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Modulation of Renal Disease in MRL/lpr Mice by Suberoylanilide Hydroxamic Acid

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Epigenetic regulation of gene expression is involved in the development of many diseases. Histone acetylation is a posttranslational modification of the nucleosomal histone tails that is regulated by the balance of histone deacetylases and histone acetyltransferases. Alterations in the balance of histone acetylation have been shown to cause aberrant expression of genes that are a hallmark of many diseases, including systemic lupus erythematosus. In this study, we determined whether suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor: 1) inhibits inflammatory mediator production in vitro and 2) modulates lupus progression in vivo. Mesangial cells isolated from 10-wk-old MRL/lpr mice were stimulated with LPS/IFN-γ and incubated with SAHA. TNF-α, IL-6, NO, and inducible NO synthase expression were inhibited by SAHA. We then treated MRL/lpr mice with daily injections of SAHA from age 10 to 20 wk. The animals treated with SAHA had decreased spleen size and a concomitant decrease in CD4+CD8+ (double-negative) T cells compared with controls. Serum autoantibody levels and glomerular IgG and C3 deposition in SAHA-treated mice were similar to controls. In contrast, proteinuria and pathologic renal disease were significantly inhibited in the mice receiving SAHA. These data indicate that SAHA blocks mesangial cell inflammatory mediator production in vitro and disease progression in vivo in MRL/lpr mice. The Journal of Immunology, 2004, 173: 4171–4178.
HDI MODULATION OF LUPUS

HDI s inhibited LPS-induced TNF-α, IL-1β, IL-12, and IFN-γ in human PMBCs.

Mesangial cells are specialized cells within the glomerulus that are vasoactive as well as being active immunologically. Mesangial cells: 1) act as specialized smooth muscle cells engaged in the regulation of glomerular hemodynamics; 2) produce extracellular matrix and provide structural support to the glomerular tuft; and 3) through their phagocytic properties uptake and clear macromolecules such as immune complexes. Mesangial cells, similar to macrophages, produce NO, superoxide, and other inflammatory mediators in response to LPS, IFN-γ, and IL-1β (16, 26).

Because mesangial cells play a pivotal role in the initiation and resolution of the inflammatory response in the glomerulus in lupus nephritis, we sought to determine whether the administration of SAHA would block inflammatory mediator production in stimulated mesangial cells. Furthermore, we investigated whether targeting histone deacetylation in lupus pathogenesis by the administration of a specific HDI (SAHA) would modulate SLE disease progression in MRL/lpr mice. We report that SAHA blocks mesangial cell inflammatory mediator production in vitro and disease progression in vivo in MRL/lpr mice.

Materials and Methods

Mice

Eight-week-old female MRL/lpr mice were purchased from The Jackson Laboratory (Bar Harbor, ME), housed under specific pathogen-free conditions at the Ralph H. Johnson Veterans Administration Medical Center animal facility, and provided autoclaved food and sterile water ad libitum. Mice were randomly tested and were serologically negative for common murine pathogens.

Reagents

IFN-γ was purchased from BD Pharmingen (San Diego, CA); FCS, DMEM, and RPMI 1640 were from Invitrogen Life Technologies (Gaithersburg, MD); and the protein assay kit was from Bio-Rad (Hercules, CA). Anti-NO synthase 2 Ab was purchased from BD Transduction Laboratories (Lexington, KY). All other reagents, including LPS, were purchased from Sigma-Aldrich (St. Louis, MO). SAHA was a gift from Aton Pharmaceuticals (Lexington, KY). All other reagents, including LPS, were purchased from Sigma-Aldrich (St. Louis, MO). SAHA was a gift from Aton Pharmaceuticals (Lexington, KY).

Mesangial cell isolation

Because glomerulonephritis occurs in MRL/lpr mice beginning at 12–14 wk of age, glomeruli were isolated before disease onset at 8–10 wk of age. To accomplish this, mice were anesthetized with metofane and sacrificed by cervical dislocation. Renal tissue was surgically removed and kept on ice until the isolation of mesangial cells was performed. The kidneys were minced, and glomeruli were separated from the remaining renal tissue by sequential mechanical sieving (27). Glomeruli were incubated for 30 min at 37°C with collagenase and then plated in 75-cm² culture flasks in DMEM/F12 (50/50) medium containing 15% FCS, streptomycin (100 µg/ml), penicillin (100 U/ml), and α-glutamine (2 mM). Media were replaced every 48 h, while adherent mesangial cells were retained. After cells reached confluence (~2 wk), they were passaged using trypsin/EDTA. The cells exhibited typical morphologic characteristics of mesangial cells and tested uniformly positive for smooth muscle actin staining. Cells between passages 3 and 7 were used for subsequent experimental procedures.

Experimental conditions

Confluent cells in triplicate six-well cell culture dishes were washed twice with DMEM devoid of phenol red, as phenol red interferes with nitrate/nitrite (N/N) measurements. After the final wash, DMEM plus 10% FCS without phenol red was added, containing various reagents described for each experiment. At the end of each incubation, supernatants were collected and analyzed for N/N production. Cell death was assessed by trypan blue exclusion and stained with DAPI to visualize nuclei. The cells were washed with PBS containing 0.05% Tween (PBS-T). Sera were added to each well in serial dilutions, starting at a 1/100 dilution, and incubated for 45 min at room temperature. After washing with PBS-T, HRP-conjugated goat anti-mouse IgG (γ-chain specific; Sigma-Aldrich) was added and incubated for 45 min. After thoroughly washing, 0.1 M citrate buffer (pH 4) containing 0.01% H₂O₂ and 3,3′,5,5′-tetramethylbenzidine (Sigma–Aldrich) was added. Results were denoted as the absorbance at 1/100 serum dilution. Anti-glomerular basement membrane antibodies (anti-GBM) and total IgG levels were measured by ELISA, as previously described (29).

Pathology

Mice were sacrificed by cervical dislocation following isoflurane anesthesia, and the kidneys were removed. One kidney was fixed with buffered formalin, embedded in paraffin, sectioned, and stained with H&E. The slides were read and interpreted by a renal immunopathologist (F. Ruiz).
Results
SAHA inhibits inflammatory mediator production from stimulated mesangial cells

Because mesangial cells are the principal resident immunoregulatory cells in the kidney, we sought to determine whether SAHA inhibits inflammatory mediator production from stimulated mesangial cells. We, and others, previously showed that MRL/lpr mesangial cells exhibit a heightened response to inflammatory stimulation compared with mesangial cells from control strains (7, 27).

We treated mesangial cells with various concentrations of SAHA 2 h before and during stimulation with LPS (1 µg/ml) and IFN-γ (50 U/ml). Twenty-four hours after the addition of LPS/IFN-γ, supernatants were collected to determine cytokine production, and cellular supernatants were assayed for N/N production by ELISA in the supernatants of stimulated mesangial cells with and without various concentrations of SAHA (Fig. 2). Similar to our findings that NO was concentration dependently inhibited by SAHA, we observed decreased production of IL-6 and TNF-α in stimulated mesangial cells treated with SAHA. IL-6 and TNF-α production were both completely inhibited with a concentration of SAHA of 10 µM. Taken together, these results indicate that SAHA significantly decreased IL-6, TNF-α, and NO production by stimulated MRL/lpr mouse mesangial cells.

In vivo administration of SAHA

Based on our in vitro data, we tested the hypothesis that SAHA would delay the progression of lupus disease activity and reduce disease manifestations. At 10 wk of age, we began daily i.p. injections of SAHA (25 or 50 mg/kg body weight (BW) in 40 µl of DMSO, five times per week) into female MRL/lpr mice. As a positive treatment control, we injected 25 mg/kg cyclophosphamide weekly. For a negative control, MRL/lpr mice were given daily injections of 40 µl of DMSO, 5 days per week.

FIGURE 1. Effect of different concentrations of SAHA on NO production by MRL/lpr mesangial cells. A, Mesangial cells were treated with increasing concentrations of SAHA 2 h prior to stimulation with LPS (1 µg/ml) and IFN-γ (50 U/ml). After 24 h of stimulation, total cellular protein was isolated and iNOS expression was determined by Western blot. B, Supernatant from MRL/lpr mesangial cells treated with increasing concentrations of SAHA 2 h prior to stimulation with LPS (1 µg/ml) and IFN-γ (50 U/ml). After 24 h of stimulation, cellular supernatants were assayed for N/N production. The bar represents the mean ± SD of triplicates.
Animals were monitored throughout the study for general health. We monitored animals for diarrhea and weight loss due to drug treatment or disease progression. At the concentrations of SAHA used in this study, there were no general adverse effects noted in the animals. Although previous reports have shown that SAHA administration to animals at concentrations from 25 to 100 mg/kg/day has resulted in antitumor effects, in our studies we observed adverse effects of SAHA when given at the 100 mg/kg/day dose or above (22). Throughout the duration of the study, the untreated controls, and cyclophosphamide-, and 25 and 50 mg/kg BW SAHA-treated animals gained weight consistently with no differences in weight gain between any of the treatment groups (data not shown). There was also no difference in the development of skin rash between the treated and control mice.

SAHA had no effect on autoantibody production

As MRL/lpr mice age, they develop hypergammaglobulinemia and exhibit high concentrations of autoantibodies directed against several nuclear and extracellular autoantigens, including dsDNA and GBM. To determine whether treatment with SAHA modified autoantibody production, serum levels of anti-dsDNA and anti-GBM autoantibodies were quantified. Compared with vehicle-treated controls and cyclophosphamide-treated animals, the levels of both anti-dsDNA and anti-GBM autoantibodies in the serum of SAHA-treated mice were similar at ages 10, 14, 18, and 20 wk (Fig. 3, A and B). Thus, SAHA treatment did not affect autoantibody production. Cyclophosphamide, however, also had minimal effect on autoantibody production in this experiment.

SAHA decreases spleen weight

At age 20 wk, the animals were sacrificed, and the spleen weights were determined. Compared with vehicle-treated controls, the SAHA (25 and 50 mg/kg BW)-treated mice had significantly smaller spleens (p < 0.05) (Fig. 4), although there was no change in splenic architecture after SAHA treatment. The cyclophosphamide-treated animals also had spleen weights that were significantly decreased compared with the untreated controls (p < 0.01).

SAHA decreases CD3+CD4−CD8− cells in spleen

To investigate the mechanism for the lower spleen weights in the SAHA-treated animals, we analyzed B and T cells’ splenic composition by flow cytometry. The flow cytometry results showed that the predominant phenotype of T cells in the untreated control MRL/lpr mice was CD3+CD4−CD8−, double-negative T cells (DN T cells), as expected (Fig. 5). The cyclophosphamide-treated animals had a significant decrease in the number of T cells that were DN. Interestingly, the SAHA-treated animals also showed a significant decrease in the number of DN T cells compared with control untreated animals. However, there was no further decrease of DN T cells with the 50 mg/kg dose of SAHA as compared with the 25 mg/kg dose. The proportion of CD4+ and CD8+CD3−
positive cells was not different between the groups. Additionally, there were no differences between the B cell populations in any of the groups, as judged by staining for CD19 and CD21 (data not shown). These results indicate that one effect of SAHA treatment in MRL/lpr mice is inhibiting the proliferation of DN T cells.

**SAHA decreases MHC II expression in stimulated mesangial cells**

In addition to LPS/IFN-γ stimulation increasing the production of inflammatory mediators, LPS/IFN-γ enhances MCH class II expression (30). To evaluate whether SAHA administration inhibits the up-regulation of MHC II, stimulated mesangial cell cultures (10^6 cells) were treated with various concentrations of SAHA (Fig. 6; Table II). Our results showed that MHC II expression was significantly up-regulated in mesangial cells treated with LPS/IFN-γ for 24 h as compared with unstimulated (control) mesangial cells (p < 0.05). The addition of SAHA to the stimulated cultures caused a dose-dependent decrease in MHC II expression that was observed in the stimulated cells. SAHA treatment alone had no effect on MHC II expression as compared with unstimulated mesangial cells.

We next sought to determine whether the inhibition of inflammatory mediation production and decrease of MHC II expression by SAHA treatment were due to a nonspecific toxic effect or by affecting cellular apoptosis. Once again, mesangial cell cultures were treated with various concentrations of SAHA for 2 h before and during stimulation of LPS/IFN-γ for 24 h (Fig. 7; Table II). In unstimulated mesangial cells, the amount of cell death attributed to either apoptosis, as judged by annexin V staining, or necrosis, as determined by propidium iodide (PI) uptake, was low. The administration of LPS/IFN-γ did not induce a significant increase in cell death by either apoptosis or necrosis. However, when the cells were given SAHA at 100 μM concentration, there was a significant increase in the number of cells that died by necrosis. SAHA administration at 10 μM resulted in slight increase in necrosis, but it was not significant. Furthermore, at a 1 μM concentration of SAHA, there was no increase in cell death as compared with the unstimulated controls.

**Measurement of protein excretion**

To determine whether SAHA altered the progression of renal disease, we quantified protein excretion in the urine that was collected over 24-h intervals at 10, 12, 14, 16, and 18 wk of age. At 10 wk, the protein levels in each group were low, but as the mice aged, protein concentrations, as measured by protein dipstick and albumin ELISA in the urine, began to increase. By 18 wk of age in the control mice, 7 of the 10 animals had urinary protein concentrations of 30 mg/dl or greater compared with 3 of 8 in the 25/mg/kg SAHA group and 2 of 10 in the cyclophosphamide group (Table I). The levels of proteinuria in the 25 mg/kg BW SAHA-, 50 mg/kg BW SAHA-, and cyclophosphamide-treated animals were consistently lower compared with the untreated controls. These results were confirmed by ELISA analysis of 24-h urinary albumin excretion (data not shown).

**Renal pathology**

To assess further the effect of SAHA on renal disease, kidney sections were stained with H&E and scored by a pathologist that was blinded to the treatment groups. The cyclophosphamide-treated mice had significantly reduced renal scores compared with the untreated controls (Fig. 8). The SAHA-treated mice (25 and 50 mg/kg BW) also had renal scores that were significantly less than the untreated control mice. These results indicate that SAHA administration inhibited proliferative renal disease in MRL/lpr mice. In addition to H&E staining, one kidney was frozen, sectioned, and stained with fluorescein-conjugated anti-mouse IgG or C3. There were no differences in staining intensity or staining pattern in the various treatment groups for either Ig or complement renal deposition (data not shown).
Discussion

The goals of these experiments were 2-fold: first, to determine whether an HDI (SAHA) can inhibit the inflammatory mediators produced by stimulated mesangial cells from MRL/lpr mice, and second, to determine whether treating MRL/lpr mice with SAHA would abrogate renal disease. In the first set of studies, we used mesangial cells from 10-wk-old (predisease) mice. Mesangial cells from MRL/lpr mice are hyperresponsive to inflammatory stimulation (27). Our in vitro studies demonstrated that SAHA blocked NO, TNF-α, and IL-6 production by stimulated MRL/lpr mesangial cells in a concentration-dependent manner. We next sought to translate our in vitro findings into an in vivo setting. MRL/lpr mice were treated with either vehicle or two concentrations of SAHA (25 or 50 mg/kg daily). As a positive control, one group of mice received cyclophosphamide (25 mg/kg weekly). The results from our in vivo studies demonstrated that SAHA inhibited DN T cell proliferation similar to cyclophosphamide and reduced renal pathology scores as compared with vehicle-treated control. Neither cyclophosphamide nor SAHA significantly inhibited renal deposition of Ig or complement nor blocked autoantibody production during the time course of these experiments.

Since the discovery of HDACs, 11 mammalian HDACs have been reported. The mechanisms by which HDACs and HDIs alter gene transcription are currently an area of active investigation. Individual HDACs associate with specific target DNA. Recently, Yu et al. (31) reported that primarily HDAC2, but also other HDAC isoforms, are recruited to, and interact with NF-κB p65, leading to the induction of the iNOS enzyme in response to inflammatory stimuli. The addition of TSA, another HDI, inhibited cytokine induction and iNOS transcription without a change in inflammatory mediators by MRL/lpr mesangial cells by SAHA.

Our in vivo studies were performed to determine whether SAHA would prevent or delay the onset of renal disease in MRL/lpr mice. The concentrations of SAHA used were based on studies by Butler et al. (22) that showed an inhibition of a prostate cancer xenograft in nude mice with 25 and 50 mg/kg BW of SAHA. SAHA would prevent or delay the onset of renal disease in MRL/lpr mice. The concentrations of SAHA used were based on studies by Butler et al. (22) that showed an inhibition of a prostate cancer xenograft in nude mice with 25 and 50 mg/kg BW of SAHA injected daily. Butler et al. showed the concentration needed to increase acetylation of both H3 and H4 in vitro was 2 μM. Furthermore, the authors observed robust acetylation of H3 and H4 at 5 μM. In our previous studies, we described acetylation of H3 and

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*a Mesangial cells from MRL/lpr mice were treated with increasing concentrations of SAHA (0.1–100 μM) 2 h prior to stimulation with LPS (1 μg/ml) and IFN-γ (50 U/ml). After 24 h of stimulation, mesangial cells were analyzed by FACS after staining with FITC-conjugated MHC II or FITC-conjugated annexin V. Results are expressed as mean percentage of expression of triplicates. The MHC II data show the mean percentage of expression ± SD of cells that express MHC II (%)."
FIGURE 8. Renal pathology scores from MRL/lpr mice treated with SAHA (25 or 50 mg/kg BW in DMSO), vehicle (DMSO), or cyclophosphamide for 10 wk beginning at 10 wk of age. At the time of sacrifice (20 wk), the kidneys were removed, then sectioned before staining with H&E. A. Representative photomicrograph shows a glomerulus of the kidney of a control-, SAHA (25 mg/kg BW)-, SAHA (50 mg/kg BW)-, and cyclophosphamide-treated mouse. B. Graph showing the results of the kidney slides that were graded for glomerular inflammation, proliferation, crescent formation, and necrosis. Scores from 0 to 4 − were assigned for each of these features, and then added together to yield a final renal score.

FIGURE 7. Representative flow cytometry profiles for annexin V and PI staining in mesangial cells from MRL/lpr mice were treated with SAHA 2 h prior to stimulation with LPS (1 μg/ml) and IFN-γ (50 U/ml). After 24 h of stimulation, annexin V-FITC and PI intensities were determined.

H4 using concentrations of 7.5 and 10 μM (25). Additionally, Butler et al. showed, in vivo, that a dosage of 25 or 50 mg/kg SAHA increased total acetylation of H3 and H4 (22). Therefore, we believe it can be concluded that a 5 μM concentration of SAHA administered in vitro is equivalent to a 25 mg/kg dose of SAHA in vivo.

We observed a decrease in the spleen weight primarily due to a decrease in the number of CD3 + CD4 + CD8 - (DN) T cells. DN T cells increase significantly in MRL/lpr mice as they age and produce inflammatory mediators including IFN-γ (41). However, it remains controversial which immune factors, including IFN-γ, are implicated in lymphoaccumulation in MRL/lpr mice. MRL/lpr mice that lack IFN-γ have reduced lymphadenopathy and splenomegaly (42). Further studies suggested that mice lacking IFN-γ exhibited reduced lymphadenopathy due to a decrease in DN T cells (43, 44). Conversely, Haas et al. (45) found that MRL/lpr mice lacking the IFN-γ receptor exhibited no decrease in the expansion of DN T cells compared with WT MRL/lpr mice. Thus, the mechanism for the decrease in DN T cells in MRL/lpr mice treated with SAHA is unclear, but is most likely secondary to decreased production of inflammatory mediators such as IFN-γ.

In patients with SLE, purified T cells treated with TSA showed a significant reduction in gene expression of CD40L and IL-10 stimulated by phorbol esters and ionomycin, while gene expression and synthesis of IFN-γ were up-regulated (46). In our studies, we found that SAHA blocks the increase in MHC II expression in stimulated mesangial cells. Inhibiting MHC II expression, but not MHC I, has been shown to effect glomerulonephritis (47–49). In this respect, SAHA may regulate disease by decreasing MHC II. Contrary to our results, Wagner et al. (50) demonstrated TSA up-regulated MHC I, MHC II, and CD40 gene expression. The differences in our findings may be attributed to the different cell type, time stimulated, or concentrations used or to differential effects of TSA and SAHA.

The circulating levels of anti-dsDNA and anti-GBM serum autoantibodies were not reduced, nor was IgG or complement deposition in the kidney with treatment with SAHA, yet renal disease was reduced (51). The uncoupling of autoimmune production, immune complex deposition, and proliferative renal disease has been reported for a number of therapeutic interventions and genetic manipulations in MRL/lpr mice (3). These studies indicate that therapies targeted at postimmune complex deposition in lupus can be effective in preventing pathologic outcomes such as renal disease.

One surprising outcome of these studies was that the cyclophosphamide treatment had no significant effect on autoantibody production contrary to previously published reports. We did, however, observe a trend toward decreased autoantibody production in the cyclophosphamide-treated mice. We believe that as the mice aged, the trend in autoantibody production would have continued, eventually reaching significance, as has been the case in previous studies by our laboratory and others in which cyclophosphamide inhibited autoantibody production and proteinuria when started before disease expression. We were encouraged that SAHA was as effective as cyclophosphamide and TSA (25); however, the lack of a more profound effect of cyclophosphamide in this experiment emphasizes the disease and treatment response variability inherent in experiments using MRL/lpr mice. We did not assess survival in this study, but chose to sacrifice the mice before the time (24–26 wk) when MRL/lpr mice typically begin to die. Choosing this early time point allowed us to assess the effects of HDIs on pathologic renal disease. Additional treatment trials are planned to determine whether SAHA treatment affords a survival benefit in MRL/lpr mice.
In summary, the studies reported in this work demonstrate that SAHA inhibits inflammatory mediator production in vitro by MRL/lpr mesangial cells, providing evidence for a local renal effect of SAHA on lupus nephritis. We also demonstrated modulation of renal disease and splenomegaly in MRL/lpr mice by SAHA. Further studies are needed to delineate the most effective therapeutic regimen and the precise mechanisms of the anti-inflammatory properties of SAHA in lupus.

References


