

Epigenetic control of tissue-type plasminogen activator synthesis in human endothelial cells

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Received 30 September 2010; revised 24 December 2010; accepted 26 January 2011; online publish-ahead-of-print 31 January 2011

Time for primary review: 28 days

Aims

Tissue-type plasminogen activator (t-PA) is produced by endothelial cells (EC) and is responsible for the removal of intravascular fibrin deposits. We investigated whether expression of t-PA by EC is under epigenetic control.

Methods and results

Methylation analysis of the proximal t-PA promoter revealed a stretch of unmethylated CpG dinucleotides from position –121 to +59, while upstream CpG dinucleotides were all methylated. In contrast, in human primary hepatocytes, which express t-PA at much lower levels than EC, the proximal promoter was partially methylated. Treatment of EC with the non-specific histone deacetylase (HDAC) inhibitors butyrate and trichostatin and with MS275, a specific inhibitor of class I HDAC, resulted in a time- and dose-dependent increase in t-PA expression. Garcinol and anacardic acid, inhibitors of the histone acetyl transferases CBP/p300 and PCAF, reduced basal and HDAC inhibitor-induced t-PA expression, whereas curcumin, an inhibitor of CBP/p300 only, had no effect. We performed chromosome immunoprecipitation analysis of the t-PA promoter using antibodies specific for acetylated histone H3 or H4 and observed an increase in H3 acetylation of 10 ± 3 and 44 ± 14 -fold in EC treated with trichostatin or MS275, respectively, and in H4 acetylation of 7.7 ± 1.4 and 16 ± 3 -fold, respectively.

Conclusion

The proximal t-PA promoter is unmethylated in human EC and partially methylated in human primary hepatocytes. Expression of t-PA by EC is repressed by HDACs in a mechanism that involves de-acetylation of histone H3 and H4.

Keywords

Tissue-type plasminogen activator • Endothelial cell • Histone acetylation • DNA methylation

1. Introduction

Activation of the fibrinolytic system is the principal mechanism by which intravascular fibrin deposits are removed. Tissue-type plasminogen activator (t-PA) converts plasminogen into the protease plasmin, which efficiently degrades fibrin into fibrin degradation products. The vascular endothelium is an important source of circulating t-PA. It releases t-PA in a constitutive manner and in a regulated manner. In human forearm perfusion studies, acute release of t-PA was demonstrated in response to 1-desamino-8-arginine-vasopressin¹ and in a rat hind limb perfusion system, acute t-PA secretion was induced by a variety of secretion agonists.² The storage of tissue-type plasminogen in the vascular endothelium and its regulated release after endothelial activation by a variety of secretagogues, such as thrombin, assures that fibrin deposited in front of an intact endothelium is rapidly removed. Thus, experimental induction of disseminated intravascular coagulation in chimpanzees or baboons by injection of a mixture of activated coagulation factor Xa and phospholipids was followed within minutes by a massive (>100-fold) increase

in plasma t-PA concentrations. The amount of t-PA released was sufficient to rapidly restore blood circulation even under conditions where all fibrinogen had been converted into fibrin.^{3,4} The rapidity of the t-PA release response suggested the presence of an endothelial storage pool for t-PA. Indeed, studies *in vitro* and *in vivo* have shown that t-PA is stored in endothelial cells (EC) in Weibel–Palade bodies and in distinct small granules, and is released by thrombin, histamine, phorbol ester, and the cAMP-inducing agent forskolin.^{5–8} Biosynthesis and storage of t-PA in EC is increased by a number of factors such as vascular endothelial growth factor, basic fibroblast growth factor, thrombin, retinoic acid and forskolin and phorbol ester, which activate the protein kinase A and the protein kinase C, respectively.^{8–12} In addition, trichostatin and butyrate, which are non-specific histone deacetylase (HDAC) inhibitors increase t-PA expression in EC,^{8,13} which implies a role for epigenetic control of t-PA gene expression.

The PA system not only plays an important role in the removal of incipient fibrin deposits, but also in a variety of important physiological and pathological conditions such as development, tissue remodelling,

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tumour invasion and metastasis, inflammation, neuronal plasticity, and blood–brain barrier function.

Epigenetic events, as characterized by cell-type specific modifications in the pattern of DNA methylation and of histone modifications at the gene promoter are of major importance for understanding gene regulation. In particular, epigenetics provides a mechanism by which cells can respond to their environment. Recent studies illustrate the importance of epigenetics as a link between the environment and the risk of cardiovascular disease.^{14–16} Epigenetic events are not only important for cardiovascular disease, but also for tumour angiogenesis and cancer treatment as revealed by the potent anti-angiogenic effects of HDAC inhibitors^{17,18} and for the understanding of inflammation.¹⁹

In view of the many activities of epigenetic drugs on the vascular system in the context of cardiovascular disease or cancer and in view of the role of t-PA in dissolution of fibrin deposits and in many other physiological and pathological situations where the PA system is involved, it is important to have a better understanding of the epigenetic aspects of t-PA expression by EC. The aim was to study epigenetic control of t-PA expression by EC. We focused our study on the methylation state of the t-PA promoter and on the role of HDAC and of histone acetyl transferases (HATs) in the regulation of t-PA expression. We observed that inhibition of class I HDAC led to an increase in t-PA expression by EC, which was accompanied by an increase in acetylation of histones H3 and H4 associated with the t-PA promoter. Inhibition of the HATs p300/CBP and PCAF reduced basal t-PA expression and abrogated the effect of HDAC inhibition. A study of CpG dinucleotide methylation at the t-PA promoter revealed an unmethylated stretch of DNA from –121 to +59 in EC, whereas in human hepatocytes, which express less t-PA, the same t-PA promoter region is partially methylated.

2. Methods

2.1 Reagents

Butyrate and trichostatin were from Sigma Aldrich (Schnelldorf, Germany). MS275, anacardic acid, curcumin, and garcinol were from Enzo Life Sciences AG (Lausen, Switzerland).

2.2 Cell culture

Umbilical cords were obtained from the local maternity ward with informed consent from the parents and with approval from the hospital Ethics Committee. The investigation conforms with the principles outlined in the Declaration of Helsinki. Human umbilical cord-derived endothelial cells (HUVEC) were isolated as described²⁰ and cultured in EGM-2 medium (Cambrex, Walkersville, MD, USA). The cells were expanded in EGM-2 medium, containing VEGF, IGF, FGF-B, EGF, ascorbic acid, gentamycin, and heparin, supplemented with 2% foetal bovine serum. Cells were passaged by trypsin-EDTA (Seromed Biochrom KG, Berlin, Germany) treatment and used at passage 1–3. Stimulation of the cells was done in the same medium, but without the addition of growth factors. Human primary hepatocytes were purchased and grown in HCM bulletkit medium, both from Lonza (Walkersville, MD, USA). HuH7 and HepG2 cells were grown in Dulbecco's modified Eagle's medium with 10% foetal bovine serum (Gibco Invitrogen, Carlsbad, CA, USA).

2.3 Quantitative reverse transcriptase real-time PCR

Total cellular RNA was isolated using Trizol reagent (Invitrogen) and reverse transcribed using the Improm-II reverse transcriptase system

from Promega (Madison, WI, USA). Quantitative reverse transcriptase real-time PCR (qPCR) was performed as described previously²¹ using the $\Delta\Delta\text{CT}$ method and GAPDH as the control housekeeping gene for comparison of the effect of agonists on t-PA expression in HUVEC. For comparison of t-PA mRNA levels between HUVEC and hepatocytes or hepatoma cells a panel of three control housekeeping genes was used: 18S rRNA, human $\beta 2$ -microglobulin and elongation factor 1 alpha subunit. The oligonucleotide sequences used for qPCR are given in Table 1.

2.4 Measurement of t-PA antigen concentrations

t-PA antigen concentrations were measured by ELISA, as described previously.⁸

2.5 Chromosome immunoprecipitation analysis

The chromosome immunoprecipitation (ChIP) assay was performed according to the manufacturer's instructions using the reagents provided (Upstate Biotechnology, Temacula, CA, USA). HUVEC were treated for 6 h with 3 μM of trichostatin or MS275 or control medium. About $3 \cdot 10^6$ cells at passage 2 were used per assay. Chromatin was cross-linked to DNA by adding formaldehyde to the medium to a final concentration of 1% and incubating at 37°C during 10 min. After two washes with cold phosphate buffered saline containing proteases inhibitors (PMSF 1 mM, aprotinin 1 $\mu\text{g mL}^{-1}$, and pepstatin 1 $\mu\text{g mL}^{-1}$), cells were scraped and harvested by a brief centrifugation (4 min 600 g 4°C). Cells pellets were resuspended in SDS lysis buffer containing protease inhibitors (200 μL per $1 \cdot 10^6$ cells) and incubated on ice for 10 min. The lysate was sonicated to shear the DNA to a length between 200 and 1000 bp with cooling on ice between cycles and then centrifuged for 10 min at 13 000 g at 4°C. The supernatant was diluted 10-fold in ChIP buffer containing protease inhibitors, a 100 μL aliquot of the diluted supernatant was removed to serve as input sample. The chromatin solution was pre-cleared by adding 75 μL of a 50% protein A sepharose slurry containing salmon sperm DNA and left on a rocking platform for 30 min at 4°C. Chromatin solutions were recovered by 1 min centrifugation at 200 g and 4°C and incubated overnight on a rocking platform with 10 μL of rabbit anti-acetyl histone H3 (1 mg/mL) or anti-acetyl histone H4 (Upstate Biotechnology) at 4°C. 'Mock' samples were prepared by incubation of the chromatin solution under the same conditions with 10 μL of non-relevant rabbit IgG (1 mg/mL). Then 60 μL of a 50% protein A sepharose/ssDNA slurry was added and incubated on a rocking platform for 1 h at 4°C. After centrifugation at 200 g, agarose beads were washed and the immune complexes extracted twice with 250 μL of elution buffer (SDS 1%, 0.1 M NaHCO_3). Then 20 μL of 5 M NaCl was added to the eluate and the input fractions and the formaldehyde cross-links were reversed by heating at 65°C for 4 h. After treatment by proteinase K, tris–HCl and EDTA for 1 h at 45°C, the DNA was purified. The recovered DNA was suspended in H_2O and analysed by qPCR using the primers given in Table 1. The primers amplified a 191 bp DNA fragment corresponding to the region –1037 to –846 with respect to the transcription initiation site of the t-PA gene. The ChIP results are expressed in percent of the input fraction.

2.6 DNA methylation analysis

A quantity of 500 ng of extracted DNA was modified with sodium bisulfite, which converts all unmethylated cytosines to uracil, whereas methylated cytosines remain unchanged. Bisulfite conversion and desulfonation were carried out according to the procedure of the manufacturer (Invitrogen). The resulting DNA was amplified by nested PCR using three primer sets to cover three overlapping regions of the proximal t-PA promoter. In short, 100 ng of bisulfite-treated DNA was subjected to 35 cycles of PCR amplification in a volume of 50 μL using the outer primers described in Table 1. Three microlitres of the PCR product were used as template

Table 1 Oligonucleotide primers used in this study

			Location ^a
GAPDH mRNA qPCR		Forward GGTGAAGGTCGGAGTCAAC Reverse CCATGGGTGGAATCATATTG	
t-PA mRNA qPCR		Forward CCGGCTACGGCAAGCA Reverse AGCGGCTGGATGGGTACA	
18S rRNA qPCR		Forward AGTCCCTGCCCTTTGTACACA Reverse GATCCGAGGGCCTCACTAAAC	
hB2M mRNA qPCR		Forward TGCTCGCGTACTCTCTTT Reverse TCTGCTGGATGACGTGAGTAAAC	
EEF1α1 mRNA qPCR		Forward AGCAAAAATGACCCACCAATG Reverse GGCCTGGATGGTTCAGGATA	
t-PA (CHIP analysis)		Forward GTCGGTCATCCCACAATTTT Reverse CAGGTCACGTGAGGATCAGA	–1037 to –846
t-PA promoter 1	Outer	Forward TTTGAAAAGGTGTTAGTAAG Reverse ACCACTAAAAAACAAAACC	–705 to –382
	Inner	Forward TAAGGGAAATGGTTTGTTTA Reverse CTACRATAAAAAATACCCCCATA	–721 to –416
t-PA promoter 2	Outer	Forward TTTGGGTTTATTTAAGGGGATGT Reverse AAAAATTTTCTCTCCAACCCTAAAC	–577 to +156
	Inner	Forward GAGGTTATTTATTGTAGTTTTGTATTTAT Reverse CAACTCTAAACTCCCCACAACCTC	–535 to +118
t-PA promoter 3	Outer	Forward TTAGGATTTTAAAGGAAGATGATTTTAA Reverse AAAAAACAAACCCCAAAATACAA	–176 to +222
	Inner	Forward AAAGGAAGATGATTTTAAAGGTTTATTT Reverse AAAATTTTCTCTCAACCCTAAACT	–166 to +155

^aThe gene location is given with respect to the transcription initiation site according to the NCBI Reference Sequences for t-PA: NM_033011.2 and NM_000930.3. Gene abbreviations: t-PA, tissue-type plasminogen activator; hB2M, human β2 microglobulin; EEF1α1, elongation factor 1 alpha subunit. qPCR, quantitative reverse transcriptase real-time PCR. CHIP analysis, chromosome immunoprecipitation analysis.

for another 35 cycles of nested PCR amplification in a volume of 50 μL using the inner primers listed in Table 1. After primary and secondary amplification the PCR products were purified with the High Pure PCR Product Purification kit (Roche, Basel Switzerland) and sequenced directly to compare the degree of cytosine methylation.

2.7 Statistics

The significance of differences was determined by Student's *t*-test.

3. Results

3.1 DNA methylation state at the t-PA promoter in HUVEC and in liver-derived cells and expression of t-PA

We analysed the methylation state of the proximal t-PA promoter in HUVEC, in human primary hepatocytes, and in two human hepatoma cell lines, HuH7 and HepG2. The analysis was done by DNA sequencing of bisulfite-treated DNA, which converts unmethylated C's into uracil and has no effect on methylated C's. The t-PA promoter region was amplified by nested PCR using primers for bisulfite-treated DNA and then sequenced. The methylation state of a specific CpG dinucleotide residue was determined from the relative peak heights in the sequence profile and for each CpG dinucleotide position expressed as % (C/C + T). We observed that in EC the t-PA promoter was almost completely (>95%) methylated at positions –647, –618, –548, –537, –452, –421, and –366 (95.4% ± 4.3, average ± SD), while at positions –121, –105, –81, –51, +27,

+42, +50, and +59 no methylated CpG dinucleotide was detected (Figure 1). In comparison, the methylation state of the t-PA promoter in HuH7 human hepatoma cells was quite different. In the region –647 to –366 CpG dinucleotide methylation was significantly lower than in HUVEC (average: 70.8% ± 13.1) ($P < 0.001$, paired *t*-test), while in the region –121 to +59 CpG dinucleotide methylation was significantly higher (average: 61.1% ± 19.0) ($P < 0.0002$) (Figure 1). Intermediate CpG dinucleotide methylation was observed for human hepatocytes and HepG2 hepatoma cells. Interestingly, in human hepatocytes CpG dinucleotides 121 and 105 were ~80% methylated, whereas in HUVEC these CpG dinucleotides were fully unmethylated. Also, in hepatocytes partial methylation was observed of CpG dinucleotides at 27 and 42, which are located just downstream of the transcription initiation site.

We compared t-PA antigen release and t-PA mRNA between HUVEC and the liver-derived cells. Release of t-PA antigen and level of t-PA mRNA were 11.9 and 5.5-fold higher, respectively, in HUVEC than in the primary human hepatocytes ($P < 0.01$) (Figure 2). Expression of t-PA in HepG2 and HuH7 hepatoma cells was 3- and 10-fold lower, respectively, than in hepatocytes (Figure 2).

3.2 Effect of histone deacetylase inhibitors on t-PA expression by endothelial cells

To investigate whether HDACs are involved in the repression of t-PA expression in HUVEC, cells were treated with different concentrations of sodium butyrate or trichostatin, which are selective for both class I and class II HDAC. After 24 h we observed for both

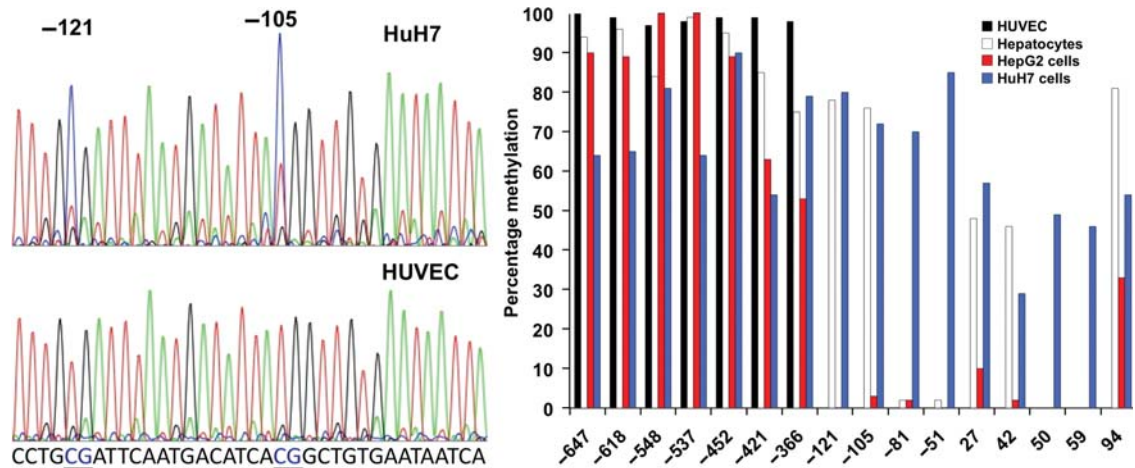


Figure 1 DNA methylation status of the t-PA promoter and t-PA mRNA levels in HUVEC and human liver-derived cells. The methylation state of the t-PA promoter in HUVEC, in human primary hepatocytes or in two human hepatoma cells, HepG2 or HuH7 cells, was determined by direct sequencing of bisulfite treated, PCR-amplified DNA. Left: Illustration of the approach to determine the CpG dinucleotide methylation state at the t-PA promoter. The figure shows the DNA sequence profile of bisulfite-treated DNA for to the t-PA promoter region at positions -125 to -90 with respect to the transcription initiation site. Note that the DNA profile of HuH7 cells shows a majority of C's (blue) and a minority of T's (red) at CpG dinucleotide positions -121 and -105 implying that the majority of CpG dinucleotides at this position are methylated. In contrast, for HUVEC, the DNA profile shows no C's at positions -121 , and -105 . Bisulfite conversion of C's outside a CpG dinucleotide context was complete as shown by a comparison of the DNA sequence profiles and the corresponding genomic DNA sequence below, with CpG dinucleotides underlined and marked in blue. Right: The degree of CpG dinucleotide methylation in the region -647 to $+94$ of the proximal t-PA was estimated by comparison of the ratio of cytidine and thymidine peak heights at each CpG dinucleotide position, as illustrated at the figure at the left. Note the absence of DNA methylation for HUVEC in the region -121 to $+59$, the persistent DNA methylation for HuH7 cells and the intermediate methylation hepatocytes and HepG2 cells in this region. Black: HUVEC; white: hepatocytes; red: HepG2 cells; blue: HuH7 cells.

inhibitors a dose-dependant increase in t-PA antigen release (Figure 3). The effect of trichostatin on t-PA mRNA levels was transient, with a peak at 10 h, whereas the effect of butyrate on t-PA mRNA was stable for at least 24 h (Figure 3). To determine which HDAC class was the target of inhibitors stimulating t-PA expression, HUVEC were treated with MS275, a selective inhibitor of the Class I HDAC. A time response curve of the t-PA mRNA in cells treated with $3 \mu\text{M}$ revealed a maximal response at 24 h (data not shown). The concentration of MS275 that gave a half maximal increase in t-PA (EC₅₀) was calculated using the four parameter model and Prism vs. 5.0 software for Macintosh. For the effect of MS275 on t-PA antigen release and t-PA mRNA, the value of EC₅₀ was 1.88 ± 0.31 and $2.01 \pm 0.12 \mu\text{M}$, respectively (Figure 4). These values are compatible with known IC₅₀'s for HDAC1, 2, and 3, but not for HDAC8.

3.3 Effect of HDAC inhibitors on histone acetylation at the t-PA promoter

HDAC and HAT act not only on histones, but also on a variety of other proteins that may influence gene regulation. To determine whether HDAC inhibition had a direct effect on histone acetylation at the t-PA promoter, we performed chromatin immunoprecipitation experiments on DNA isolated from HDAC inhibitor-treated EC and from control EC, by using antibodies directed at acetylated histones H3 or H4, followed by qPCR using primers specific for the region -1037 to -846 with respect to the transcription initiation site of the t-PA gene. Results are expressed as percentage of input DNA. Immunoprecipitation of t-PA promoter DNA with non-relevant antibody, used as negative control, was negligible.

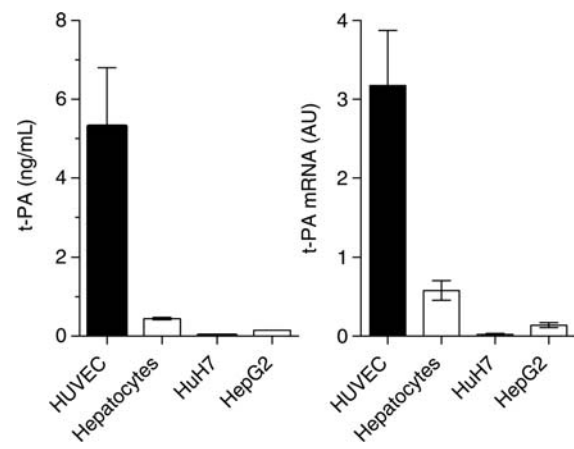


Figure 2 Comparison of basal t-PA expression in HUVEC and human liver-derived cells. Antigen levels of t-PA in 24 h conditioned medium of HUVEC, human primary hepatocytes, HepG2 or HuH7 cells was measured by ELISA (left) and t-PA mRNA levels by qPCR (right). The results give mean values \pm SEM ($n = 4$), for a representative experiment.

Acetylation of histone H3 and H4 associated with the t-PA promoter region in EC was low in non-treated cells (0.46 ± 0.38 and $1.34 \pm 0.84\%$, respectively). After treatment with trichostatin or MS275, 10 ± 3 ($n = 4$) and 44 ± 14 -fold more t-PA promoter DNA was associated with acetylated H3 histone, respectively, and 7.7 ± 1.4

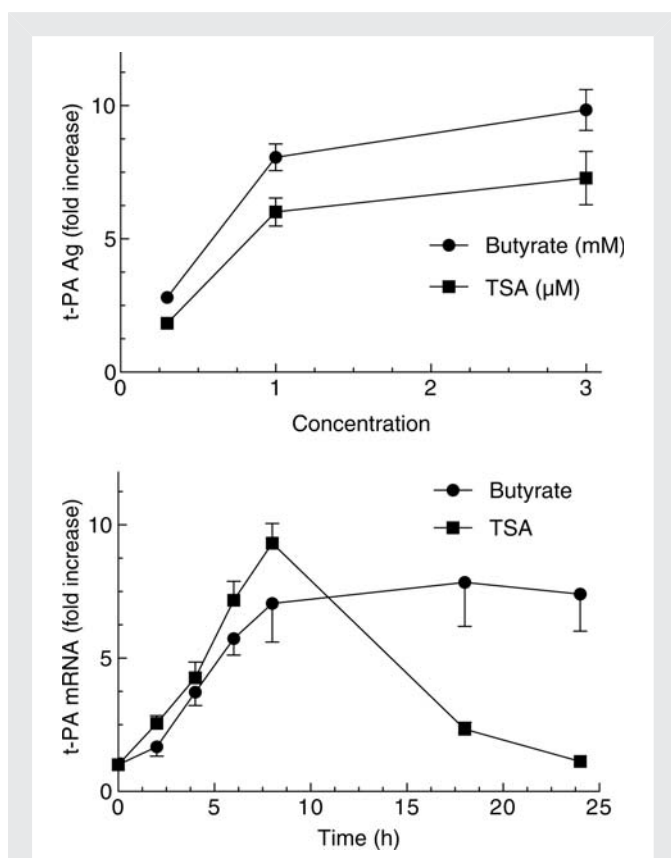


Figure 3 Effect of histone deacetylase inhibitors on t-PA expression by HUVEC. Top: The cells were treated for 24 h with different concentrations of the non-specific HDAC inhibitors butyrate or trichostatin (TSA) and t-PA antigen levels determined in cell supernatants by ELISA. The results are shown as means \pm SEM for three independent experiments. Bottom: time response curves for the effect of butyrate (3 mM) or trichostatin (1 μ M) on t-PA mRNA levels in HUVEC. The figure shows a representative experiment of three independent experiments.

and 16 ± 3 -fold more t-PA promoter DNA associated with acetylated H4 histone, respectively, when compared with DNA from non-treated cells (Figure 5).

3.4 Effect of histone acetyltransferase inhibitors on t-PA expression by endothelial cells

The increased acetylation at the t-PA promoter in EC treated with HDAC inhibitors suggested the involvement of HAT. To determine which HAT might have been involved we treated the EC with garcinol (15 μ M) and with anacardic acid (20 μ M), inhibitors of both p300/CBP and of PCAF as well as with curcumin (30 μ M), an inhibitor of p300/CBP but not of PCAF. Levels of t-PA mRNA were measured in EC treated with these HAT inhibitors alone or in combination with MS275 (3 μ M). With garcinol and with anacardic acid, we observed a reduction in basal and MS275-induced t-PA mRNA levels, whereas curcumin had no effect (Figure 6).

4. Discussion

DNA methylation and histone modifications represent the major epigenetic mechanisms regulating gene transcription. The results

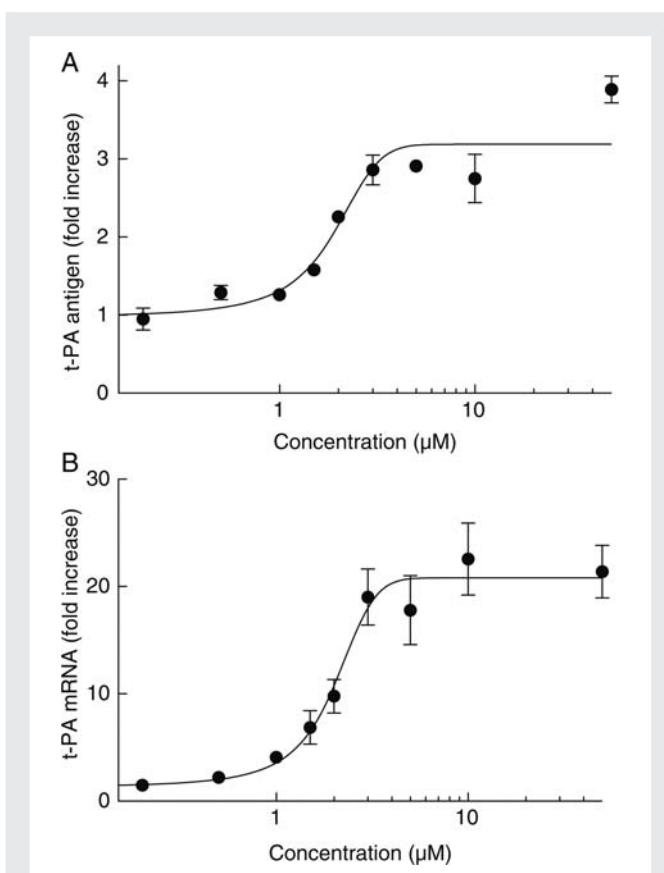


Figure 4 Effect of the class I-specific HDAC inhibitor MS275 on t-PA expression by HUVEC. The cells were treated for 24 h with different concentrations of the class I-specific HDAC inhibitor MS275 and the effect on t-PA release (top) and mRNA levels (bottom) determined by ELISA and quantitative PCR. The results are shown as means \pm SEM for three independent experiments.

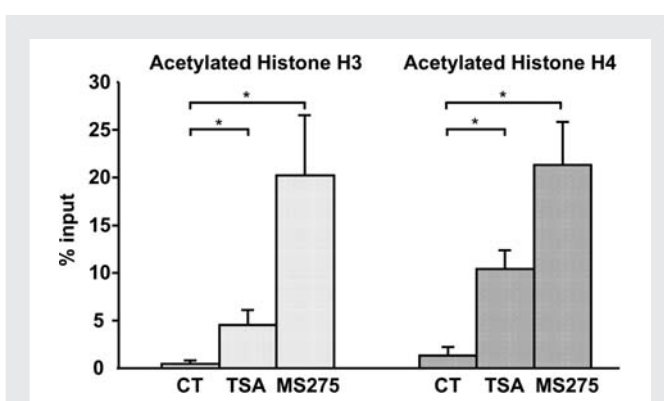


Figure 5 Histone acetylation at the t-PA promoter of HUVEC treated with HDAC inhibitors. HUVEC were treated for 6 h with trichostatin (TSA, 3 μ M) or MS275 (3 μ M). Chromatin was isolated and immunoprecipitated using antibodies specific for acetylated histones H3 or histone H4. Associated t-PA promoter DNA was determined by quantitative PCR using primers for the region -1037 to -846 with respect to the transcription initiation site of the t-PA gene. The PCR results were normalized with respect to input DNA. The results are shown as means \pm SEM for four independent experiments. * $P < 0.005$, Student's *t*-test.

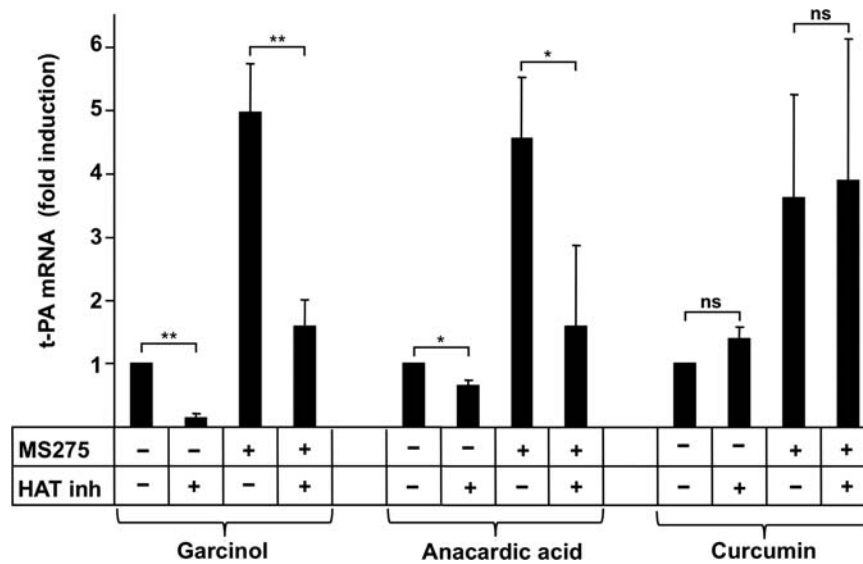


Figure 6 Effect of histone acetyltransferase inhibitors on t-PA expression by endothelial cells. The cells were treated for 24 h with different combinations of the HDAC inhibitor MS275 (3 μ M) and the histone acetyl transferase inhibitors garcinol (15 μ M), anacardic acid (20 μ M), and curcumin (30 μ M) as indicated below the figure. t-PA mRNA levels were determined by quantitative PCR. Note the inhibitory effect of garcinol and anacardic acid on basal and MS275 induced t-PA mRNA and the lack of effect of curcumin. The results are shown as means \pm SEM for three independent experiments. Significance of differences: * P < 0.05; ** P < 0.01; ns, non-significant.

presented in this study imply that t-PA expression is under epigenetic control and repressed by HDAC in non-stimulated EC. An analysis of the CpG dinucleotide methylation state of the t-PA promoter revealed a stretch from -121 to +59 in which the CpG dinucleotides were unmethylated. In contrast, in human primary hepatocytes this stretch was methylated and basal t-PA expression in these cells was much lower than that in EC. Lowest levels of t-PA expression occurred in HuH7 human hepatoma cells, in which CpGs in its promoter region were more extensively methylated. These results are in agreement with the concept that methylated CpG dinucleotides at the proximal promoter are associated with gene inactivation (Cedar, Nat Rev Genet 2008).²²

We observed that HDAC inhibitors increase the expression of t-PA by human EC. Our results confirm and extend a previous observation that the non-specific HDAC inhibitors trichostatin and butyrate increase t-PA gene transcription in EC.¹³ In particular, our results provide clear evidence that HDAC inhibitors have a direct effect and modify the acetylation state of histones at the t-PA promoter. Taken together, this suggests that in resting EC t-PA expression is repressed by HDACs. MS275, an inhibitor directed only towards class I HDAC, was the strongest inducer of t-PA mRNA with a half maximum effect at 2 μ M. This implies an important role for HDAC1, HDAC2, or HDAC3. HDAC remove acetyl groups not only from histones H3 or H4, but also may deacetylate other proteins that have an effect on cell signalling.²³ Our finding that acetylation of histone H3 and histone H4 associated with the t-PA promoter was increased in HUVEC after treatment with MS275 or trichostatin suggests a direct effect of HDAC inhibitors on the t-PA promoter. Treatment of EC with garcinol and anacardic acid, which are inhibitors of the HATs P300/CBP and of PCAF, reduced basal and MS275-induced expression of t-PA. In contrast, curcumin, an inhibitor of P300/CBP but not of PCAF,²⁴ had no effect on basal or

MS275-induced t-PA expression. This suggests a role for PCAF rather than P300/CBP in regulation of t-PA expression.

Several regulatory elements have been identified within the t-PA promoter. Among these are a TRE-element (TGACTCA) at position -113 to -106 of the t-PA promoter which is essential for basal t-PA gene transcription.²⁵ and, in EC, binds the AP-1 family members junD and fra-2.²⁶ Two CpG dinucleotides are found in the immediate vicinity of this TRE, one is located immediately downstream, the other located 8 bp upstream of this TRE. In EC these two CpG dinucleotides are fully unmethylated, while in primary hepatocytes these are >75% methylated (see Figure 1). It remains to be established to what extent the difference in DNA methylation of these two CpG dinucleotides are responsible for the 5- to 10-fold difference in t-PA expression between HUVEC and primary hepatocytes. Other regulatory elements in the proximal t-PA promoter are likewise close to CpG dinucleotide residues. Thus a CTF/NF1 binding site (TCAGCCTGGCCCGAA) at position -92 to -77²⁶ contains a CpG dinucleotide (position -81) that is unmethylated in EC and human hepatocytes and 70% methylated in HuH7 cells (see Figure 1). An AP2-like binding site (CCCCACCCCC) at position +62 to +71 is located just downstream of a triplet of CpG dinucleotides that are unmethylated in EC and partially methylated in hepatoma cells and HuH7 cells. This site is known to be required for basal t-PA expression and to recruit the transcription factor Sp1.^{25,27} Recruitment of co-repressor complexes to these methylated CpG dinucleotide residues are likely to interfere with the binding of AP-1 family members to the TRE element, which would explain the lower expression of t-PA in hepatocytes and HUH7 cells. In contrast, an Sp-1 binding site coincides with the CpG dinucleotide at position -366, and was found to mediate the increased expression of t-PA induced by quercetin in HUVEC.²⁸ In HUVEC, this position is fully methylated. Further work needs to be done to determine whether

the close proximity of CpG dinucleotides with almost all known transcription factor binding sites within the t-PA promoter is fortuitous or has a functional relevance.

A limitation of the present study is the use of human EC in culture. For primary early passage EC or hepatocytes, the methylation state of the promoter is unlikely to be different from that *in vivo*. Other epigenetic aspects that may impact on t-PA expression such as activity of HDACs and HATs may be different from those *in vivo*. As HDAC inhibitors are being developed for therapy of cancer and brain disorders,^{29,30} it is likely that therapeutic use of inhibitors of DNA methylation or of HDAC inhibitors has an impact on expression of t-PA *in vivo*. Our finding, that hepatocytes cultured *in vitro* are capable of t-PA production, raises the question as to what extent these cells contribute to plasma t-PA concentrations.

In conclusion, regulation of t-PA in EC is under epigenetic control. Methylation of the proximal t-PA promoter is associated with a reduced expression of t-PA in hepatocytes and in HuH7 hepatoma cells when compared with EC. Class I histone deacetylases repress t-PA expression in EC, whereas the HAT PCAF has the opposite effect. A better understanding of the epigenetic control mechanisms modulating t-PA expression may help to establish to what extent epigenetic approaches that are being developed for therapies of cancer or cardiovascular disease modify the vascular fibrinolytic system. Further studies are needed to determine the tissue-specific aspects of the epigenetic control of t-PA gene regulation.

Conflict of interest: none declared.

Funding

This work was supported by the Swiss National Science Foundation [grant no. 320000-118125].

References

1. Wall U, Jern S, Tengborn L, Jern C. Evidence of a local mechanism for desmopressin-induced tissue-type plasminogen activator release in human forearm. *Blood* 1998;**91**:529–537.
2. Tranquille N, Emeis JJ. The role of cyclic nucleotides in the release of tissue-type plasminogen activator and von Willebrand factor. *Thromb Haemost* 1993;**69**:259–261.
3. Giles AR, Nesheim ME, Herring SW, Hoogendoorn H, Stump DC, Heldebrandt CM. The fibrinolytic potential of the normal primate following the generation of thrombin *in vivo*. *Thromb Haemost* 1990;**63**:476–481.
4. Kruithof EK, Mestries JC, Gascon MP, Ythier A. The coagulation and fibrinolytic responses of baboons after *in vivo* thrombin generation—effect of interleukin 6. *Thromb Haemost* 1997;**77**:905–910.
5. Emeis JJ, van den Eijnden-Schrauwen Y, van den Hoogen CM, de Priester W, Westmuckett A, Lupu F. An endothelial storage granule for tissue-type plasminogen activator. *J Cell Biol* 1997;**139**:245–256.
6. Rosnoble C, Vischer UM, Gerard RD, Irminger JC, Halban PA, Kruithof EK. Storage of tissue-type plasminogen activator in Weibel-Palade bodies of human endothelial cells. *Arterioscler Thromb Vasc Biol* 1999;**19**:1796–1803.
7. Datta YH, Youssoufian H, Marks PW, Ewenstein BM. Targeting of a heterologous protein to a regulated secretion pathway in cultured endothelial cells. *Blood* 1999;**94**:2696–2703.
8. Huber D, Cramer EM, Kaufmann JE, Meda P, Massé JM, Kruithof EK *et al*. Tissue-type plasminogen activator (t-PA) is stored in Weibel-Palade bodies in human endothelial cells both *in vitro* and *in vivo*. *Blood* 2002;**99**:3637–3645.
9. Dichet D, Quertermous T. Thrombin regulation of mRNA levels of tissue plasminogen activator and plasminogen activator inhibitor-1 in cultured human umbilical vein endothelial cells. *Blood* 1989;**74**:222–228.
10. Kooistra T, Bosma PJ, Toet K, Cohen LH, Griffioen M, van den Berg E *et al*. Role of protein kinase C and cyclic adenosine monophosphate in the regulation of tissue-type plasminogen activator, plasminogen activator inhibitor-1, and platelet-derived growth factor mRNA levels in human endothelial cells. Possible involvement of proto-oncogenes c-jun and c-fos. *Arterioscler Thromb* 1991;**11**:1042–1052.
11. Bulens F, Nelles L, Van den Panhuyzen N, Collen D. Stimulation by retinoids of tissue-type plasminogen activator secretion in cultured human endothelial cells: relations of structure to effect. *J Cardiovasc Pharmacol* 1992;**19**:508–514.
12. Pepper MS, Rosnoble C, Di Sanza C, Kruithof EK. Synergistic induction of t-PA by vascular endothelial growth factor and basic fibroblast growth factor and localization of t-PA to Weibel-Palade bodies in bovine microvascular endothelial cells. *Thromb Haemost* 2001;**86**:702–709.
13. Arts J, Lansink M, Grimbergen J, Toet KH, Kooistra T. Stimulation of tissue-type plasminogen activator gene expression by sodium butyrate and trichostatin A in human endothelial cells involves histone acetylation. *Biochem J* 1995;**310**:171–176.
14. Ordovás JM, Smith CE. Epigenetics and cardiovascular disease. *Nat Rev Cardiol* 2010;**7**:510–519.
15. Yan MS, Matouk CC, Marsden PA. Epigenetics of the vascular endothelium. *J Appl Physiol* 2010;**109**:916–926.
16. Bruneau BG. Epigenetic regulation of the cardiovascular system: introduction to a review series. *Circ Res* 2010;**107**:324–326.
17. Ellis L, Hammers H, Pili R. Targeting tumor angiogenesis with histone deacetylase inhibitors. *Cancer Lett* 2009;**280**:145–153.
18. Marks PA, Xu WS. Histone deacetylase inhibitors: Potential in cancer therapy. *J Cell Biochem* 2009;**107**:600–608.
19. Dinarello CA. Anti-inflammatory agents: present and future. *Cell* 2010;**140**:935–950.
20. Dunoyer-Geindre S, Kruithof EK, Galve-de Rochemonteix B, Rosnoble C, Gruenberg J, Reber G *et al*. Localization of beta2-glycoprotein 1 in late endosomes of human endothelial cells. *Thromb Haemost* 2001;**85**:903–907.
21. Fish RJ, Kruithof EK. Short-term cytotoxic effects and long-term instability of RNAi delivered using lentiviral vectors. *BMC Mol Biol* 2004;**5**:9.
22. Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet* 2009;**10**:295–304. Review.
23. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* 2007;**26**:5541–5552.
24. Balasubramanyam K, Varier RA, Altaf M, Swaminathan V, Siddappa NB, Ranga U *et al*. Curcumin, a novel p300/CREB-binding protein-specific inhibitor of acetyltransferase, represses the acetylation of histone/nonhistone proteins and histone acetyltransferase-dependent chromatin transcription. *J Biol Chem* 2004;**279**:51163–51171.
25. Medcalf RL, Rüegg M, Schleuning WD. A DNA motif related to the cAMP-responsive element and an exon-located activator protein-2 binding site in the human tissue-type plasminogen activator gene promoter cooperate in basal expression and convey activation by phorbol ester and cAMP. *J Biol Chem* 1990;**265**:14618–14626.
26. Arts J, Herr I, Lansink M, Angel P, Kooistra T. Cell-type specific DNA-protein interactions at the tissue-type plasminogen activator promoter in human endothelial and HeLa cells *in vivo* and *in vitro*. *Nucleic Acids Res* 1997;**25**:311–317.
27. Costa M, Shen Y, Maurer F, Medcalf RL. Transcriptional regulation of the tissue-type plasminogen-activator gene in human endothelial cells: identification of nuclear factors that recognise functional elements in the tissue-type plasminogen-activator gene promoter. *Eur J Biochem* 1998;**258**:123–131.
28. Pan W, Chang MJ, Booye FM, Grenett HE, Bradley KM, Wolkowicz PE *et al*. Quercetin induced tissue-type plasminogen activator expression is mediated through Sp1 and p38 mitogen-activated protein kinase in human endothelial cells. *J Thromb Haemost* 2008;**6**:976–985.
29. Sebova K, Fridrichova I. Epigenetic tools in anticancer therapy. *Anticancer Drugs* 2010;**21**:565–577. Review.
30. Narayan P, Draganow M. Pharmacology of epigenetics in brain disorders. *Br J Pharmacol* 2010;**159**:285–303.