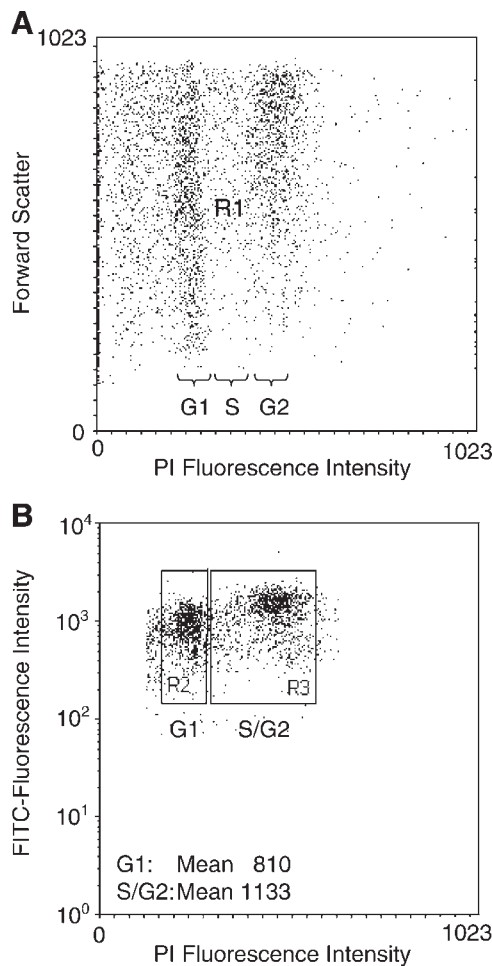
The trypsin/EDTA-treated cells were washed twice in PBS, centrifuged at 200 *g*, adjusted at a concentration of 10<sup>7</sup> cells/ml and fixed in 1 ml ice-cold PBS containing 9.25% (vol/vol) formaldehyde and 45% (vol/vol) acetone. The fixation procedure did not affect the epitopes and was immediately stopped by adding 9-ml ice-cold FACS buffer containing 0.1% BSA and 0.1% sodium azide in PBS. After centrifugation at 200 *g*, the pellet was washed with 10 ml ice-cold FACS buffer and resuspended with 1-ml buffer. The fixed endothelial cells were permeabilized by treatment with 0.1% saponin (Sigma) for 30 min on ice. To remove RNA, cells were treated with 10  $\mu$ g/ml RNase for 15 min after the beginning of treatment with saponin. Following the addition of 2 ml FACS buffer, the cells were centrifuged again and resuspended carefully. 10<sup>6</sup> cells/ml were incubated with the specific antibodies for 30 min on ice and were washed twice in 2 ml FACS buffer. For fluorescence labeling, the cells were incubated with FITC-conjugated F(ab')<sub>2</sub>-specific secondary antibodies for 30 min on ice. For DNA labeling, propidium iodide was added to a final concentration of 50  $\mu$ g/ml. After two washing steps, the cells were measured by two-color flow cytometry using a FACScan (Becton-Dickinson, Heidelberg, Germany) and Lysis II software.

## RESULTS

### Cell cycle-dependent protein expression of prothrombotic and antithrombotic molecules

To investigate cell cycle-regulated protein expression during the G1 and S/G2 phase, we stained chromo-

somal DNA of HUVEC to differentiate between 2n and 4n cells. Cells were then additionally labeled with specific antibodies against different hemostatic proteins. **Figure 1** shows the cell cycle-dependent expression of thrombomodulin in HUVEC. The cells were first gated according to their propidium iodide fluorescence and size in the forward scatter (Fig. 1A). Corresponding to their red fluorescence intensity reflecting various amounts of DNA, subpopulations at G1 and S/G2 were distinguished. Double staining with propidium iodide and antithrombomodulin Ab indicated that endothelial cells expressed considerably higher amounts of thrombomodulin in the S/G2 than in the



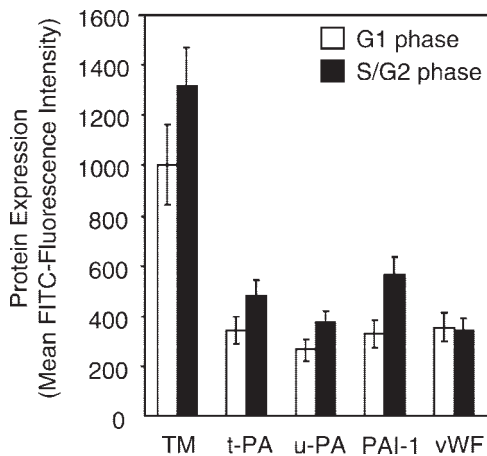
**Figure 1.** Differential expression of thrombomodulin during human umbilical vein endothelial cell (HUVEC) cycling. *A*) Principle of cell cycle analysis.  $1 \times 10^5$  propidium iodide (PI)-stained cells were live-gated using sideward and forward scatter characteristics. Cells were analyzed using forward scatter and red fluorescence to detect the cells in the G1, S, and G2 phase according their DNA amount. These cells were gated in R1. *B*) The R1-gated cells were further gated in R2 and R3 related to their cell cycle phase to determine thrombomodulin expression detected by a specific murine Ab and corrected by nonspecific Ab binding. R2 contains cells in the G<sub>1</sub> phase and R3 contains cells in the S and the G<sub>2</sub> phase of the cell cycle. The mean FITC-fluorescence in gate R2 (G<sub>1</sub> phase) was 810 and in gate R3 (S/G<sub>2</sub> phase) 1133 arbitrary units. A representative example with a 40% increase of thrombomodulin expression in the S/G<sub>2</sub> phase is shown.

G<sub>1</sub> phase. The mean FITC fluorescence intensity increased by 40% (Fig. 1B).

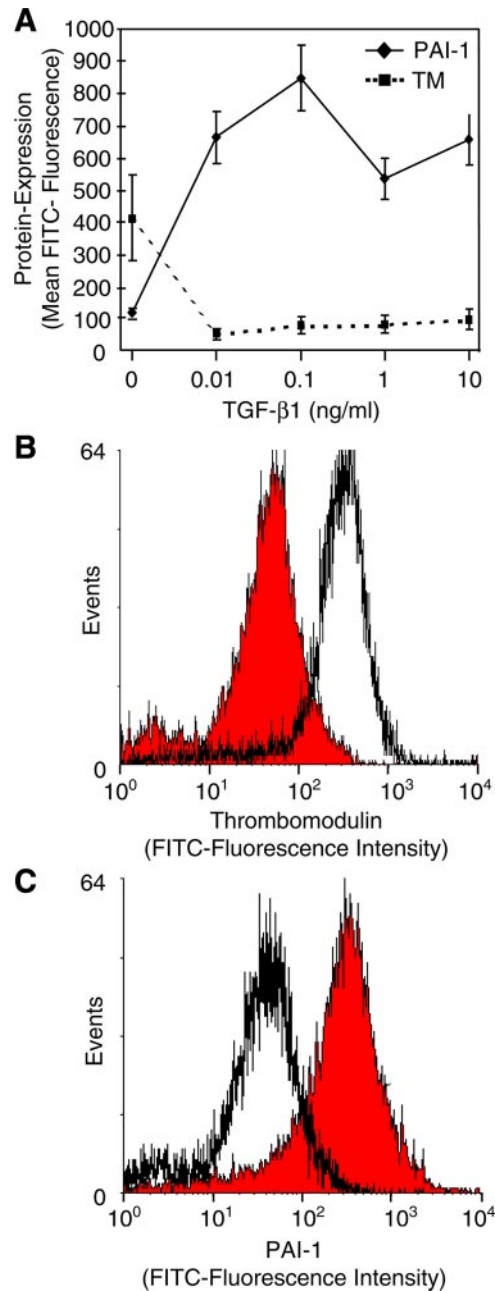
To investigate hemostatic properties of growing HUVEC, we measured the expression of several prothrombotic and antithrombotic molecules during the G<sub>1</sub> and S/G<sub>2</sub> phase. The expression of thrombomodulin, t-PA and u-PA increased between 30% and 40% in replicating tetraploid cells, as compared to G<sub>1</sub>-arrested diploid cells (Fig. 2). The permeabilization procedure did not affect the expression concentration of the proteins in our system. The cell cycle-dependent expression of PAI-1 increased by up to 72% in the S/G<sub>2</sub> phase compared to cells in the G<sub>1</sub> phase. Interestingly and in contrast to the other proteins, expression of vWF remained unchanged during the cell cycle.

### Effect of TGF-β1 on the expression of PAI-1 and thrombomodulin

TGF-β1 is known to control the expression of various genes in endothelial cells (13). To analyze its regulatory effect on the expression level of thrombomodulin and PAI-1, we tested different TGF-β1 concentrations on exponentially growing HUVEC (Fig. 3A). Even at low concentrations of 0.01 ng/ml, TGF-β1 treatment decreased the expression of thrombomodulin. This decrease was ninefold compared to TGF-β1-untreated cells (Fig. 3B). In contrast to its inhibitory effect on thrombomodulin expression, TGF-β1 triggered PAI-1 expression in exponentially growing endothelial cells (Fig. 3A). At a concentration of 0.1 ng/ml, TGF-β1 led to a eightfold increase in PAI-1 expression compared to



**Figure 2.** Cell cycle-dependent expression of prothrombotic and antithrombotic molecules. Exponentially growing endothelial cells were fixed and double-stained with propidium iodide and antibodies against thrombomodulin (TM), t-PA, u-PA, PAI-1, or vWF. The enhanced protein expression measured by increase of mean FITC fluorescence intensity in the G<sub>2</sub> phase was 1314 for thrombomodulin, 483 for t-PA, 374 for u-PA, 569 for PAI-1, and 348 arbitrary units for vWF in comparison to the G<sub>1</sub> phase with corresponding values of 1002, 343, 265, 329, and 355 arbitrary units, respectively. The open bars represent diploid cells in the G<sub>1</sub> phase, solid bars replicating tetraploid cells in the S/G<sub>2</sub> phase. Means ± SD of 4 independent experiments are shown.



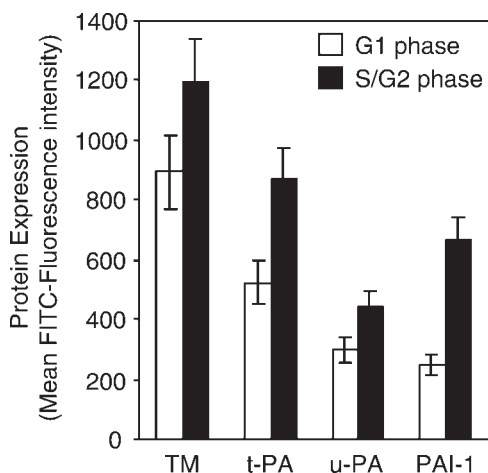
**Figure 3.** Effect of TGF-β1 on the expression concentration of thrombomodulin and PAI-1 in exponentially growing endothelial cells. A) Increasing concentrations of TGF-β1 (0.01 to 10 ng/ml) were tested during an incubation of 24 h for the effect on protein expression. The effect of TGF-β1 was measured in 3 independent experiments. The results are shown as means of FITC fluorescence intensity ± SD (B). TGF-β1 at a concentration of 0.01 ng/ml resulted in a maximal decrease of thrombomodulin expression as measured by FITC-labeled Ab and flow cytometry. The open histogram represents HUVEC kept in the absence of TGF-β1, the closed histogram in the presence of TGF-β1. The intracellular and cell surface expression of thrombomodulin measured by the FITC fluorescence decreased, resulting in a left shift of the histogram. C) TGF-β1 at 0.1 ng/ml led to a maximal increase of PAI-1 expression measured as indicated above. The open and closed histograms represent HUVEC in the absence and presence of TGF-β1, respectively. The intracellular and cell surface expression of PAI-1 increased, resulting in a right shift of the histogram.

untreated cells (Fig. 3C). At higher concentrations, TGF- $\beta$ 1 did not further increase PAI-1 expression.

### TGF- $\beta$ 1-dependent protein expression of prothrombotic and antithrombotic molecules during the cell cycle phases

TGF- $\beta$ 1 can arrest endothelial cells in the G<sub>1</sub> phase. When cells have passed the G<sub>1</sub>/S transition, they are no longer responsive to TGF- $\beta$ 1-dependent cell cycle control (5). We therefore used cells in the S/G<sub>2</sub> phase to compare the expression of hemostatically relevant molecules in the G<sub>1</sub> and S/G<sub>2</sub> phase following incubation with TGF- $\beta$ 1. On treatment with TGF- $\beta$ 1, the cell cycle-dependent expression of thrombomodulin was elevated by ~34% in the S/G<sub>2</sub> compared to the G<sub>1</sub> phase. Expression of t-PA and u-PA showed an increase of 66% and 49%, respectively, when replicating tetraploid cells were treated with 1 ng/ml TGF- $\beta$ 1 in comparison to the equally treated diploid cells (Fig. 4). When the combined effects of TGF- $\beta$ 1 and cell cycle-dependent protein expression were studied, an increase of PAI-1 expression by 165% was observed. In contrast and consistent with Fig. 2, there was no change in the expression concentration of the vWF under the same experimental conditions (data not shown).

To stress the potential physiological relevance of the S/G<sub>2</sub> phase and the role of TGF- $\beta$ 1 in modulating the expression of prothrombotic and antithrombotic molecules in growing HUVEC, we compared their expression during the cell cycle in the presence and absence



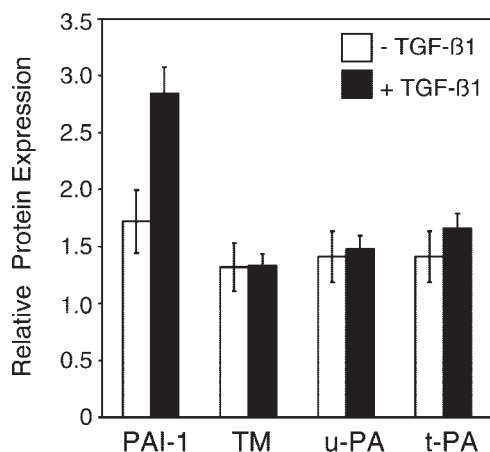
**Figure 4.** Effect of TGF- $\beta$ 1 on the expression of antithrombotic and prothrombotic molecules in exponentially growing endothelial cells. HUVEC treated for 24 h with 1 ng/ml TGF- $\beta$ 1 were fixed and stained with propidium iodide and specific antibodies against thrombomodulin, t-PA, u-PA, PAI-1, and vWF. The enhanced protein expression measured by mean FITC-fluorescence in the G<sub>2</sub> phase in comparison to the G<sub>1</sub> phase increased from 894 to 1198 for thrombomodulin, from 524 to 871 for t-PA, from 299 to 445 for u-PA, and from 250 to 663 arbitrary units for PAI-1. The open bars represent resting diploid cells (G<sub>1</sub> phase) and the solid bars replicating tetraploid cells (S/G<sub>2</sub> phase). Means  $\pm$  SD of 4 independent experiments are shown.

of TGF- $\beta$ 1. The ratio of cells expressing prothrombotic and antithrombotic molecules in the S/G<sub>2</sub> phase of TGF- $\beta$ 1-treated and untreated HUVEC represents the hemostatic capacity during endothelial growth. **Figure 5** demonstrates that cells in the S/G<sub>2</sub> phase rather selectively augment expression of PAI-1 on treatment with 1 ng/ml of TGF- $\beta$ 1. The protein expression ratio (G<sub>2</sub>/G<sub>1</sub>) increased from 1.7 to 2.9. In contrast, this ratio was almost not affected by TGF- $\beta$ 1 with regard to thrombomodulin, t-PA, and u-PA. Thus, these data demonstrate that TGF- $\beta$ 1 specifically modulates cell cycle-controlled expression of PAI-1.

### DISCUSSION

In the present study, we performed cell cycle analyses and studied the expression of hemostatically relevant proteins during the G<sub>1</sub> and S/G<sub>2</sub> phase of exponentially growing endothelial cells. The expression of antithrombotic molecules including u-PA, t-PA and thrombomodulin increased approximately up to 40% in the S/G<sub>2</sub> phase as compared to the G<sub>1</sub> phase. The expression of the prothrombotic molecule PAI-1 was even increased by ~70% in S/G<sub>2</sub> phase. However, not all hemostatically relevant proteins that were analyzed were apparently under cell cycle control. For instance, vWF was constitutively expressed throughout the cell cycle. Thus, the potential to influence the hemostatic properties of growing endothelium in a cell cycle-dependent manner appears to be restricted to a specific subset of proteins. The cell cycle-independent expression of vWF underlines the specific regulation of thrombomodulin, u-PA, t-PA and especially PAI-1. The observed expression profile in the S/G<sub>2</sub> phase of thrombomodulin, t-PA, u-PA, and PAI-1 may be the result of different regulatory mechanisms that include cell cycle-regulated induction of gene transcription, transcript stabilization, protein translation, or still other processes.

TGF- $\beta$ 1 is known to modulate the expression of several endothelial proteins. The observed repression of thrombomodulin by TGF- $\beta$ 1 has been previously documented by Ohji et al. (14). The TGF- $\beta$ 1-dependent increase of PAI-1 in exponentially growing HUVEC shown here for the first time is consistent with data obtained in microvascular endothelial cells (13) and mesothelial cells (15). Despite these reports, little is known to what extent TGF- $\beta$ 1 modulates protein expression during the cell cycle progression. The protein expression of thrombomodulin, t-PA, u-PA, and PAI-1 increased by 34%, 66%, 48%, and 165%, respectively, in the S/G<sub>2</sub> phase compared to the G<sub>1</sub> phase of TGF- $\beta$ 1-treated cells. No cell cycle-mediated repression on TGF- $\beta$ 1 treatment was observed. Thus, the decreased expression of thrombomodulin on treatment of exponentially growing cells with TGF- $\beta$ 1 appears to affect cells preferentially in the G<sub>1</sub> phase. There was no net induction of thrombomodulin expression by TGF- $\beta$ 1 in the S/G<sub>2</sub> phase, yet the cell cycle transition



**Figure 5.** Effect of TGF- $\beta$ 1 on G2/S phase-specific protein expression. Exponentially growing HUVEC were treated for 24 h with 1 ng/ml TGF- $\beta$ 1, and were then stained with propidium iodide and specific antibodies against thrombomodulin, t-PA, u-PA, PAI-1, or vWF. Thrombomodulin, t-PA, u-PA and PAI-1 expression was measured in relation to the cell cycle phases G1 and G2. The ordinate indicates the ratio of the corresponding protein expression in the G2 vs. G1 phase. The results are shown as means  $\pm$  SD of mean FITC fluorescence intensity from 4 independent experiments.

to the S/G2 phase alone enhanced the expression of thrombomodulin.

PAI-1 expression showed an extensive net increase in the S/G2 phase on treatment with TGF- $\beta$ 1. The effect of TGF- $\beta$ 1 on the relative protein expression of t-PA and u-PA was slightly increased. Thrombomodulin revealed no increase under the same conditions. Thus, among the pro- and antithrombotic proteins determined in this study, modulation of hemostatic properties of growing endothelial cells in a TGF- $\beta$ 1- and cell cycle-dependent manner is restricted to PAI-1.

The molecular mechanisms that govern the different TGF- $\beta$ 1 effects during cell cycle progression have not been identified. In particular, with regard to the expression of PAI-1 demonstrated here, it is still unclear whether its modulation occurs at a transcriptional or translational concentration. Interestingly, Boehm et al. (16) reported an increase of PAI-1 specific mRNA in a cell cycle-dependent manner on stimulation of quiescent normal (N) rat kidney epithelial cells with TGF- $\beta$ 1. This finding together with the observed actinomycin D sensitivity imply that transcriptional control is involved in TGF- $\beta$ 1-induced effects on the expression of PAI-1 (16). Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) is another important regulator of PAI-1 expression under physiological and pathological conditions. Recently, a 5' distal TNF- $\alpha$  responsive enhancer of the *PAI-1* gene has been identified (17). This enhancer, located 15 kb upstream of the transcription start site, contains a conserved NF- $\kappa$ B binding site that mediates the response to TNF- $\alpha$  (17). Apart from these findings, other recent observations suggest that internal ribosome entry site (IRES)-dependent translation and RNA/protein interaction-dependent polyadenylation contribute to the modulation of cell cycle-

related protein expression, preferentially in the G2/M transition (18).

Taken together, the TGF- $\beta$ 1- and cell cycle-dependent synergism on expression of PAI-1 may be the result of distinct underlying signal transduction pathways and transcription factors. The DNA regulatory sequences in the *PAI-1* promoter that are responsible for the TGF- $\beta$ 1-induced increase of *PAI-1* protein have been previously studied in detail, suggesting that AP1 or Smad4/Smad3 binding to the PAI-1 promoter might be responsible for TGF- $\beta$ 1-dependent regulation (10,11,19). As shown for epidermal keratinocytes (20), the *PAI-1* promoter is also under cell cycle control. However, it is virtually unknown how TGF- $\beta$ 1-induced regulatory mechanisms interact with cell cycle-dependent gene expression of *PAI-1*.

Inhibition or deficiency of PAI-1 is associated with hyperfibrinolysis or abnormal bleeding phenotypes, as demonstrated in patients or in *PAI-1*<sup>-/-</sup> mice (21–24), while animals transgenic for the *PAI-1* gene develop venous thrombosis (25). Thus, synthesis and activity of PAI-1 are crucial for the hemostatic balance. The enhanced expression of PAI-1 that is triggered by TGF- $\beta$ 1 selectively in the S/G2 phase might be a new aspect of hemostatic functions of endothelial cells during angiogenesis and wound healing. To demonstrate the physiological relevance of the up-regulation of PAI-1 in response to TGF- $\beta$ 1, further experimental studies are required. Reports from other laboratories also stress the significance of pathological effects of increased TGF- $\beta$ 1 concentrations in hemostasis and vascular dysfunction. Grainger et al. (26) showed that genetically determined elevations of TGF- $\beta$ 1 are related to a -509 C-T promoter polymorphism and result in vascular failure. Vascular and hemostatic dysfunctions related to abnormal concentrations of TGF- $\beta$ 1 were also demonstrated in different mouse models. For instance, constitutive overexpression of hepatic TGF- $\beta$ 1 in transgenic mice was associated with an increased incidence of thrombotic events (27).

TGF- $\beta$ 1 arrests cells at the G1/S transition (5) but can also modulate protein expression specifically in S/G2 phase. On the basis of our results, we therefore suggest that the TGF- $\beta$ 1-dependent increase of protein expression in the G2/S phase augments the prothrombotic and antifibrinolytic state of growing endothelial cells. Pharmacological concentrations of t-PA used for thrombolysis of platelet-rich arterial thrombi can fail in areas where endothelial cell growth takes place and where TGF- $\beta$ 1 is present. Therefore, strategies to inhibit or suppress the effects of PAI-1 in the S/G2 phase of the cell cycle could enhance thrombolysis. EJ

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