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Histone deacetylase inhibitors enhance endothelial cell sprouting angiogenesis *in vitro*

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Abstract

Background—Treatment with histone deacetylases inhibitors (HDACi) such as valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) has been shown to improve survival following lethal insults through mechanisms that are incompletely understood. Cell survival under adverse conditions requires a healthy network of capillaries to ensure adequate oxygen delivery. Angiogenetic activation of endothelial cells to migrate and to form sprouts is associated with characteristic changes in gene expression profiles. As HDACi can modulate expression of various genes involved in angiogenic activity, we investigated the effect of these agents on capillary-like sprout-formation in this study.

Methods—Human umbilical vein endothelial cells (HUVECs) were cultured as multicellular spheroids within a type I collagen matrix, which promotes formation of sprouts resembling angiogenesis *in vitro*. Human umbilical vein endothelial cells (HUVECs) were cultured as multicellular spheroids within a type I collagen matrix, which promotes formation of sprouts (*in vitro* angiogenesis). Cells were cultured under following conditions: Control (no growth factors); VPA (1mM); VEGF (Vascular endothelial growth factor, 10 ng/ml); VPA+ VEGF; SAHA (5 mM) and SAHA+VEGF. After 24 hours of treatment the length of spheroid sprouting and cell migration was assessed quantitatively. The levels of acetylated histone H3, phosphor-ERK1/2 and β -Catenin in HUVECs were measured by Western blotting at 6 hours after treatment.

Results—High levels of acetylated histone H3 were detected in VPA and SAHA treated-groups. Compared to VEGF alone treated group ($2379 \pm 147.1 \mu\text{m}$), the spheroid sprouting was 1.7 times increased with VPA and VEGF combined treatment ($3996 \pm 192.5 \mu\text{m}$) ($p < 0.01$). Cell migrations did not show significant difference after addition of VPA, whereas SAHA suppressed migration. Expression of β -Catenin was significantly increased by VPA and SAHA treatments. Addition of VPA greatly enhanced expression of phosphor-ERK1/2.

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Conclusions—Exposure of HUVECs to VPA and SAHA increased the expression of β -catenin and enhanced spheroid sprout formation in vitro. Modulation of HDAC dependent pathways may offer a novel approach to alter angiogenic processes and provide a useful therapeutic target.

Keywords

Endothelial cells; angiogenesis; Histone deacetylase inhibitor

INTRODUCTION

The growth of the vascular system during development, tissue repair or in disease conditions involves the sprouting, migration and proliferation of endothelial cells in a process termed angiogenesis. Angiogenic activation of endothelial cells (EC) to migrate and to form sprouts is associated with characteristic changes in gene expression profiles (1). A balance between pro-angiogenic and anti-angiogenic growth factors and cytokines tightly controls angiogenesis (2).

The cellular actions of vascular endothelial growth factor (VEGF) need to be coordinated to guide vascular patterning during sprouting angiogenesis (3). A number of signal transduction molecules are activated or modified in response to VEGF stimulation, such as extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein kinase (4). In endothelial cells, ERK phosphorylation is known to promote cell survival and angiogenesis (5, 6). Wnt/ β -catenin pathway is involved in various aspects of EC biology and in angiogenesis, such as proliferation and vessel assembly (7). In a previous study we have reported that valproic acid, a histone deacetylase inhibitor, upregulates β -Catenin expression in vivo (8). β -Catenin activates the expression of vascular endothelial growth factor (VEGF)-A and VEGF-C in endothelial cells, and promotes capillary formation and endothelial cell differentiation into network structures (9).

Histone deacetylases (HDACs) constitute a family of enzymes that regulate gene transcription by modifying the acetylation level of histones and nonhistone proteins (10, 11). In particular, protein kinase D-dependent phosphorylation of HDAC5 and HDAC7 play an important role in vascular endothelial growth factor (VEGF)-induced angiogenesis in vitro (12, 13). Inhibition of HDAC causes histone hyperacetylation and modulates angiogenic genes expression. However, the precise mechanisms through which HDAC inhibitors (HDACi) regulate angiogenic sprouting processes is still unclear. In this study, therefore, we examined the effect of two different HDACi valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) on EC spheroid sprouting in relation to modulation of β -catenin expression.

MATERIALS AND METHODS

Cell culture

Pooled human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Verviers (Verviers) and cultured in endothelial basal medium (EGM-2; Lonza) with completed supplement kit or in M199 medium supplemented with 10 ng/mL VEGF (Sigma), 200 mM Glutamin, 5000 U/mL heparin, 1.25 μ g/mL thymidine, 5% FBS (Sigma), 22 and 100 μ g/mL penicillin and streptomycin.

Spheroid sprouting assay

The assay was performed as described previously (14) with following modifications. HUVEC spheroids were generated overnight in hanging-drop culture consisting of 500 cells

in EBM-2 medium, 10 % FCS and 20% methylcellulose (Sigma Biochemicals). Spheroids were embedded in collagen type I gels. The spheroid containing gel was rapidly transferred into prewarmed 24-well plates and allowed to polymerize (30 minutes), then 100 μ l endothelial basal medium with or without VEGF (10 ng/ml) was added on top of the gel. After 24 hours, the pictures were taken using microscope, and *In vitro* capillary sprouting was quantified by measuring the cumulative sprout length per spheroid using ImageJ software (National Institutes of Health, Bethesda, MD). To obtain a measure of the cumulative sprout length per spheroid, every sprout from 10 spheroids was assessed and from these data the mean cumulative sprout length per spheroid was calculated.

Cell migration assay: Scratch assay

Cells were seeded into 6 cm dishes previously labeled with a traced line. Once at confluence, cells were serum-starved in medium containing 0.5% FCS over night, and then scratch injury was applied using a cell scraper (width approximately 10 mm). After injury, the monolayer was gently washed with PBS. Cell migration from the edge of the injured monolayer was examined and photographed 24 hours after scratching. The numbers of endothelial cells that moved across the injury line were counted as a standard marker of migration. Data were analyzed as a percentage of migration in untreated endothelial cells.

Western blot analysis

HUVECs were harvested after 6 hours incubation with following conditions, 1) no VEGF, 2) 10 ng/ml VEGF, 3) 10 ng/ml + 1 mM VPA, 4) 10 ng/ml + 5 mM SAHA. Cell lysates were prepared using a whole lysis kit (BD), according to manufacturer's instructions. Protein concentration was determined by bicinchoninic acid (BCA) method, and equal amounts (15 μ g) of samples were loaded per lane. Proteins were detected using specific antibodies against acetylated histone H3 (Lys9) (07-352, Millipore), p-ERK1/2 (Thr202/Tyr204) (#4377, Cell Signaling), and β -Catenin (13-8400, Invitrogen) and were visualized by enhanced chemiluminescence Western blotting detection system kit (Amersham). The results were scanned and quantified using ImageJ software (NIH).

Immunocytochemistry

HUVECs were prepared in 12 well dishes one day prior to experiment. After 6 hours incubation with following conditions, 1) no VEGF, 2) 10 ng/ml VEGF, 3) 10 ng/ml + 1 mM VPA, 4) 10 ng/ml + 5 mM SAHA, the cells were fixed in 4% paraformaldehyde for 20 min, and washed three times in PBS. Cultures were incubated together with polyclonal anti-phospho-ERK antibody (Cell Signaling, #4376, 1:200) and monoclonal anti- β -catenin antibody (Invitrogen, #138400, 1:400) in blocking solution (1% BSA in PBS) at 4°C overnight. Cells were washed three times in PBS and incubated with the blocking solution including FITC-conjugated donkey anti-mouse IgG (Sigma) and TRITC-conjugated donkey anti-rabbit IgG (Sigma) at room temperature for 2 hours. Signals were examined using an Olympus microscope (BX71; Olympus) equipped with FITC and rhodamine filter set. All images were collected from microscope image system under the same conditions.

Statistical analyses

Data are presented as mean plus or minus SEM. As appropriate, one-way analysis of variance (ANOVA) and independent samples T test were performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California). A p value of ≤ 0.05 was considered to be significant.

RESULTS

HDAC inhibitor upregulated acetylated histone 3 level

We first examined the protein acetylation level in brain tissue after treatment with VPA or SAHA. The significantly increased protein level of acetylated histone 3 at lysine 9 (AcH3K9) was detected by western blot in both VPA and SAHA treated groups (Fig. 1).

Sprouting of EC spheroids is enhanced by HDAC inhibitor

Spheroids generated from HUVECs with a defined cell number of 500 cells per spheroid were embedded into type I collagen gels and incubated for 24 hours in the absence or presence of HDACi with VEGF. There was almost complete absence of spheroid sprouting in group without VEGF (cumulative sprout length $101 \pm 30 \mu\text{m}$) (Fig. 2A, Control). A slightly increased sprouting was showed in VPA ($337 \pm 52 \mu\text{m}$) (Fig. 2A, VPA) or SAHA ($706 \pm 122 \mu\text{m}$) (Fig. 2A, SAHA) alone treatment groups, but the increase did not reach statistical difference in VPA group. As expected, addition of VEGF significantly increased spheroid length to $1755 \pm 122 \mu\text{m}$ (Fig. 2A, VEGF). Compared to VEGF alone treated group, the treatment of VEGF combined VPA ($2777 \pm 189 \mu\text{m}$) (Fig. 2A, VEGF + VPA) or SAHA ($2417 \pm 150 \mu\text{m}$) (Fig. 2A, VEGF + SAHA) greatly enhanced the HUVECs sprouting further; the increase was highly significant (Fig. 2B).

EC migration enhanced by VPA but not by SAHA

To examine the functional effects of HDACi on HUVECs, we tested the in vitro scratch (wound) assay. Confluent monolayers HUVECs were subjected to scratch wounds, and then incubated with the HDACi for 24 hr. Compared to VEGF absence control group, EC migration increased to 168 % (Fig. 3A, Control and VEGF, Fig. 3B) after VEGF treatment. Compared with control and VEGF alone treated groups, the EC migrations did not show significant difference after addition of VPA (Fig. 3A, VPA and VEGF + VPA, Fig. 3B). Interestingly, with SAHA treatment, the cell migration was reduced in both groups with or without VEGF (Fig. 3A, SAHA and VEGF + SAHA, Fig. 3B).

Expressions of phosphor-ERK and β -catenin enhanced by HDAC inhibitor

With Western blot analysis for phosphor-ERK, only a weak band was detectable in VEGF absence control group, while that the signal increased to 1.6 folds with incubation of 10 ng/ml VEGF after 6 hours (Fig. 4). Compared with VEGF only treatment, phosphor-ERK expression was greatly enhanced (3.7 folds) with the addition of VPA (Fig. 4). In contrast, expression of phosphor-ERK was decreased to almost control level after addition of SAHA. Consistently the enhancement of phospho-ERK signals were detected in the immunocytochemistry (Fig. 6, red). No significant changes were detected in total ERK expressions.

At 6 hours after VEGF treatment, no significant difference was observed in the expression of β -catenin compared with the control. However, the expression of β -catenin increased significantly after treatment with VPA or SAHA (Fig. 5, 6, green). In the immunostaining, the increased β -catenin expressions were showed in the relatively large endothelial cells. No statistical difference was shown between VPA and SAHA treatments.

DISCUSSION

The current study suggests that HDACi may play an important role in the endothelial cell sprouting process. We observed that: (i) acetylation of histone 3 was clearly increased 6 hours after VPA or SAHA treatment, (ii) endothelial cell spheroid sprouting was greatly enhanced after treatment with VPA and SAHA, when combined with VEGF, (iii) VPA

treatment did not alter endothelial cell migration, whereas SAHA treatment slightly suppressed the migration, (iv) The expression of p-ERK was increased by VPA treatment, but not by SAHA, (v) the expression of β -catenin was increased after VPA and SAHA treatment.

The cellular actions of VEGF need to be coordinated to guide vascular patterning during sprouting angiogenesis (3). Our results show that VEGF promotes EC sprouting and migration (Fig. 2 and 3). Acetylation of chromatin proteins and transcription factors is part of a complex signaling system that is involved in the control of gene expression (15, 16). Ha and colleagues have reported that the nuclear export of HDAC5 regulates VEGF-induced MEF2 transcriptional activation in ECs (12). Another recent study reported that over expression of HDAC5 decreases sprout formation, indicating that HDAC5 is a negative regulator of angiogenesis (17). Consistently, we have shown in current study that spheroid sprouting was greatly promoted by addition of VPA or SAHA (Fig. 2). These observations indicate that HDAC inhibitor modulates angiogenic gene expression in the endothelial cells (18), which contributes to capillary-like sprouting of endothelial cells (17). We also discovered in this study that VPA and SAHA treatments upregulated the β -Catenin signal (Fig. 5). This is consistent with our previous studies that have demonstrated that VPA administration increases transcription of β -catenin gene as well the protein level (8). The canonical Wnt/ β -catenin pathway has been reported to be crucial for angiogenesis (19). This has also been confirmed in human umbilical vein ECs (HUVECs) infected with a kinase-mutant form of GSK3 β , leading to increased β -catenin signaling and downstream VEGF expression (9). In our study, we found that VPA or SAHA amplified the VEGF angiogenic response (Fig. 2), suggesting that this mechanism might be involved.

VPA is considered a first-generation HDAC inhibitor, it inhibits HDACs 1–5 and HDAC 7 in a concentration of 1 mM (20), which is within the range of patient's plasma levels (21). Whereas, the second-generation HDAC inhibitor SAHA inhibits HDACs 4–7 and HDACs 9–10 (22). A recent study implicates HDAC7 as a key modulator of endothelial cell migration, and HDAC7 silencing caused an inhibition of endothelial cell migration by increasing PDGF-B expression (23). In our study, SAHA treatment was associated with significantly suppressed VEGF induced cell migration, but not with VPA treatment (Fig. 3). On the other hand, only VPA treatment increased the expression of phosphor-ERK signal (Fig. 5). This is similar to previous studies that have reported that VPA increases phosphor-ERK expression (5, 20), which is likely induced by a mechanism independently of HDAC inhibition (5). The phosphorylation of ERK in endothelial cells is generally thought to be a proangiogenic event (24). Therefore, VPA induced phosphor-ERK may partially contribute to EC migration, which is consistent with our observation that VPA had higher migration level than SAHA group.

Although we showed that HDACi altered various parameters of angiogenesis *in vitro*, we cannot be entirely sure what mechanisms are specifically involved. Global inhibition of HDAC activity inhibits angiogenesis by modifying expression of proangiogenic and antiangiogenic factors (23, 25, 26). It is likely that accumulated multiple effects of HDAC inhibitor lead to different specific angiogenic response in different biological conditions and status. But how individual subtype of HDAC signals responses to different angiogenic processes? What is the long-term effect? Different inhibitors block very different classes of HDAC (27). We tested pan-HDACi in the current study. It is possible that using more specific inhibitors or a combination treatment would generate a more robust response. These questions are the focus of future studies. Our current study only provides proof of concept focused on EC sprouting.

In conclusion, we have shown that exposure to VPA and SAHA *in vitro* increase the expression of β -catenin, thereby enhancing HUVECs spheroid sprout formation. Additionally, as a proangiogenic signal, phosphor-ERK1/2 induced by VPA may contribute to an enhanced angiogenic response. Modulation of HDAC pathway may therefore be a useful therapeutic approach to alter angiogenic processes.

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Figure 1. Western blot for acetylated histone 3 at lysine 9 (AcH3K9) in ECs at 6 hours after treatment with VPA or SAHA. AcH3K9 level clearly increased after VPA or SAHA treatment. N=3.

Fig. 2A

Fig. 2B

Figure 2. Sprouting of HUVEC spheroid in collagen gels. After 24 h of treatment with HDAC inhibitor, spheroid sprouting was analyzed. (Control) Low baseline sprouting was observed. (VEGF) in the presence of VEGF, the sprouting increased significantly (2B). Increasing of HUVEC sprouting in VPA (VPA) or SAHA (SAHA) alone groups were not significant (2B). Treatment of VEGF combined VPA (VEGF + VPA) or SAHA (VEGF + SAHA) greatly enhanced the HUVECs sprouting (2B). ** $p < 0.01$.

Fig. 3A

Fig. 3B

Figure 3.

Migration of HUVECs in an *in vitro* scratch assay. Compared with control and VEGF alone treated groups, the EC migrations did not show significant difference after addition of VPA (VPA and VEGF + VPA, 3B). With SAHA treatment, the cell migration was reduced in both groups with or without VEGF (SAHA and VEGF + SAHA, 3B). * $p < 0.05$.

Figure 4. Western blot for p-ERK and ERK at 6 hours after treatment with VPA or SAHA. Uper panel and bar graph: Presence of VEGF significantly induced p-ERK expression, and the expression of p-ERK greatly increased by addition of VPA. However, the expression of p-ERK was decreased to control level by addition of SAHA. Lower panel: No significant changes were detected in total ERK expressions. * $p < 0.05$.

Figure 5.

Western blot for β-catenin at 6 hours after treatment with VPA or SAHA. Compared with the control, no significant difference was observed after VEGF treatment. The expression of β-Catenin became increased significantly after treatment with VPA or SAHA. * $p < 0.05$.

Figure 6.

Double immunostaining for p-ERK (red) and β -catenin (green) at 6 hours after the treatment with VPA or SAHA. VEGF induced p-ERK signal was greatly enhanced by the addition of VPA. Enhanced β -catenin immunoreactivity was detected in the cells treated with VPA or SAHA. Scale bar: 20 μ m.