Valproate inhibition of histone deacetylase 2 affects differentiation and decreases proliferation of endometrial stromal sarcoma cells

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Abstract

Covalent modifications of histone proteins, in particular deacetylation of lysine residues, are important for the regulation of gene transcription both in normal and malignant cells. These processes are controlled by histone acetyltransferases and histone deacetylases (HDAC) and have up to now not been described in solid mesenchymal tumors. The present study shows differences in the HDAC1 and HDAC2 expression in endometrial stromal sarcomas (ESS) and a cognate cell line (ESS-1) compared with nonneoplastic endometrial stroma. We show for the first time that HDAC2 expression is consistently increased in ESS. In contrast, HDAC1 expression is generally lower than HDAC2 both in nonneoplastic stroma and in ESS, suggesting that these two proteins, although closely related, are regulated in different ways. *In vitro* experiments with an ESS cell line showed that valproate, an inhibitor of the class I HDACs, led to significant HDAC2 decrease and to cell differentiation. HDAC2 inhibition in ESS-1 cells caused significant changes in the cell cycle by inhibiting G_1-S transition and influencing expression of $p21^{WAF1}$ and cyclin D1. Moreover, in ESS-1 cells, increased expression of the $p21^{WAF1}$ was associated with reduction of HDAC2 expression after transfection with small interfering RNA directed against HDAC2. Our results suggest that HDAC2 might be considered as potential drug target in the therapy of ESS and that HDAC inhibitors should be further evaluated in clinical trials in ESS. [Mol Cancer Ther 2006;5(9):2203–10]

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Introduction

Chromatin architecture is strongly influenced by post-translational modifications of the core histones, the highly conserved proteins involved in the basic structural unit of chromatin. In addition to their structural role, the core histones play an important role in the modulation of chromatin structure, chromatin function, and regulation of gene expression (1–4). Various covalent modifications (methylation, acetylation, phosphorylation, ubiquitination, etc.) of histone proteins are involved in the regulation of gene transcription (5). In particular, acetylation and deacetylation of ε-amino groups in lysine residues on core histones alter nucleosomal conformation and modulate chromatin structure, chromatin function, and gene expression by regulating the accessibility of transcriptional regulatory proteins to chromatin templates. The equilibrium of histone acetylation is controlled by histone acetyltransferases and histone deacetylases (HDAC). Their balance seems to be essential for normal cell growth, and imbalances are often associated with carcinogenesis and cancer progression (6). In general, decreased level of histone acetylation is associated with transcriptional repression (2, 7).

The HDAC family consists of ~20 isoenzymes, which are members of up to four different classes. The class I enzymes HDAC1, HDAC2, HDAC3, and HDAC8 are homologues of the yeast RPD3 protein and the class II enzymes HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10 are homologues of yeast Hda1. The third class consists of Sir2-like proteins, sirtuins, whereas HDAC11 seems to represent a new class of HDACs (8). Up to now, altered HDAC expression has been described in different tumors and it is connected with regulation of different tumor-specific genes involved in growth control and cell differentiation (9, 10).

Thus far, several structurally different substances have been identified to inhibit HDACs. HDAC inhibitors are able to activate differentiation processes, to inhibit G_1-S transition by arresting cells in the G_1 phase, and/or to induce apoptosis in transformed and cancer cells (11–13). Accurately defined molecular mechanisms of these processes are currently under intensive study. Whereas novel, more potent inhibitors are developed, some substances which have been already used for other purposes are rediscovered as HDACs inhibitors. To the latter belongs valproic acid, which has been used during the last decades as anticonvulsant and mood stabilizer (14). Recently, it has been shown by Goettlicher et al. (11, 12) that valproic acid may affect the concentration of HDAC2 by inducing its proteasomal degradation.

In the past, myeloic leukemia has been frequently used as a model system for studying aberrant transcriptional

repression and therapeutic interventions directed against aberrant chromatin modifications (15, 16). However, it is now obvious that alterations in the structure or expression of histone acetyltransferases and HDACs occur also in a variety of nonhematopoietic cancer cells (17–22). Until now, no investigations of these processes in solid mesenchymal tumors have been reported.

The present study was designed to investigate differences in HDAC1 and HDAC2 expression in endometrial stromal sarcomas (ESS), which are rare mesenchymal malignancies with poorly understood pathogenesis (23), and a cognate ESS-1 cell line compared with nonneoplastic endometrial stroma. We also tried to define possible therapeutic effects of sodium valproate, a HDAC inhibitor that preferentially inhibits HDAC2 enzyme, in ESS. We therefore examined whether this substance is able to mediate processes involved in cell differentiation, cell cycle arrest, and apoptosis in ESS-1 cells. According to the results of this study, HDACs might be considered as potential drug targets for therapy of ESS and possibly other mesenchymal tumors.

Materials and Methods

Tissue Samples

Tumor material was obtained from patients with ESS (n = 22) and undifferentiated endometrial sarcoma (UES; n = 2). The diagnosis and classification of ESS/UES cases was confirmed independently by two pathologists (F.M. and H.D.) according to the new WHO classification (24). Normal endometrial stromal tissues (curettage material) were used as control. The general characteristics of tumors used in this study have been described in more detail recently (25). All investigations were approved by the Institutional Review Board of Medical University of Graz (Graz, Austria).

Cell Culture

All media and chemicals used in the cell culture experiments were purchased from Sigma (Sigma-Aldrich Handels GmbH, Vienna, Austria). The ESS-1 cells (cell line of a human ESS) were purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). This cell line, established by Gunawan et al. (26), shows mainly a mesenchymal phenotype and focal multilayered growth pattern and is not tumorigenic in nude mice. For *in vitro* experiments, the cells were grown in RPMI 1640 supplemented with heat-inactivated FCS (10%, v/v), 2.2 g/L sodium bicarbonate, 100 units/mL penicillin, and 100 μg/mL streptomycin and cultured under standard conditions (37°C, 5% CO_2, and 95% humidity).

Flow Cytometry

ESS-1 cells were seeded into 12-well plates (5×10^5 per well) 1 day before treatment. On the next day, the medium was changed and the cells were treated with RPMI 1640 containing 5 mmol/L sodium valproate (sodium 2-propylpentanoate; Sigma-Aldrich) that was used instead of valproic acid, which is poorly soluble in water. Control cells were incubated in growth medium without valproate. At indicated time points, both control and treated cells were harvested (0.25% trypsin-EDTA), resuspended in 500 μL PBS (pH 7.4), fixed with 5 mL ice-cold methanol, and stored at 4°C. Propidium iodide (PI) staining was done using DNA-Prep stain [PI (50 μg/mL) and RNase (4 KU/mL; type III-A, bovine pancrease)] according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA). Finally, the cells were analyzed on Coulter EPICS XL-MCL using System II3.0 data analyzing software provided by the producer (Beckman Coulter).

Small Interfering RNA Transfection

ESS-1 cells were trypsinized and resuspended in RPMI 1640 at a concentration of 1×10^5 per mL (six-well plates). Transfection complexes were prepared using chemically synthesized small interfering RNA (siRNA) molecules (two different siRNAs against HDAC2, nonsilencing siRNA and positive control siRNA against lamin; 20 nm each; Qiagen, Vienna, Austria) and siPORT NeoFX transfection agent (Ambion, Vienna, Austria) according to protocols recommended by the producers. After transfection, cells were harvested (at 24, 48, and 72 hours) and analyzed by real-time reverse transcription-PCR with SYBR Green detection using QuantiTect Primer Assays (Qiagen). Data were normalized to the glyceraldehyde-3-phosphate dehydrogenase signal. Percentage of remaining expression was calculated as the amount of HDAC2 mRNA in cells transfected with HDAC2-siRNA compared with that of cells transfected with the nonsilencing siRNA. HDAC2 knockdown effects were additionally proved by Western blotting.

Western Blot Analysis

ESS-1 cell lysates were fractionated by SDS-PAGE for 1 hour at 150 V, transferred to nitrocellulose membrane, and incubated with specific antibodies. If not stated otherwise, all antibodies were purchased from DAKO (Copenhagen, Denmark). The following antibodies and concentrations were used: rabbit anti-HDAC1 (1 μg/mL); rabbit anti-HDAC2 (1 μg/mL); mouse anti-$p21^{WAF1}$ (0.5 μg/mL); mouse anti-cyclin D1 (1.7 μg/mL); mouse anti-Bcl-2 (1.9 μg/mL); and mouse anti-β-tubulin (1 μg/mL). As secondary antibodies, we used swine anti-rabbit and rabbit anti-mouse horseradish peroxidase–labeled antibodies at final concentration of 1 μg/mL. Specific protein bands were visualized by enhanced chemiluminescence assay (Amersham Biosciences, Buckinghamshire, England). All Western blots were probed for β-tubulin to show equal loading of protein samples. Blots were quantified by densitometric analysis using Multi-ImageLight Cabinet and ChemiImager 5500 software (Alpha Innotech Corp., San Leandro, CA).

Apoptosis Detection Assays

Caspase-3 activity was assayed using the caspase-3 apoptosis detection kit sc-4263 AK (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions. The reaction was monitored on a Hitachi F-2500 fluorescence spectrophotometer (Hitachi High Technologies Corp., Tokyo, Japan) using excitation/

emission wavelength of 400/510 nm. Finally, levels of emission of valproate-treated and untreated cells were compared.

Double staining with Annexin V and PI was done using the BD PharMingen Annexin V-FITC Apoptosis Detection kit II (BD Biosciences, Schwechat, Austria) according to the manufacturer's instructions. The cells were monitored on a BD FACSCalibur (BD Biosciences) using CellQuest Pro data analyzing software provided by the producer.

Immunohistochemistry

Representative serial sections (2-μm thick) of formalin-fixed, paraffin-embedded material were preheated at 65°C for 30 minutes, deparaffinized in xylol, rehydrated in ethanol (descending concentrations), and finally washed in double-distilled water and PBS (pH 7.4). Endogenous peroxidase was blocked by incubation in 1% H_2O_2 (dissolved in methanol) for 15 minutes and sections were additionally washed with Tris buffer (3 × 5 minutes). Afterwards, tissue sections were incubated with primary (60 minutes/room temperature) and secondary (30 minutes/room temperature) antibodies. The following antibodies and dilutions were used: rabbit anti-HDAC1 (final concentration of 2 μg/mL); swine anti-rabbit horseradish peroxidase (1:100; DAKO); rabbit anti-HDAC2 (final concentration of 1.5 μg/mL); and swine anti-rabbit horseradish peroxidase (1:100). Negative controls were done using mouse IgG1. All sections were additionally stained with hematoxylin. Description and scoring of immunohistochemistry were done independently by two experienced pathologists (F.M. and H.D.). Intensity of staining was graded on an arbitrary scale and reported as negative (0), weakly positive (+1), moderately positive (+2), and strongly positive (+3).

Statistical Analysis

Statistical analysis was done using SPSS statistical software, version 11.5 (SPSS, Inc., Chicago, IL). Differences between neoplastic and nonneoplastic tissues in HDAC1 and HDAC2 expression were tested by Mann-Whitney *U* test. Distribution of different HDAC1 and HDAC2 expression according to the grading (0, +1, +2, and +3) was tested using Fisher's exact test. Spearman's test was used to test for correlation in HDAC1 and HDAC 2 expression. All tests were two sided and a $P < 0.05$ was considered statistically significant. If not stated otherwise, mean of at least three independent experiments ± SD is shown.

Results

HDAC2 Expression Is Highly Increased in ESS and UES

The expressions of HDAC1 and HDAC2 were analyzed in 24 endometrial stromal tumors (22 ESS and 2 UES) and 20 nonneoplastic endometria, the latter group consisting of 6 proliferative, 7 secretory, and 7 inactive (atrophic) types. Both HDAC1 and HDAC2 showed similar expression patterns in tumors and normal endometria, although their intensity of expression did not correlate ($P = 0.26$). As shown with representative samples in Fig. 1, HDAC2 nuclear expression was highly increased in tumor tissue compared with nonneoplastic endometrial stroma ($P < 0.01$). None of the 24 tumor samples was scored as negative for HDAC2 expression. Five cases were scored weakly positive (+1), 9 cases were moderately positive (+2), and 10 were strongly positive (+3) for HDAC2. Both UES cases showed strong HDAC2 expression.

Among the 20 nonneoplastic endometria, 2 (10%) were scored as negative for HDAC2 both in endometrial stromal and glandular cells. Eleven (55%) cases were scored as weakly (+1) and 6 (30%) cases as moderately (+2) positive, but HDAC2 immunoreactivity was more intense in endometrial glandular than in stromal cells. Among these 17 cases, the stromal component of 6 (35.3%) cases was negative. Only one specimen was strongly positive (+3), both in stromal and in glandular cells. In 7 (35%) cases of nonneoplastic endometria, myometrial components were also present; in 5 cases, HDAC2 was negative; in 2 cases, HDAC2 was only weakly positive (+1) (data not shown). Different functional states (proliferation, secretion, and inactivity) did not influence HDAC2 expression. HDAC2 expression of all tissue samples is summarized in Fig. 2.

HDAC1 Expression in ESS Is Rather Low

HDAC1 expression in both tumor tissue and healthy endometrial stroma was generally weaker than that of HDAC2 ($P < 0.01$). None of the tumor samples was scored as +3 for HDAC1 expression and only two cases were scored as +2. Thirteen samples were weakly positive (+1) and 9 were negative. Normal endometrial stroma was HDAC1 negative in seven cases, whereas, in 13 cases, it was only weakly positive (+1). Overall, HDAC1 expression was not different between tumor and nonneoplastic tissue ($P = 0.4$). Different functional states (proliferation, secretion, and inactivity) did not influence HDAC1 expression. The myometrial component was present in seven cases and was HDAC1 negative in all. Both UES cases showed weak HDAC1 expression (+1). HDAC1 expression of all tissue samples is summarized in Fig. 2.

Valproate Inhibits HDAC2 Expression in ESS-1 Cells

To study the effects of valproate on the HDAC2 concentration *in vitro*, ESS-1 cells were treated with 5 mmol/L sodium valproate dissolved in RPMI 1640. As shown in Fig. 3, HDAC2 was markedly down-regulated in valproate-treated ESS-1 cells. Already 24 hours after onset of the treatment, HDAC2 in treated cells decreased to 53% of the initial concentration, as determined by immunoblotting and densitometric analysis. On the other hand, HDAC1 concentration in ESS-1 cells was virtually unaffected by valproate treatment (Fig. 3).

HDAC2 Inhibition by Valproate in ESS-1 Cells Leads to Differentiation and Arrested Cell Proliferation

Treatment of ESS-1 cells with 5 mmol/L valproate resulted in changes both in cell density and morphology. Compared with untreated cells, which were polygonal and/or irregular shaped with indistinct cell borders, valproate-treated cells were spindle shaped and showed more cytoplasmic extensions (data not shown). About cell density, 32 hours after incubation with valproate, ESS-1

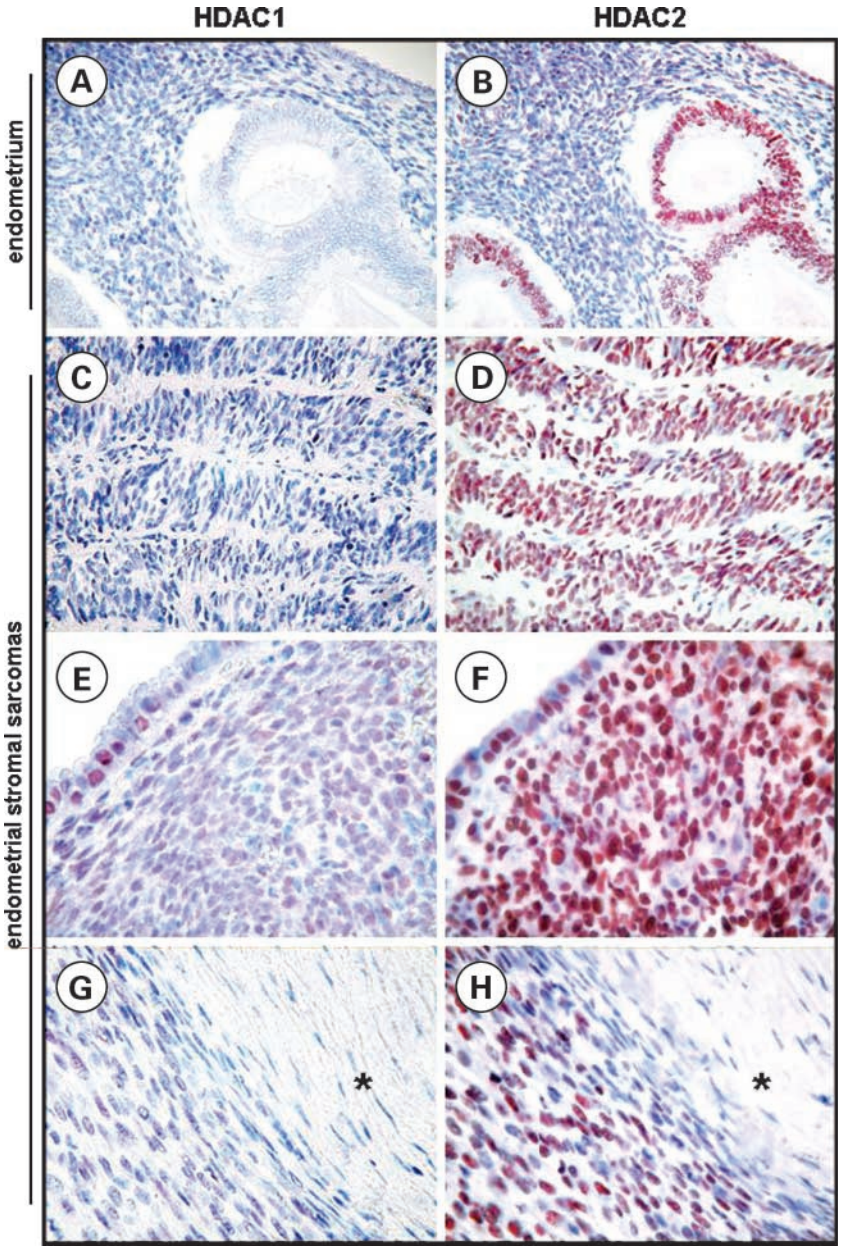

Figure 1. HDAC1 and HDAC2 expression in nonneoplastic endometrial stroma and in ESS. **A,** complete lack of HDAC1 immunoreactivity in nonneoplastic endometrium. **B,** endometrial gland cells show intensive nuclear staining for HDAC2. **C** and **D,** an example of ESS with sex cord-like pattern showing negative reaction for HDAC1 but intense and diffuse nuclear positivity for HDAC2. **E,** HDAC1 is only weakly and focally positive in tumor cells. **F,** an intense and diffuse positive HDAC2 immunoreaction typical for ESS. **G** and **H,** example of ESS with very weak and focal reactivity for HDAC1 but intense positive reaction for HDAC2. Note that the myometrial component (*asterisks*) is negative for both HDAC1 and HDAC2. Magnifications, ×200 (**A – D**) and ×400 (**E – H**).

cells showed significantly (P = 0.0052) lower cell density than untreated ones and this feature became even more pronounced with time (Fig. 4A).

Flow cytometry after incubation with 5 mmol/L valproate showed typical G_1 arrest and a concomitant decrease of the cell number in S phase compared with the untreated ESS-1 cells (Fig. 4B). For example, 48 hours after treatment, 74% of treated cells were in G_1 phase compared with 41% of cells growing in medium without valproate. At the same time, 15% of treated cells were in S phase compared with 46% of untreated cells.

To determine whether valproate-treated ESS-1 cells undergo apoptosis, we analyzed the expression level of different cell cycle– and apoptosis-related proteins $p21^{WAF1}$, cyclin D1, and Bcl-2 by Western blotting. As shown in Fig. 5A, valproate led to up-regulation of the $p21^{WAF1}$ expression, a well-known multifunctional mediator of cytostatic signals, the expression of which in untreated cells was negligible and could be detected only after prolonged film exposure time. On the other hand, as determined by densitometry, the levels of cyclin D1 and of the antiapoptotic protein Bcl-2 were markedly reduced by valproate treatment compared with untreated cells showing a reduction by 75% and 38%, respectively. Fluorometric caspase-3 activity assay revealed no changes between untreated and valproate-treated ESS-1 cells (Fig. 5B). Additionally, Annexin V assay with subsequent flow cytometric analysis has been done 24, 48, and 72 hours

after valproate treatment but revealed no significant changes compared with untreated cells (Fig. 5C).

HDAC2-siRNA Knockdown Leads to p21^{WAF1} Increment and Arrested Cell Proliferation

To prove whether lowering of HDAC2 expression specifically leads to cell cycle arrest in ESS-1 cells, we did knockdown experiments using the siRNA technology. Following siRNA transfection, ESS-1 cells were analyzed both by real-time reverse transcription-PCR and by Western blotting (Fig. 6). Two different siRNAs directed against HDAC2 were used, HDAC2-2-siRNA showing slightly better knockdown effects (Fig. 6A). Pronounced knockdown effects (85%) were also reflected in Western blot analyses (Fig. 6B). The expression of HDAC1, which belongs to the same HDAC class, remained unaffected, proving the specificity of this method. Moreover, the expression of p21^{WAF1} in ESS-1 cells transfected with HDAC2-siRNA was noticeably increased. Both nonsilencing and positive control (lamin) siRNA did not cause any changes, indicating that p21^{WAF1} increment was specifically associated with HDAC2 down-regulation rather than being affected by the transfection itself. Cell cycle analysis of transfected cells showed effects similar to those found after valproate treatment (i.e., G_1 arrest and S-phase decrement; data not shown). Annexin V apoptosis detection assay revealed no significant changes in HDAC2-siRNA-transfected cells (Fig. 5C).

Discussion

Imbalances in the equilibrium of histone acetylation are associated with carcinogenesis and cancer progression. About the growing list of tumors showing increased expression of HDACs, the question on whether this is a widespread phenomenon in carcinogenesis is of considerable importance.

In our study, we analyzed and compared HDAC1 and HDAC2 expression in ESS. These results show for the first time that HDAC2 is markedly up-regulated in these tumors compared with nonneoplastic endometrial stroma. Although HDAC1 and HDAC2 are closely related proteins,

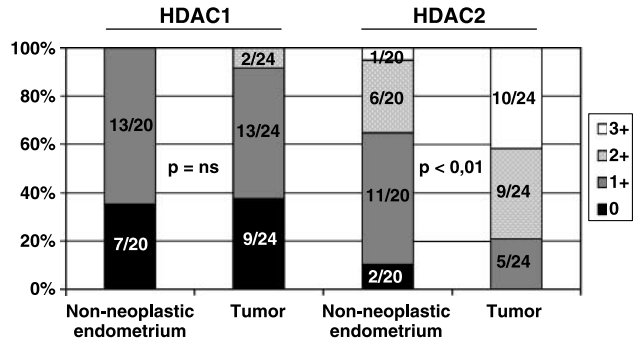

Figure 2. Summary of HDAC1 and HDAC2 scores in ESS (n = 24) and in nonneoplastic endometrial stroma (n = 20). $P < 0.05$ was considered statistically significant. *ns,* nonsignificant.

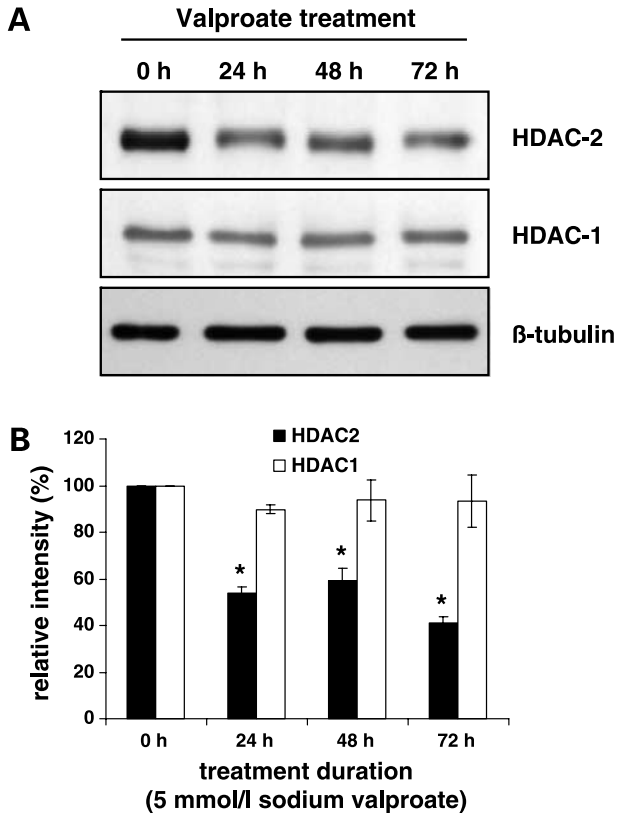

Figure 3. Valproate decreases HDAC2 but not HDAC1 protein levels in ESS-1 cells. **A,** to asses the specificity, ESS-1 cells were incubated with 5 mmol/L valproate and, at indicated time points, cell lysates were analyzed by Western blotting using antibodies against HDAC1 and HDAC2. β-Tubulin immunoblotting was used as a loading control. **B,** HDAC2 protein level in valproate-treated ESS-1 cells is significantly decreased already 24 h after onset of the treatment. P = 0.02, 0.019, and 0.019 at 24, 48, and 72 h, respectively. HDAC1 concentration remains largely unaffected. P = 0.11, 67, and 0.75 at 24, 48, and 72 h, respectively. Representative immunoblots. *Columns,* mean of three independent experiments with similar results; *bars,* SD.

HDAC1 expression in ESS was not increased. This is a strong argument that HDAC1 and HDAC2, which share 85% homology on protein basis, are regulated in different ways not only in colorectal polyps and colonic cancer, as published by Huang et al. (13), but also in other types of neoplasms, including ESS.

In this study, we also showed variations in HDAC1 and HDAC2 expression in normal endometrial stroma. The comparison of HDAC1 and HDAC2 scores in nonneoplastic endometria revealed that these proteins share a similar localization pattern. They were primarily expressed in the endometrial gland cells, whereas their expression in stromal cells was much less pronounced. Although both HDAC1 and HDAC2 belong to the class I HDACs, which are constitutively expressed in most cell types, they are not expressed at the same intensity in nonmalignant endometrial stroma and in ESS. This correlates with data published by de Ruijter et al. (27), showing serial analysis of gene

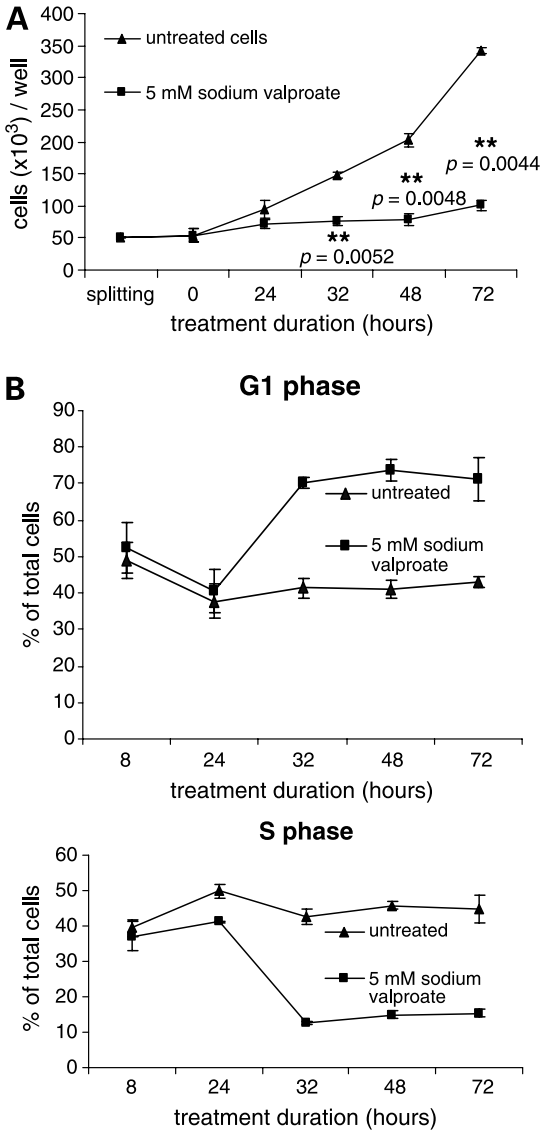

Figure 4. Treatment of ESS-1 cells with 5 mmol/L valproate influences cell density and induces G_1 arrest in ESS-1 cells. **A,** cell density changed significantly ($P < 0.005$) 48 h after valproate has been added to the growth medium. **B,** flow cytometry data show clear-cut changes in the cell cycle in valproate-treated ESS-1 cells. Significant changes (G_1 phase, $P = 0.06$; S phase, $P = 0.04$) were observed after valproate treatment for 24 h and this is further accentuated during the following 48 h. Exposure to valproate markedly increased the proportion of cells in the G_1 phase and decreased the proportion of cells in the S phase.

expression data based on the Academisch Medisch Centrum Human Transcriptome Map.[3] They showed that the average expression of HDAC2 in various normal tissues is slightly higher than that of HDAC1. Because differences in expression seem to be also tissue specific, they should be further investigated to elucidate their potential role in gene expression in normal and neoplastic cells of different origins. Our finding that neither localization nor expression levels varied markedly between proliferative, secretory, and inactive endometria suggests that HDAC1 and HDAC2 expression in nonneoplastic endometrium is not affected by the hormonal status.

Whereas, in normal endometrial stroma, the expression of both HDAC1 and HDAC2 was rather low, HDAC2 expression was moderately to highly increased in ~80% of endometrial stromal tumors analyzed. On the contrary, HDAC1 was about equally expressed in ESS and nonneoplastic endometrial stroma. About the scarcity of this malignancy and significance of our statistical analysis, we are highly convinced that the number of samples analyzed in our study is sufficient to recognize a well-defined trend

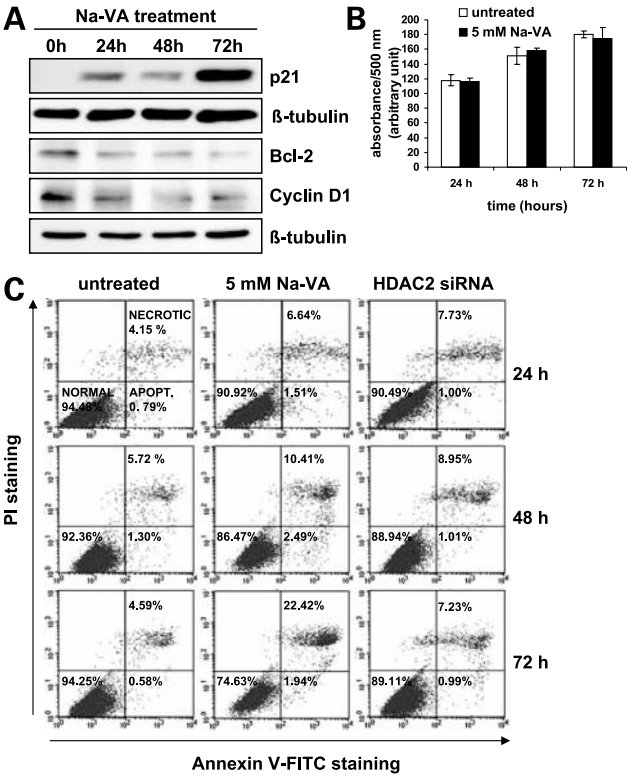

Figure 5. HDAC2 inhibition by valproate increases expression of $p21^{WAF1}$ in ESS-1 cells. **A,** after valproate treatment, ESS-1 cell lysates were analyzed by immunoblotting. An increase in the $p21^{WAF1}$ expression was visible 24 h after starting valproate treatment and further rose thereafter (72-h time point). The expression of antiapoptotic protein Bcl-2 and cell cycle – related protein cyclin D1 decreased continuously during the valproate treatment. **B,** caspase-3 activity in untreated and valproate-treated ESS-1 cells remains unchanged. **C,** fluorescence-activated cell sorting analysis after double staining with Annexin V-FITC and PI. ESS-1 cells were treated as indicated, stained using BD PharMingen Annexin V-FITC Apoptosis Detection kit II, and analyzed. Flow cytometric analysis clearly differentiates normal (living) cells with low Annexin V and low PI staining and some necrotic cells with high Annexin V and high PI staining. No apoptosis has been detected. Representative data of three experiments. Numbers are the percentage of all gated cells.

[3] http://www.amc.uva.nl.

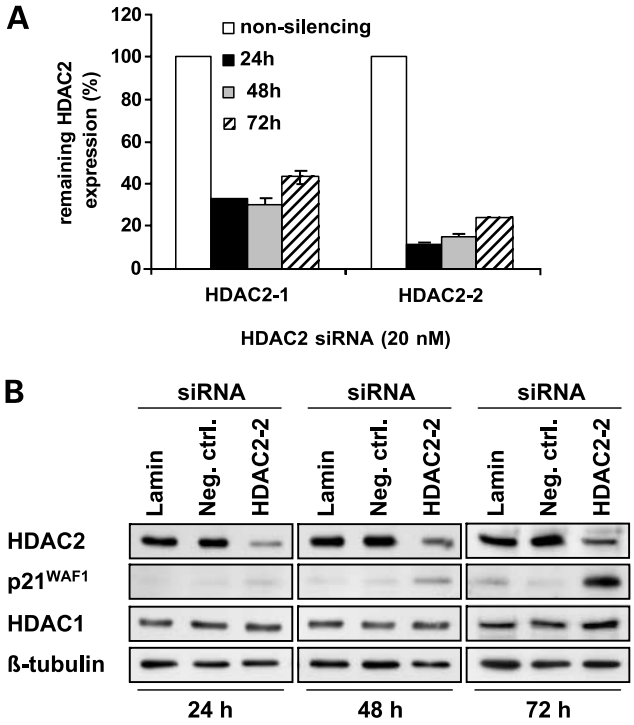

Figure 6. **A,** transfections with two different siRNAs directed against HDAC2 (HDAC2-1 and HDAC2-2; 20 nmol/L each) specifically decreased the RNA amount of HDAC2 in ESS-1 cells as shown by real-time reverse transcription-PCR. Data were normalized to the glyceraldehyde-3-phosphate dehydrogenase signal. As negative control, nonsilencing siRNA has been used. **B,** after siRNA transfection (data for HDAC2-2 are shown), specific HDAC2 decrement on the protein basis is clearly visible. Twenty-four h after siRNA transfection of ESS-1 cells, $p21^{WAF1}$ concentration is slightly increased and is further accentuated over time. HDAC1, which belongs to the class I HDACs, remains unaffected by siRNA transfection. β-Tubulin immunoblotting was used as a loading control.

of HDAC2 overexpression in ESS compared with nonneoplastic endometrial stroma. Both UES cases involved in this study showed moderate to strong HDAC2 reactivity, but the low number of samples does not allow making a final statement about HDAC2 expression in different subtypes of ESS (i.e., ESS versus UES).

Inhibition of HDACs results in hyperacetylation of histones, which is followed by relaxation of DNA conformation and finally by transcriptional activation of different genes. Thus far, several compounds belonging to structurally distinct classes have been identified as HDAC inhibitors. These compounds are able to induce differentiation, to arrest the cell cycle in G_1 and/or G_2 phase thus inhibiting cell proliferation, and to activate the apoptosis program in transformed or cancer cells. Major emphasis is currently placed on the elucidation of the underlying molecular mechanisms. In xenograft models, several HDAC inhibitors revealed antitumor activity with only few side effects. Antitumor effects have also been shown for valproate, a specific inhibitor of class I HDACs, which mainly affects HDAC2 (12). Although valproate inhibits HDAC2 in millimolar range and is therefore less efficient than, for example, trichostatin A, it inhibits HDAC2 more specifically and shows only mild side effects within the therapeutic range in animal experiments and in clinical practice (28). It has been shown that valproate might also inhibit HDAC1 in some cells (29), but this effect seems to be cell type specific because, in our cell model, HDAC1 expression remained unaffected during valproate treatment. Our *in vitro* experiments with the ESS-1 cell line showed that the valproate leads not only to the expected HDAC2 reduction but also to cell differentiation and pronounced G_1 arrest. This is additional evidence that induction of differentiation by valproate is not limited to hematopoietic cells but also includes other cell lines and solid tumors, as also reported by Goettlicher et al. (11). Because after valproate treatment there were no changes in the caspase-3 activity and Annexin V staining in ESS-1 cells, we postulate that the observed decrease in cell number is primarily caused by inhibition of cell proliferation and induction of cell differentiation rather than by apoptosis.

Cellular differentiation and cell cycle control are associated with an increase in $p21^{WAF1}$ expression (30, 31). As described by several authors, $p21^{WAF1}$ can influence G_1 progression in both positive and negative manner (32–34). $p21^{WAF1}$ is a cyclin-dependent kinase inhibitor, which, in complex with some other proteins, decreases cyclin-dependent kinase activity, thereby leading to growth arrest. Our results show that valproate causes time-dependent increment of $p21^{WAF1}$ expression in ESS-1 cells. There are data suggesting that, at least in colonic cancer cells, HDAC2 down-regulation does not influence $p21^{WAF1}$ expression (35). Our data imply that these processes are cell and tissue specific and depend on methods and/or substances used for HDAC inhibition. Using siRNA technology, we were able to show that $p21^{WAF1}$ expression in HDAC2-siRNA-transfected ESS-1 cells is increased in time-dependent manner, supporting the concept that, in our system, HDAC2 specifically contributes to cell cycle arrest. It still remains to be answered whether $p21^{WAF1}$ expression is controlled directly by inhibition of HDACs or by some other factors involved in these complex interactions. Additionally, we showed here that aberration of $p21^{WAF1}$ expression in ESS-1 cells was associated with a decrease of the antiapoptotic protein Bcl-2 and of cyclin D1, an adult proto-oncogene. Consequently, we observed arrest in the G_1 phase and decreased cell proliferation but no apoptosis. Similar effects were also reported for other HDAC inhibitors (36, 37). This suggests a potential of valproate and probably also other HDAC inhibitors in the therapy of ESS.

ESS are rare malignancies; therefore, one can hardly expect that specific therapeutic agents will be developed with the intention to cure them selectively. However, this should not preclude ESS as possible target of chemotherapeutic agents already in use or tested in clinical trials for treatment of other more common malignancies. Taken together, our findings suggest that HDACs might be considered as targets in the therapy of endometrial stromal tumors with valproate being particularly effective in this respect.

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References

1. Annunziato AT, Hansen JC. Role of histone acetylation in the assembly and modulation of chromatin structures. Gene Expr 2000;9:37–61.

2. Grunstein M. Histone acetylation in chromatin structure and transcription. Nature 1997;389:349–52.

3. Strahl BD, Allis CD. The language of covalent histone modifications. Nature 2000;403:41–5.

4. Wade AP. Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. Hum Mol Genet 2001;10:693–8.

5. Spencer VA, Davie JR. Role of covalent modifications of histones in regulating gene expression. Gene 1999;240:1–12.

6. Waterborg JH. Dynamics of histone acetylation *in vivo*. A function for acetylation turnover? Biochem Cell Biol 2002;80:363–78.

7. Struhl K. Histone acetylation and transcriptional regulatory mechanisms. Genes Dev 1998;12:599–606.

8. Gray SG, Ekstroem TJ. The human histone deacetylase family. Exp Cell Res 2001;262:75–83.

9. Mai A, Massa S, Rotili D, et al. Histone deacetylation in epigenetics: an attractive target for anticancer therapy. Med Res Rev 2005;25:261–309.

10. Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. J Cell Physiol 2000;184:1–16.

11. Goettlicher M, Minucci S, Zhu P, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 2001;20:6969–78.

12. Kramer OH, Zhu P, Ostendorff HP, et al. The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. EMBO J 2003;22:3411–20.

13. Huang BH, Laban M, Leung CHW, et al. Inhibition of histone deacetylase 2 increases apoptosis and $p21^{Cip1/WAF1}$ expression, independent of histone deacetylase 1. Cell Death Differ 2005;12:395–404.

14. Grimes CA, Jope RS. Cholinergic stimulation of early growth response-1 DNA binding activity requires protein kinase C and mitogen-activated protein kinase activation and is inhibited by sodium valproate in SH-SY5Y cells. J Neurochem 1999;73:1384–92.

15. Grignani F, De Matteis S, Nervi C, et al. Fusion protein of the retinoic acid receptor-α recruits histone deacetylase in promyelocytic leukemia. Nature 1998;391:815–8.

16. Lin RJ, Nagy L, Inoue S, Shao W, Miller WH, Jr., Evans RM. Role of the histone deacetylase complex in acute promyelocytic leukemia. Nature 1998;391:811–4.

17. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.

18. Marks PA, Richon VM, Breslow R, Rifkind RA. Histone deacetylase inhibitors as new cancer drugs. Curr Opin Oncol 2001;13:477–83.

19. Marks PA, Miller T, Richon VM. Histone deacetylases. Curr Opin Pharmacol 2003;3:344–51.

20. Timmermann S, Lehrmann H, Polesskaya A, Harel-Bellan A. Histone acetylation and disease. Cell Mol Life Sci 2001;58:728–36.

21. Wang C, Fu M, Mani S, Wadler S, Senderowicz AM, Pestell RG. Histone acetylation and the cell-cycle in cancer. Front Biosci 2001;6:610–29.

22. Zhu P, Martin E, Mengwasser J, Schlag P, Janssen KP, Gottlicher M. Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. Cancer Cell 2004;5:455–63.

23. Hrzenjak A, Tippl M, Kremser ML, et al. Inverse correlation of secreted frizzled-related protein 4 and β-catenin expression in endometrial stromal sarcomas. J Pathol 2004;204:19–27.

24. Hendrickson MR, Tavassoli FA, Kempson RL, McCluggage WG, Haller U, Kubik-Huch RA. Mesenchymal tumours and related lesions. In: Tavassoli FA, Devilee P, editors. World Health Organization classification of tumours: pathology and genetics of tumours of the breast and female genital organs. Lyon: IARC Press; 2003. p. 233–6.

25. Hrzenjak A, Moinfar F, Tavassoli FA, et al. JAZF1/JJAZ1 gene fusion in endometrial stromal sarcomas: molecular analysis by RT-PCR optimized for paraffin-embedded tissue. J Mol Diagn 2005;7:388–95.

26. Gunawan B, Braun S, Cortes MJ, Bergmann F, Karl C, Fuzesi L. Characterization of a newly established endometrial stromal sarcoma cell line. Int J Cancer 1998;77:424–8.

27. de Ruijter AJM, van Gennip AH, Caron HN, Kemp S, van Kuilenburg ABP. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J 2003;370:737–49.

28. Warrell RP, Jr., He LZ, Richon V, Calleja E, Pandolfi PP. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. J Natl Cancer Inst 1998;90:1621–5.

29. Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 2001;276:36734–41.

30. El Deiry WS, Tokino T, Waldman T, et al. Topological control of $p21^{WAF1/CIP1}$ expression in normal and neoplastic tissue. Cancer Res 1995;55:2910–9.

31. Parker SB, Eichele G, Zhang P, et al. p53-independent expression of $p21^{Cip1}$ in muscle and other terminally differentiating cells. Science 1995;267:1024–7.

32. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G_1-phase progression. Genes Dev 1999;13:1501–12.

33. Weinberg WC, Denning MF. $p21^{WAF1}$ control of epithelial cell cycle and cell fate. Crit Rev Oral Biol Med 2002;13:453–64.

34. Massague J. G_1 cell-cycle control and cancer. Nature 2004;432:298–306.

35. Zhu P, Huber E, Kiefer F, Goettlicher M. Specific and redundant functions of histone deacetylases in regulation of cell cycle and apoptosis. Cell Cycle 2004;3:115–7.

36. Takai N, Kawamata N, Gui D, Said JW, Miyakawa I, Koeffler P. Human ovarian carcinoma cells: histone deacetylase inhibitors exhibit antiproliferative activity and potently induce apoptosis. Cancer 2004;101:2760–70.

37. Lallemand F, Courilleau D, Sabah M, Redeuilh G, Mester J. Direct inhibition of the expression of cyclin D1 gene by sodium butyrate. Biochem Biophys Res Commun 1996;229:163–9.

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