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SAHA SUPPRESSES PERITONEAL FIBROSIS IN MICE

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HDACI FOR PERITONEAL FIBROSIS

IO et al.

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[ABSTRACT]

Objective: Long-term peritoneal dialysis causes peritoneal fibrosis in submesothelial areas. However, the mechanism of peritoneal fibrosis is unclear. Epigenetics is the mechanism to
induce heritable changes without any changes in DNA sequences. Among epigenetic modifications, histone acetylation leads to the transcriptional activation of genes. Recent studies indicate that histone acetylation is involved in the progression of fibrosis. Therefore, we examined the effect of suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, on the progression of peritoneal fibrosis in mice.

Methods: Peritoneal fibrosis was induced by the injection of chlorhexidine gluconate (CG) into the peritoneal cavity of mice every other day for 3 weeks. SAHA, or a dimethylsulfoxide and saline vehicle, was administered subcutaneously every day from the start of the CG injections for 3 weeks. Morphologic peritoneal changes were assessed by Masson's trichrome staining, and fibrosis-associated factors were assessed by immunohistochemistry.

Results: In CG-injected mice, a marked thickening of the submesothelial compact zone was observed. In contrast, the administration of SAHA suppressed the progression of submesothelial thickening and type III collagen accumulation in CG-injected mice. The numbers of fibroblast-specific protein-1–positive cells and vxa-smooth muscle actin–positive cells were significantly decreased in the CG + SAHA group compared to that of the CG group. The level of histone acetylation was reduced in the peritoneum of the CG group, whereas it was increased in the CG + SAHA group.

Conclusions: Our results indicate that SAHA can suppress peritoneal thickening and fibrosis in mice through up-regulation of histone acetylation. These results suggest that SAHA may have therapeutic potential for treating peritoneal fibrosis.

KEY WORDS: Suberoylanilide hydroxamic acid, SAHA, histone deacetylase inhibitor, peritoneal fibrosis, chlorhexidine gluconate.
Although peritoneal dialysis (PD) is a beneficial treatment for patients with end-stage renal diseases, long-term PD causes morphologic and functional changes in the peritoneum (1). Characteristic pathologic findings in the peritoneum, related to long-term PD, include marked peritoneal fibrosis and massive accumulation of collagen in submesothelial areas (2, 3). In particular, some patients with peritoneal fibrosis develop encapsulating peritoneal sclerosis (EPS) associated with high mortality, which is one of the most critical complications in PD. However, the mechanism underlying peritoneal fibrosis in PD patients remains poorly understood and no effective therapy is available at present. To investigate the mechanism of peritoneal fibrosis and effective therapy, animal models of peritoneal fibrosis are used. Among them, chlorhexidine gluconate (CG) is often used to induce peritoneal fibrosis (4). This is a reversible model (5), therefore, repeat injections of CG need to be continued during the course of the experiment to induce persistent chemical irritation and tissue damage (6). In fact, following injection of CG, many pathologic findings in the peritoneum of PD patients, including increased expression of collagen III, xxa-smooth muscle actin (xxaSMA), macrophage infiltration, were also observed in previous studies (4,7). These similarities in alterations of the peritoneal membranes between experimental CG models and human PD patients strongly suggest that the CG model is a reasonable candidate model to examine the efficacy of various potential therapeutic reagents for regulating peritoneal sclerosis.

Epigenetics is defined as the mechanism to induce heritable change in the pattern of gene expression without any changes in DNA sequences (8). Epigenetic regulations depend on DNA methylation, histone modification, RNA interference, and so on. It has been shown that epigenetic modifications are involved in various human diseases, for example, cancer, hypertension, diabetes, and atherosclerosis (8). Moreover, a recent study has revealed that hypermethylation of rat sarcoma (RAS) protein activator like-1 (RASAL1) is associated with
the perpetuation of fibroblast activation and fibrogenesis in the kidney, leading to glomerular and interstitial fibrosis (9).

Among epigenetic regulation, global histone acetylation alters chromatin structure and regulates the dynamics of gene expression. The level of acetylation reflects a balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) protein families (10). Recently, it was demonstrated that HDACs are related to the progression of tissue fibrosis in multiple organs including kidney, heart and lung (11). A report showed that HDAC levels were increased and histone acetylation was decreased in kidneys injured by unilateral ureteral obstruction (UUO), which is a model of fibrosis (12). In addition, it has been reported that HDAC inhibitors (HDACi) are potentially therapeutic for fibrotic disorders because they have anti-fibrotic activity. Among HDACi, suberoylanilide hydroxamic acid (SAHA) has nonspecific HDAC-inhibition activity, affecting all classes of HDACs, and has received approval from the US Food and Drug Administration for treatment of refractory cutaneous T-cell lymphoma (13). SAHA exhibits relatively low toxicity towards normal cells compared to other HDACi (13). It was reported that SAHA attenuated mesangial collagen IV deposition in a mouse model of diabetic nephropathy (14). In addition, SAHA also attenuated collagen deposition in the ventricle and cell infiltration throughout the interstitium; thereby, improving cardiovascular remodeling in deoxycorticosterone acetate-salt hypertensive rats (15). Furthermore, previous reports indicate that inflammation plays an important role in the progression of organ fibrosis, and angiogenesis is associated with the development of fibrosis (16,17). It has been also reported that SAHA suppressed inflammation and angiogenesis (18,19). Therefore, we used SAHA as an HDACi in our present study.
Generally, increased levels of histone acetylation are associated with transcriptional activation (20). Some reports indicate that the expression of fibrosis inhibitory factors can be increased by promoting histone acetylation (21,22). As the effects of inhibitory factors for fibrosis, Yu et al. reported that bone morphogenetic protein 7 (BMP-7) and hepatocyte growth factor (HGF) ameliorated high-glucose–induced epithelial-to-mesenchymal transition (EMT) of peritoneal mesothelial cells (23). Inhibitors of DNA binding/differentiation 2 (Id2), which plays a role in inhibiting TGF-xxb–induced EMT in human renal epithelial cells, were shown to be upregulated, together with BMP-7, by HDACi (21).

In the present study, we hypothesized that HDACi could inhibit peritoneal fibrosis by promoting histone acetylation, following the increase of a fibrosis inhibitory factor; we focused on BMP-7. In this study, we investigated first the changes in histone acetylation in the CG-induced fibrotic peritoneum of mice. Then, we determined the effects of HDACi on fibrotic changes induced by CG.

Our results raise the possibility that HDACi may be a useful therapeutic agent for attenuating the progression of peritoneal fibrosis.
METHODS

ANIMAL MODELS

The experiments described in this study were conducted on 10-week-old male ICR mice (Japan SLC Inc., Shizuoka, Japan). They were housed in standard rodent cages in a light- and temperature-controlled room in the Biomedical Research Center, Center for Frontier Life Sciences, Nagasaki University. They had free access to laboratory food and tap water. The experimental protocol was inspected by the Animal Care and Use Committee of Nagasaki University, and approved by the President of Nagasaki University (Approval number: 1004050846).

EXPERIMENTAL PROTOCOL

Peritoneal fibrosis was induced by intraperitoneal injection of 0.1% CG in 15% ethanol dissolved in saline, as described previously, but with a slight modification (16,24). Mice received injections of 0.1% CG in 15% ethanol dissolved in saline (CG) or 15% ethanol dissolved in saline only at a dose of 0.3 mL/body into the peritoneal cavity every other day for 3 weeks. SAHA (10009929; Cayman Chemicals, MI, USA), which was dissolved in a vehicle (dimethylsulfoxide:saline = 1:1), or a vehicle alone, was administered subcutaneously, as previously reported (25). SAHA was administered at a dose of 25 mg/kg body weight every day for 3 weeks. Mice were assigned to 4 groups: (1) mice injected 15% ethanol dissolved in saline intraperitoneally and vehicle subcutaneously (n = 5), defined as the control group; (2) mice injected 15% ethanol dissolved in saline intraperitoneally and SAHA subcutaneously (n = 5), defined as SAHA group; (3) mice injected CG intraperitoneally and vehicle subcutaneously (n = 10), defined as CG group; and (4) mice injected CG intraperitoneally and SAHA subcutaneously, defined as CG + SAHA group (n = 10). The
dose and interval for administration of SAHA were selected on the basis of pilot studies conducted to determine the effects of different doses on the thickness of the submesothelial area. SAHA at a dose of 12.5 mg/kg body weight did not reduce peritoneal thickness compared with that in non-treated CG mice. SAHA at doses of 25 or 50 mg/kg body weight reduced peritoneal thickness to the same degree. Therefore, we selected 25 mg/kg body weight in the present study. No mice were excluded from the study once they were treated.

Mice were sacrificed at day 21 after the first CG injection, and peritoneal tissues were dissected out carefully. To avoid direct damage to the peritoneum due to repeated injections, CG was injected at the lower part of the peritoneum, whereas the upper portion of the parietal peritoneum was used for the examination. The tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) immediately after sampling, and were embedded in paraffin. For morphological examination of the peritoneum, 4-xxmm thick, paraffin-embedded tissues were stained with Masson’s trichrome staining.  

**IMMUNOHISTOCHEMISTRY**

Paraffin-embedded tissue sections were stained immunohistochemically using an indirect method (26). The following antibodies were used for immunohistochemistry: (1) rabbit anti-type III collagen antibody diluted 1:400 (LB-1393; LSL Co., Tokyo, Japan); (2) rabbit anti-human phosphorylated-Smad2/3 antibody diluted 1:50 (sc-11769-R; Santa Cruz Biotechnology, Santa Cruz, CA, USA); (3) rabbit anti-human fibroblast-specific protein-1 (FSP-1) antibody diluted 1:100 (A5114; DAKO, Glostrup, Denmark); (4) mouse anti-human xxaSMA antibody diluted 1:50 (A2547; Sigma-Aldrich, St.Louis, US); (5) rat anti-mouse F4/80 antibody diluted 1:50 (MCA 497; Serotec, Kidlington, UK); and (6) goat anti-mouse PECAM/CD31 antibody diluted 1:50 (sc-1506; Santa Cruz Biotechnology).
After deparaffinization, the sections were treated with 0.3% H₂O₂ for 20 min to inactivate endogenous peroxidase activity. For the immunohistochemical analysis of phosphorylated-Smad2/3 and CD31, the sections were treated in a microwave oven (MI-77; Azumaya, Tokyo, Japan) at 95°C for 5 or 15 min in 10 mmol/L citrate buffer (pH 6.0), before H₂O₂ treatment for antigen retrieval. For the immunohistochemical analysis of type III collagen, FSP-1, F4/80 and xxaSMA, the sections were treated with proteinase K (P2308; Sigma, St Louis, MO) for 15 min at 37°C, before H₂O₂ treatment for antigen retrieval. The sections were incubated for 30 min with a blocking solution (5% normal goat serum, 5% fetal calf serum, 5% bovine serum albumin (BSA), and 20% normal swine serum in PBS) at room temperature. The sections were then exposed to the primary antibody, which was diluted in the blocking buffer. For phosphorylated-Smad2/3 and CD31 staining, the sections were stained with the avidin–biotin complex by using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) after reacting with the primary antibody overnight at 4°C. For xxaSMA, the sections were exposed to a complex of anti-xxaSMA antibody and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin antibody (P0161; Dako) diluted 1:50 for 30 min at room temperature. Then, sections were incubated with the Envision⁺ System-HRP Labelled Polymer Anti-Rabbit (K4002, Dako) for 30 min at room temperature. For type III collagen, FSP-1 and F4/80, after reacting with the primary antibodies for 1 h or overnight at room temperature, the sections were incubated with HRP-conjugated swine anti-rabbit immunoglobulin antibody (P0399; Dako) or rabbit anti-rat immunoglobulin antibody (P0450; Dako), diluted 1:50, for 30 min at room temperature. The reaction sites were visualized by treating the sections with H₂O₂ and 3,3′-diaminobenzidine tetrahydrochloride (26). Finally, the sections were counterstained with methyl green and mounted. For all specimens, negative controls were prepared using normal IgG instead of the primary antibody.

IMMUNOFLUORESCENCE STAINING
The following antibodies were used for immunofluorescence staining: (1) rabbit anti-human acetyl-histone H3 (Lys9) antibody, diluted 1:100 (#9671; Cell Signaling Technology, Danvers, MA, USA), and (2) goat anti-human BMP-7 antibody diluted 1:25 (SC-9305; Santa Cruz Biotechnology).

After deparaffinization, the sections were incubated for 30 min or 1 h with a blocking solution consisting of 500 μg/mL normal goat IgG and 1% BSA in PBS at room temperature. The sections were then incubated with the primary antibodies, which were diluted in the same blocking solution. The sections were incubated with either Alexa Fluor dye (Molecular probes, Inc; Eugene, OR, USA) 594-labeled goat anti-rabbit IgG (A11012; Invitrogen, Paisley, UK) or Alexa Fluor dye 488-labeled rabbit anti-goat IgG (A11078; Invitrogen) for 1 h, stained with 4,6-diamidino-2-phenylindole (DAPI) for 1 min, and mounted. The sections were analyzed using a confocal laser scanning microscope (LSM 5 PASCAL; Carl Zeiss Co., Ltd., Obekochen, Germany), and the images were obtained. Normal rabbit or goat IgG was used as a negative control.

MORPHOMETRIC ANALYSIS

To assess the extent of peritoneal thickening, we used digitized images and image analysis software (Lumina vision; MITANI Corporation, Tokyo, Japan). We measured the thickness of the submesothelial zone above the abdominal muscle in cross-sections of the abdominal wall. The diameter of the × 200 magnification is 1 mm. For each sample, 5 such areas were selected and type III collagen-positive areas were determined. In each peritoneal sample, the numbers of phosphorylated-Smad2/3-positive cells, cells expressing FSP-1, αSMA, F4/80 or BMP-7, CD31-positive vessels and submesothelial cells were counted in 5 fields at × 200 magnification.
QUANTITATIVE RT-PCR

Quantitative real-time RT-PCR was performed as previously described (27). RNA was isolated from the peritoneum with 600 μL RNA lysis buffer using an RNeasy Mini Kit (QIAGEN, Tokyo, Japan). Complementary DNA was reverse-transcribed using a high-capacity reverse transcription kit (Applied Biosystems, Life Technologies Japan Ltd., Tokyo, Japan). Approximately 25 ng cDNA was used as a template in reactions with 0.5 mM of forward and reverse primers and SYBR Green (Applied Biosystems). Reactions were carried out using the StepOne Plus Real-Time PCR system, and quantitative comparisons were obtained using the xxCt method (Applied Biosystem). The following primers were used in reactions: xxb-actin, 5′-CATCCGTAAAGACCTCTATGCCAC-3′ (forward) and 5′-ATGGAGCCACCATCCACA-3′ (reverse) (28); BMP-7, 5′-CACTCCCTCCTCAACCCTCGGCA-3′ (forward) and 5′-TAGAGGCATCATAGGCCAGGTGCCC-3′ (reverse) (29); xxa1 chain of type I collagen (COLIxxa1), 5′-TGGTCCTGCCGGTCCTCCTG-3′ (forward) and 5′-ACACATTGGGGGTAGGAACA-3′ (reverse) (30); Fibronectin, 5′-GAAACGGCCAACTCCGTCAC-3′ (forward) and 5′-TGCCAGAACGCACACTGAGG-3′ (reverse) (31); Connective tissue growth factor (CTGF), 5′-TGCCAGAACGCACACTGAGG-3′ (forward) and 5′-ATTGCCCTCCCGGTTACAC-3′ (reverse) (31).

STATISTICAL ANALYSIS

Data were expressed as mean ± standard error mean (SEM). Differences among groups were examined for statistical significance by using repeated measures analysis of variance (Bonferroni/Dunn test). A p value of < 0.05 denoted statistically significant difference.
RESULTS

SAHA PREVENTED THE PERITONEAL FIBROSIS

Morphological changes were assessed using Masson’s trichrome staining (Figure 1). In the normal mice, the peritoneal tissue comprised a peritoneal mesothelial monolayer and an exiguity of connective tissues under the mesothelial layer (Figure 1A). In the control group, the peritoneal tissues were almost normal and without thickening of the submesothelial zone (Figure 1B). On the other hand, the peritoneal samples from the mice in the CG group had a marked thickening of the submesothelial compact zone and a heavy infiltration of various cells at day 21 (Figure 1C). However, subcutaneous injection of SAHA prevented the progression of submesothelial thickening and reduced the number of cells in the CG + SAHA group (Figure 1D). A significant difference between the CG group and the CG + SAHA group was seen in the area of the submesothelial compact zone (p < 0.05, Figure 1E). The thickening of the peritoneum was not affected following repeated injections of SAHA compared with that of the control group (data not shown).

For the analysis of collagen deposition, we conducted the immunohistochemistry for type III collagen at day 21. As shown in Figure 2, in the control group, type III collagen expression in the submesothelial compact zone was marginal and equivalent to that of normal mice (Figure 2A). In the CG group, type III collagen was diffusely expressed in the submesothelial compact zone, whereas this expression was clearly decreased in the CG + SAHA group (Figure 2B, 2C). When we examined the signal for type III collagen quantitatively, the expression of type III collagen was higher in the CG group than in the control group, but was significantly lower in the CG + SAHA group than in the CG group (Figure 2E).
The expression of FSP-1, which is a marker of fibroblasts, was examined by immunohistochemistry. A few FSP-1–positive cells were in the peritoneum of the control group (data not shown). In the CG group, the number of FSP-1–positive cells increased markedly compared to that in the control group, and the treatment of SAHA inhibited these expressions significantly (Figure 3A – 3D). Furthermore, we examined the expression of α-SMA, which is a marker of myofibroblasts, by immunohistochemistry. In the control group, a few α-SMA–positive cells were found in the peritoneum (data not shown). The CG group showed a significant increase of α-SMA–positive cells, and in the CG + SAHA group, the number of α-SMA–positive cells was markedly decreased (Figure 3E – 3H).

SAHA SUPPRESSED TRANSFORMING GROWTH FACTOR (TGF)-XXB SIGNALING

We examined TGF-β signaling by performing immunohistochemistry for phosphorylated-Smad2/3, which is known as a mediator of TGF-β signaling in regulation of the fibrotic response (Figure 4). In the control group, very few phosphorylated-Smad2/3–positive cells were observed in the peritoneum (data not shown). Treatment with CG significantly increased the number of phosphorylated-Smad2/3–positive cells in the thickened submesothelial compact zone compared with that in the control group (Figure 4A). Compared with that in the CG group, the number of positive cells was markedly reduced in the CG + SAHA group (Figure 4B, 4D).

Next, we investigated the expression of TGF-β-dependent pro-fibrotic genes in the peritoneum tissue using real-time RT-PCR (Figure 4E – 4G). In the CG group, COLIα1, fibronectin and CTGF mRNA levels were higher than the control group. On the other hand, COLIα1, fibronectin and CTGF mRNA levels showed a tendency to be lower in the SAHA group.
SAHA INCREASED THE LEVEL OF HISTONE ACETYLATION

Using immunofluorescence technique, we examined the level of H3K9 acetylation, which is representative of histone acetylation, in the cells of the submesothelial compact zone (Figure 5). H3K9 acetylation was low in the cells of the peritoneum of the control group (Figure 5A). In the CG group, moderate H3K9 acetylation was observed in the thickened submesothelial compact zone. In contrast, in the CG + SAHA group, the level of H3K9 acetylation tended to be higher than that in the CG group (Figure 5B, 5C).

SAHA INCREASED THE EXPRESSION OF BMP-7

Next, we focused on BMP-7 as an inhibitory factor for fibrosis that is upregulated by the administration of HDACi (21). As shown in Figure 6, we examined the ratio of BMP-7–positive cells to all submesothelial cells in the submesothelial compact zone. In the control group, a few BMP-7–positive cells were detected in the peritoneum (Figure 6A). Although in the CG group some BMP-7–positive cells were in the submesothelial compact zone, the ratio of BMP-7–positive cells was significantly increased in the CG + SAHA group compared with that in the CG group (Figure 6B – 6E). The BMP-7 mRNA level in the CG + SAHA group was significantly higher than levels in the CG group (Figure 6F).

SAHA SUPPRESSED INFLAMMATION AND ANGIOGENESIS

To examine anti-inflammatory and anti-angiogenic effects by SAHA in peritoneal tissues, we used immunohistochemistry for F4/80 and CD31, which are markers of macrophages or dendritic cells, and vessels respectively (Figure 7A – 7H). In the peritoneum of the CG + SAHA group, the numbers of both F4/80–positive cells and CD31–positive vessels were less than those of the CG group. We identified anti-inflammatory and anti-angiogenic effects of SAHA in models of peritoneal fibrosis.
DISCUSSION

Our results indicated that SAHA effectively ameliorated peritoneal fibrosis. In addition, we confirmed that SAHA, as an HDAC inhibitor, promoted histone acetylation in the submesothelial compact zone of the peritoneum. Our study supports the existing data and reveals the beneficial effects of HDACi on fibrotic diseases. Previous studies described the effects of HDACi in various animal models, including heart failure (9), unilateral ureteral obstruction (8), and diabetic nephropathy (32). However, to our knowledge, this is the first study to investigate whether HDACi have inhibitory effects on peritoneal fibrosis.

Because increased levels of histone acetylation are usually associated with transcriptional activation (20), we speculated that the promotion of histone acetylation could be an effective strategy for the suppression of peritoneal fibrosis. In the present study, we focused on the expression of BMP-7 among fibrosis inhibitory factors. It is an endogenous protein that belongs to the TGF-xxb superfamily and is known to prevent the development of fibrosis in various organs (33–35). BMP-7 has been suggested to suppress fibrosis by inhibiting TGF-xxb1 synthesis (36) and by controlling TGF-xxb signaling via blocking the nuclear translocation of phosphorylated Smad2/3 (37). In fact, the association between BMP-7 and histone acetylation has been already shown (22). Marumo, et al. reported that marked induction of BMP-7 and the increase of strongly positive cells for acetylated histone were observed simultaneously in the proximal tubular cells during the recovery phase following renal ischemic reperfusion in mice (22). We also confirmed that the number of BMP-7–positive cells increased in the submesothelial compact zone in the CG + SAHA group compared with that in the CG group, and our results suggested that BMP-7 is involved in the suppression of peritoneal fibrosis, where it inhibits TGF-xxb signaling. Although we do not yet understand the difference observed between the levels of BMP-7 mRNA and protein level...
in the control group, this difference could have been caused by post-transcriptional regulation of protein synthesis, including mRNA stability and translational efficiency (38).

In this study, the peritoneal samples from mice in the CG group had a marked thickening of the submesothelial compact zone and a heavy infiltration of various cells at day 21. The results of immunohistochemistry for F4/80, FSP-1 and αSMA suggested that a heavy infiltration of various cells in the peritoneum were mainly macrophages or dendritic cells, and myofibroblasts. The SAHA group had fewer inflammatory cells and myofibroblasts, in addition to displaying an inhibition of angiogenesis. Therefore, SAHA might have ameliorated peritoneal fibrosis by not only increasing the expression of BMP-7 but also inhibiting inflammation or angiogenesis.

Our study has some limitations for clinical application. First, there are some differences between CG-induced peritoneal fibrosis and human peritoneal fibrosis. CG models induce a chemical irritant but the etiology of human EPS is more complicated (4). However, most of the histological changes in CG models are similar to peritoneal fibrosis in humans — injection of CG results in progression of fibrosis and thickening of the peritoneum similar to human peritoneal fibrosis. Second, we started the administration of SAHA and CG simultaneously; the effect of SAHA as a therapeutic intervention subsequent to starting CG is unknown. Therefore, we anticipate further investigation into the therapeutic effect of SAHA. Third, the dosage of SAHA used in our study was almost 3 times that used clinically in the treatment of human cutaneous T cell lymphoma. There were no side effects such as liver or kidney dysfunction and the increase in mortality between the CG + SAHA group and the control group in our investigation. The effective and safe dose of SAHA for mice may be different from that used for humans. Further studies are needed to clarify whether SAHA can
be used to reduce peritoneal fibrosis in PD patients and to examine the suitable dose of SAHA for humans.

In conclusion, we have shown for the first time that SAHA inhibits the progression of peritoneal fibrosis in a mouse CG-induced peritoneal fibrosis model. Our result might be consistent with our hypothesis that SAHA inhibits the expression of fibrosis-associated factors via induction of BMP-7. Thus, SAHA represents a novel potential candidate for the treatment of peritoneal fibrosis.

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DISCLOSURES

All authors state that they have no conflicts of interest to declare.
REFERENCES


Figure 1 — The result of Masson's trichrome staining of peritoneal tissues. (A) In normal mice, the monolayer of mesothelial cells covered the entire surface of the peritoneum. (B) In the control group, peritoneal tissues were almost normal without thickening of the submesothelial zone. (C) The peritoneal tissues of the mice in the CG group showed marked thickening of the submesothelial compact zone and presence of numerous cells. (D) SAHA significantly prevented the progression of submesothelial thickening. (A–D), magnification 200×; bars indicate the thickness of the submesothelial compact zone. (E) Bar graph showing the thickness of the submesothelial compact zone. Data are expressed as mean±SEM; * represents p<0.05.

Figure 2 — The result of the immunohistochemical analysis for type III collagen. (A) In the control group, type III collagen expression in the submesothelial compact zone was equivalent to that of normal mice. (B) In the CG group, type III collagen was diffusely expressed in the submesothelial compact zone. (C) Type III collagen expression was clearly decreased in the CG+SAHA group. (D) The peritoneal tissue of CG+SAHA group was incubated with normal IgG instead of type III collagen antibody as a negative control. (A–D), magnification 200×; bars indicate the thickness of the submesothelial compact zone. (E) Bar graph showing the positive areas for type III collagen. Data are expressed as mean±SEM; * represents p<0.05.

Figure 3 — The result of the immunohistochemical analysis for FSP-1 and xxaSMA. (A) The number of FSP-1–positive cells increased markedly in the CG group. (B) The expression of
FSP-1 was inhibited in the CG+SAHA group. (C) The peritoneal tissue of CG+SAHA group was incubated with normal IgG instead of FSP-1 antibody as a negative control. (A-C), magnification 200×; bars indicate the thickness of the submesothelial compact zone. (D) Bar graph showing the number of FSP-1–positive cells. Data are expressed as mean ± SEM; * represents $p<0.05$. (E) In the CG group, the number of xxaSMA–positive cells was increased. (F) The number of xxaSMA–positive cells was reduced in the CG+SAHA group. (G) The peritoneal tissue of CG+SAHA group was incubated with normal IgG instead of xxaSMA antibody as a negative control. (E–G), magnification 200×; bars indicate the thickness of the submesothelial compact zone. (H) Bar graph showing the number of xxaSMA–positive cells. Data are expressed as mean±SEM; * represents $p<0.05$.

Figure 4 — The results of immunohistochemical analysis for phosphorylated Smad2/3 and TGF-b dependent profibrotic genes expression.

(A) In the CG group, a number of phosphorylated-Smad2/3 positive cells were observed in thickened peritoneal compact zone. (B) These numbers were significantly decreased in the CG+SAHA group. (C) The peritoneal tissue of CG+SAHA group was incubated with normal IgG instead of phosphorylated Smad2/3 antibody as a negative control. (A–C), magnification 200×; bars indicate the thickness of the submesothelial compact zone. (D) Bar graph showing the number of phosphorylated-Smad2/3–positive cells. Data are expressed as mean±SEM; * represents $p<0.05$. (E) $COL1xxa1$ expression levels were measured by quantitative RT-PCR relative to xxb-actin controls. Values are relative to the control group±SEM; * represents $p<0.05$. (F) Fibronectin expression levels were measured by quantitative RT-PCR relative to xxb-actin controls. Values are relative to the control group±SEM; * represents $p<0.05$. (G)
CTGF expression levels were measured by quantitative RT-PCR relative to xxb-actin controls. Values are relative to the control group±SEM; * represents $p<0.05$.

Figure 5 — The result of the immunofluorescence staining for H3K9 acetylation. (A) The control group showed low level of H3K9 acetylation in the cells of the peritoneum. (B) In the CG group, moderate level of H3K9 acetylation was evident by the thickened submesothelial compact zone. (C) In the CG+SAHA group, the level of H3K9 acetylation tended to be higher than that in the CG group. (D) The peritoneal tissue of CG+SAHA group was incubated with normal IgG instead of H3K9 acetylation antibody as a negative control. (A–D), magnification 200×; bars indicate the thickness of the submesothelial compact zone.

Figure 6 — The result of the immunofluorescence staining for BMP-7. (A) In the control group, very few BMP-7–positive cells were shown in the peritoneum. (B) The CG group showed some BMP-7–positive cells in the submesothelial compact zone. (C) The BMP-7–positive cells were increased in the CG+SAHA group. (D) The peritoneal tissue of CG+SAHA group was incubated with normal IgG instead of BMP-7 antibody as a negative control. (A–D), magnification 200×; bars indicate the thickness of the submesothelial compact zone. (E) Bar graph showing the BMP-7–positive cells. Data are expressed as mean±SEM; * represents $p<0.05$. (F) BMP-7 expression levels were measured by quantitative real-time RT-PCR relative to xxb-actin controls. Values are relative to the control group±SEM; * represents $p<0.05$. 
Figure 7 — The result of the immunohistochemical analysis for F4/80 and CD31. (A) In the CG group, a number of F4/80–positive cells were observed in thickened peritoneal compact zone. (B) These numbers were significantly decreased in the CG+SAHA group. (C) The peritoneal tissue of CG+SAHA group was incubated with normal IgG instead of F4/80 antibody as a negative control. (A–C), magnification 200×; bars indicate the thickness of the submesothelial compact zone. (D) Bar graph showing the number of F4/80–positive cells. Data are expressed as mean±SEM; * represents \( p < 0.05 \). (E) The number of CD31–positive vessels increased markedly in the CG group. (F) The number of CD31–positive vessels was reduced in the CG+SAHA group. (G) The peritoneal tissue of CG+SAHA group was incubated with normal IgG instead of CD31 antibody as a negative control. (E–G), magnification 200×; bars indicate the thickness of the submesothelial compact zone. (H) Bar graph showing the number of CD31–positive vessels. Data are expressed as mean±SEM; * represents \( p < 0.05 \).
Fig 1

**Thickness of submesothelial compact zone (μm)**

- **A**
- **B**
- **C**
- **D**
- **E**

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<th>Condition</th>
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<td>Control</td>
<td>40</td>
</tr>
<tr>
<td>CG</td>
<td>120</td>
</tr>
<tr>
<td>CG+SAHA</td>
<td>80</td>
</tr>
</tbody>
</table>

* denotes statistical significance.
Type III collagen positive area ($\times 10^3 \mu m^2$)

Fig 2
Fig 3

A

B

C

D

E

F

G

H

Control CG CG+SAHA

Number of FSP-1 positive cells

Number of αSMA positive cells

* * *
Fig 5
Fig 6

**Ratio of BMP-7 Positive cells to submesothelial cells**

A - B

Control CG CG+SAHA

BMP-7 mRNA

C - D

E - F

Fig 6
**Fig 7**

A - B: Images showing the number of F4/80-positive cells.
C: Image showing the number of CD31-positive vessels.

D: Bar graph depicting the number of F4/80-positive cells with annotations indicating significant differences.

H: Bar graph showing the number of CD31-positive vessels with annotations indicating significant differences.

Legend:
- Control
- CG
- CG+SAHA