Effects of histone deacetylase inhibitors on rat mesangial cells

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Freidkin I, Herman M, Tobar A, Chagnac A, Ori Y, Korzets A, Gafter U. Effects of histone deacetylase inhibitors on rat mesangial cells. Am J Physiol Renal Physiol 298: F426–F434, 2010. First published November 18, 2009; doi:10.1152/ajprenal.00107.2009.—Glomerular mesangial cells (MCs) proliferate and produce extracellular matrix proteins in many progressive renal diseases. Recently, histone deacetylase inhibitors (HDIs) were shown to have antiproliferative and antifibrogenic effects in some in vitro and in vivo models. Using the [3H]-thymidine incorporation test, we have found that the HDI trichostatin A (TSA) effectively inhibits MC growth at nontoxic nanomolar concentrations. Similarly, the HDI valproic acid also inhibited MC proliferation. Cell-cycle analysis indicated an arrest in G0/G1 phase in response to TSA, which was accompanied by elevation in synthesis of the cyclin-dependent kinase inhibitors (CDKIs) p21/Waf1 and p27/Kip1. TSA treatment suppressed G0/G1 phase in response to TSA, which was accompanied by elevation in synthesis of the cyclin-dependent kinase inhibitors (CDKIs) p21/Waf1 and p27/Kip1. TSA treatment suppressed α-smooth muscle actin, transforming growth factor-β1, and collagen protein synthesis by MCs and induced myofibroblast-like appearance of proliferating MCs. In the in vivo model of the anti-Thy1.1-induced glomerulonephritis, TSA and valproic acid treatments significantly suppressed proteinuria. Collectively, these data suggest a therapeutic potential for HDIs in the treatment of mesangial proliferative diseases and glomerulosclerosis.

increased proliferation of glomerular mesangial cells (MCs) leading to mesangial hypercellularity is a hallmark of many forms of glomerulonephritis (45). Excessive proliferation of MCs is associated with the expansion of extracellular matrix leading to the development of glomerulosclerosis (45). Several studies on experimental glomerular diseases demonstrated that the inhibition of MC proliferation could prevent glomerulosclerosis (9, 13, 15, 16). At the cellular level, it is usually accompanied by arrest of cell-cycle progression.

Modulation of chromatin structure through histone acetylation/deacetylation is known to be one of the major mechanisms involved in the regulation of gene expression. Two opposing enzyme activities determine the acetylation state of histones: histone acetyltransferases and histone deacetylases, respectively, acetylating or deacetylating the ε-amino groups of lysine residues located in the amino-terminal tails of the histones.

The therapeutic potential of histone deacetylase inhibitors (HDIs) is currently an area of active investigation. It has been demonstrated that these drugs induce growth arrest, inhibit proliferation, differentiation, and/or apoptosis in various in vitro and in vivo cancer models (21, 22). Previous evidence suggested that HDIs arrest human tumor cells at the G1 or G2 phase of the cell cycle by transcriptional activation of certain genes, such as the CDK inhibitor (CDK) p21/Waf1 (33). HDIs induce reversal of the transformed morphology of cells in culture. HDIs have been reported to inhibit proliferation and induce differentiation in noncancer cells like fibroblasts (39), adipocytes (51), and hepatic stellate cells (26, 38). HDIs are currently investigated as anticancer agents in phase I and II of clinical trials (3, 8, 37).

In the present study, we used the HDIs valproic acid and trichostatin A (TSA). Valproic acid is an aliphatic acid HDI, a prescribed treatment for epilepsy, bipolar disorders, and phase I and II clinical trials (3, 37). TSA is a specific HDI in vitro and in vivo, working at nanomolar concentrations. A number of studies showed that TSA inhibits the proliferation of several cancer cell lines and stimulates their differentiation (3, 8). TSA can also affect growth and differentiation of noncancer cells: 1) it suppresses myofibroblastic differentiation of colon epithelial cells (42), 2) inhibits synthesis of collagen and α-smooth muscle actin (α-SMA) synthesis in rat hepatic stellate cells (26, 38, 40), and 3) decreases expression of fibrosis-related genes in skin fibroblasts (39). TSA interferes with the transforming growth factor (TGF)-β1 signaling pathway and inhibits SMADs and TGF-β1 type II receptor synthesis (28, 43). Two HDIs, TSA and suberoylanilide hydroxamic acid, were shown to affect the immune system in systemic lupus erythematus (SLE)-like murine models (24, 36). They downregulated production of cytokines by splenocytes (24) and upregulated the CD4+ and CD25+ and Foxp3 regulatory T cells (36). These led to decreased manifestations of SLE, which included reduced anti-double-stranded DNA autoantibodies; decreased deposition of IgG and C3 in the glomeruli; and, consequently, less severe nephritis (24, 36).

Recently, it has been shown that TSA prevents epithelial-to-mesenchymal transition induced by TGF-β1 in human renal proximal tubular epithelial cells (52) and normal rat kidney epithelial cells (19). Mesangial cell proliferation is a key feature of many human glomerular diseases including IgA nephropathy, diabetic nephropathy, membranoproliferative glomerulonephritis, and lupus nephritis.

The aim of this study was to investigate the effect of HDIs on MCs and mesangial proliferative glomerulonephritis. Specific objectives were 1) to investigate whether TSA and valproic acid inhibit mesangial cell proliferation and activation in vitro; and 2) to determine their effect in vivo in the anti-Thy1.1 rat model, the most widely studied experimental mesangial proliferative glomerulonephritis, using a nontoxic dose.

MATERIALS AND METHODS

Materials. TSA and valproic acid sodium salt were obtained from Sigma-Aldrich (Rehovot, Israel).

Cell culture. Primary rat glomerular MCs were purified from Sprague-Dawley rats (Harlan Laboratories, Jerusalem, Israel) by the
standard sieving protocol as in Ref. 16. Animal experiments were performed in accordance with approved institutional protocols and approved by both the Local and National Committees for Experiments in Animals. To eliminate contamination by either epithelial or endothelial cells, the culture was performed in a medium with d-valine (6). MC culture medium consisted of DMEM (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 2 mM l-glutamine, 1 g/l glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin. The culture medium was then supplemented with 0.5% to 20% (vol/vol) FCS. When it is not differently indicated, MCs were subcultured at densities of 7.5 × 10^4 cells/cm² (low-density culture) and 3 × 10^5 cells/cm² (subconfluent culture), which are ~20–30% and 80–90% of confluence, respectively. MCs between passages 5 and 8 were used.

**Proliferation assay.** MCs were cultured in flat-bottomed 96-well plates and treated, as described in RESULTS, in the presence of 2.5 mCi/ml [3H]-thymidine (Danyel Biotech, Rehovot, Israel). Thereafter, cells were harvested on glass fiber filters with an automatic cell harvester. Radioactivity incorporated into DNA was counted in a Rackbeta 1217 Liquid Scintillation counter (KLB Wallac, Turku, Finland).

**Quantification of apoptosis by DNA fragmentation assay.** The programmed cell death (apoptosis) is characterized by nuclear DNA breakdown into multiples of ~200 bp oligonucleosomal-size fragments. The method we used to assess apoptosis is based on quantitave determination of the low molecular weight (broken) fraction of total DNA, as described by Higuchi and Aggarwal (10). MCs subcultured in 96-well microtiter plates at a density of 3 × 10^4 cells/cm² in a medium supplemented with 10% FCS were growth arrested by incubation in a medium containing 0.5% serum and 2.5 mCi/ml [3H]thymidine for 24 h, washed intensively, and treated as described in RESULTS. They were then washed again and solubilised in 250 μl of hypotonic lysis buffer [20 mM Tris/EDTA, 0.5% Triton X-100 (vol/vol), 5 mM Tris-HCl, pH 8.0]. Intact chromatin was then separated from fragmented DNA by centrifugation for 20 min at 13,000 g at 4°C. Supernatants containing cleaved DNA and the pellets dissolved in 500 μl Tris/EDTA buffer were separately transferred for scintillation counting of radioactivity (counts per minute = cpm) in a LS6000IC beta counter (Beckmann, Fullerton, CA). The percentage of cleaved DNA was calculated according to the following equation: DNA fragmentation (%) = cpm (cleaved DNA) × 100/cpm (cleaved DNA)+cpm (intact chromatin).

**Cell-cycle analysis.** MCs were harvested with trypsin, washed twice with PBS, fixed in cold 70% (vol/vol) ethanol, and stored at 4°C until use. Before flow cytometric analysis, cells were washed with PBS and centrifuged, and the cell pellets were resuspended in a solution of RNase (1 mg/ml) and propidium iodide (80 μg/ml) in PBS for 30 min. Stained cells were analyzed with flow cytometer (model FACSCalibur; BD Bioscience, Franklin Lakes, NJ). Data were acquired using CellQuest software (BD Bioscience); at least 10,000 events were collected for each histogram. The ModFitLT version 3.0 software (Verity Software House, Topsham, ME) was used to determine the percentages of cells in the G1, S, and G2 phases of the cell cycle.

**Western blot analysis.** Experiments were carried out with MCs seeded at a density of 3 × 10^5 cells/cm² in 60-mm cell culture dishes. At chosen time points, cells were rinsed twice with ice-cold PBS and scraped in 500 μl of lysis buffer [50 mM Tris - HCl (pH 8.0), 0.15 M NaCl, 0.1% SDS, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 1 mM NaV, 1 mM PMSF, 1:100 protease inhibitors cocktail (Sigma-Aldrich)]. Lysates were incubated for 30 min on ice and then centrifuged at 18,000 g for 15 min to collect supernatants. Then 20 μg total protein were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membrane. The following primary antibodies were used: mouse anti-p21/Waf1 monoclonal antibody, mouse anti-p27/Kip1 (both from BD Bioscience, Franklin Lakes, NJ), rabbit antihistone H3 antibody (Cell Signaling Technology, Danvers, MA), and rabbit antiacetyl-histone H3 (Upstate Cell Signaling Solutions, Lake Placid, NY).

The peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Protein antibody complexes were visualized by the enhanced chemiluminescence detection system (Santa Cruz Biotechnologies, Santa Cruz, CA).

**Assessment of TGF-β1.** Production of TGF-β1 was assessed in cell culture medium by a specific ELISA (R&D Systems, Minneapolis, MN). Samples of tested medium were placed into the wells of a 96-well microplate precoated with specific monoclonal anti-TGF-β1 antibody. Subsequently, an enzyme-linked polyclonal-specific antibody was added to form a “sandwich” with the immobilized TGF-β1. Following the substrate addition and color development, the intensity of the color was measured at 450 nm (correction wavelength set at 570 nm) and translated into concentration units using Sunrise/Touch Screen ELISA reader (Tecan Switzerland, Männedorf, Switzerland).

**Immunocytochemistry.** Cells were plated onto gelatin-coated eight-well slides and treated as described in the results section. Thereafter, the cells were fixed by incubation in cold acetone for 2 min, air dried, and washed in three changes of PBS. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide in PBS for 10 min. For immunocytochemistry of α-SMA, cells were incubated with anti-α-SMA Pp Ab (Zymed Laboratories, San Francisco, CA). Bound antibodies were visualized by incubation with horseradish peroxidase polymer (SuperPicTure Polymer Detection Kit, Zymed Laboratories) and peroxidase substrate 3,3′-diaminobenzidine. Nucleus counterstain was achieved with Mayer’s hematoxylin. For immunofluorescent analysis of collagen IV, aceton-fixed cells were blocked in a solution of 1% BSA and subsequently incubated with mouse anti-collagen type IV mAbs (Chemicon International, Temecula, CA). Bound antibodies were visualized by incubation with rabbit anti-mouse FITC-conjugated immunoglobulins (DakoCytomation, Glostrup, Denmark), and the nucleus was counterstained with Mayer’s hematoxylin. The data were quantified using ImageJ 1.4 (NIH, Open Source Software) (34).

**Measurement of collagen synthesis.** MCs cultured in six-well plates were growth arrested by incubation in a medium containing 0.5% serum. Cells were washed and treated, as described in RESULTS, in the presence of 5 μCi/ml l-[2,3,4,5-3H]-proline (Amersham Pharmacia, Piscataway, NJ) and 80 μg/ml β-aminopropionitrile fumarate (Sigma-Aldrich). Collagen synthesis during the treatment period was measured by assessing [3H]-proline incorporation into pepsin-resistant, salt-precipitated, extracellular/cell surface-associated proteins (46) and was corrected for cell number.

**mRNA quantitation.** Total RNA was prepared with guanidinium phenol-chloroform method using TRI Reagent (Sigma-Aldrich) and subjected to reverse transcription with RevertAid M-MuLV Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania) and a random hexamer primer. Prior to the preparation of cDNA, RNA was DNase-treated using the DNA-free kit (Ambion, Austin, TX). Portions of the resulting cDNA were subjected to PCR with a Platinum SYBR Green qPCR SuperMix-UDG Kit with ROX kit (Invitrogen, Carlsbad, CA). Quantitative real-time PCR using TaqMan probe was done using ABI Prism 7000 Sequence-Detection System (Applied Biosystems, Foster City, CA). The amplification protocol comprised incubations at 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s, and 60°C for 30 s.

The cycle threshold values for cDNAs corresponding to β-2 microglobulin mRNA and the mRNAs of interest were used to calculate the abundance of the latter relative to that of the former. The oligonucleotide primers for PCR were 5′-CCGTTTGGCAAGTGCTTCAAAC-3′ and 5′-TGATGGCCAGAATGACCCACCTGA-3′ for β-2 microglobulin gene; 5′-GACTACCTCTACCTGGTGTCGCAC-3′ and 5′-GCACACCTGCAACCTGCTA-3′ for the collagen IV gene; and 5′-CCCCGGTCTGTATGGT-3′ and 5′-CCCCGATGTCGTGATTGAAA-3′ for the TGF-β1 gene.
Experimental rat anti-Thy1.1-induced glomerulonephritis. Anti-Thy1.1 glomerulonephritis was induced by an intravenous injection of 1 mg/kg anti-Thy1.1 antibody (OX-7; Cedarlane, Ontario, Canada). Male Sprague-Dawley rats (8–10 wk old, 150–170 g) were treated as follows: daily subcutaneous injection with DMSO (50 μl) with no OX-7 administration (vehicle control group, n = 6); daily subcutaneous injection with TSA (125 μg/kg in 50 μl of DMSO) with no OX-7 administration (TSA control group, n = 3); valproic acid in the drinking water (3.5 mg/ml, ad libitum) with no OX-7 administration (valproic acid control group, n = 6); daily subcutaneous injection with TSA (125 μg/kg in 50 μl of DMSO) with OX-7 administration (TSA treatment group, n = 5); valproic acid in the drinking water (3.5 mg/ml, ad libitum) with OX-7 administration (valproic acid treatment group, n = 6).

The daily dose of TSA and valproic acid used was fourfold to eightfold less than that used in previous studies (11, 24, 36). Furthermore, the daily dose of valproic acid was comparable to the dose of 60 mg·kg⁻¹·day⁻¹ used in epileptic patients and to the 90–140 mg·kg⁻¹·day⁻¹ given in phase I and II studies in patients with advanced solid tumors (25, 37).

All rats were housed in individual metabolic cages and had free access to water (or to the valproic acid water solution) and food. The weight of rats in the various groups was similar on day 0 and on the death day (weight range 210–255 g). A 24-h urine collection was obtained for determination of protein excretion.

Statistics. Statistics were performed using SPSS 10.0 software (SPSS, Chicago, IL). Data are presented as means ± SE unless otherwise specified in the text. Comparisons between groups were performed using nonparametric Mann-Whitney U-test; P = 0.05 was considered significant.

RESULTS

TSA inhibits MC proliferation. Subconfluent rat MCs were growth arrested by incubation for 24 h in a medium containing 0.5% FCS, a treatment commonly used to obtain quiescent cells. To determine the effect of TSA on serum-induced MC proliferation, the medium was changed to the one containing 10% FCS with various concentrations of TSA for 24 h. TSA substantially reduced cell proliferation (Fig. 1A), as assessed by [³H]-thymidine incorporation. The inhibitory effect of TSA on MC proliferation was concentration dependent: at 37.5 nM TSA concentration, proliferation was reduced by 35% (P = 0.002) compared with control without TSA; treatment with 75 and 150 nM of TSA reduced MCs proliferation by 69% and 95%, respectively (P = 0.002 and 0.003, respectively). TSA inhibited proliferation of nonquiescent MCs in a similar pattern (data not shown).

The next experiment was carried out to evaluate whether the growth-inhibiting effect of TSA is influenced by FCS concentration. Quiescent subconfluent MCs were stimulated by 5, 10, or 20% FCS alone or in the presence of 75 nM TSA for 24 h. Proliferation of the subconfluent culture of MCs was similar at 5% and 10% FCS, but at 20% FCS, proliferation was 36% lower. TSA reduced MC proliferation in 5% FCS medium by 75%, by 79% in 10% FCS, and by 60% in 20% FCS (Fig. 1B). A similar pattern of inhibition was observed when TSA was applied to a low-density MC culture (data not shown).

TSA is known to induce apoptosis in different tested cancer cell lines; the apoptotic effect of TSA was less pronounced in nontransformed cells (1, 30, 47, 49). Using quantitative DNA-fragmentation assay we assessed TSA proapoptotic effect on MCs. TSA at concentrations up to 150 nM had no effect on quiescent cells (P = 0.6). Only at 300 nM was modest but significant apoptosis found (P = 0.003) (Fig. 1C). A similar pattern was also observed with nonquiescent cells (data not shown).

To ensure that effects of TSA are histone-acetylation related, we examined whether TSA at growth-inhibiting concentration affects acetylation of histone H3. Western blot analysis revealed that TSA led to a substantial rise in H3 acetylation 2 h from the beginning of treatment (Fig. 2); the effect remained significant even after 24 h of treatment.

Valproic acid inhibits MC proliferation. As depicted in Fig. 3A, valproic acid suppressed MC proliferation in a dose-response manner similar to that observed with TSA: at 1 mM by 25% (P = 0.002), at 2 mM by 38% (P = 0.002), and at 4 mM by 73% (P = 0.002). Fig. 3B illustrates valproic acid apoptotic effect on MCs. No statistically significant apoptosis was found at valproic acid concentrations up to 4 mM (P = 0.08). Only at 6 mM did it begin to be significant (P = 0.03).

TSA arrests MC cell-cycle progression. The next experiment investigated whether TSA interferes with the MC cell-cycle progression of mesangial cells. This was assessed by performing a BrdU incorporation assay (Fig. 4). TSA at concentrations up to 150 nM had no effect on BrdU incorporation.

Statistics were performed using SPSS 10.0 software (SPSS, Chicago, IL). Data are presented as means ± SE unless otherwise specified in the text. Comparisons between groups were performed using nonparametric Mann-Whitney U-test; P = 0.05 was considered significant.

**Fig. 1.** Effects of trichostatin A (TSA) on serum-induced mesangial cells (MCs) proliferation. A: quiescent subconfluent cultures of MCs were stimulated by 10% FCS alone or in combination with different concentrations of TSA for 24 h. [³H]-thymidine was added to the medium to assess cell proliferation. B: quiescent subconfluent cultures of MCs were stimulated by different concentrations of FCS alone or in combination with 75 nM TSA for 24 h. [³H]-thymidine was added to the medium to assess cell proliferation. C: apoptotic effect of TSA on MCs. The results are means ± SE of 3 (B) or 6 (A and C) different experiments. *P = 0.002, †P = 0.021, §P = 0.003 vs. control without TSA.
progression. Quiescent low-density cultures of MCs were stimulated for 24 h by 10% FCS in the presence of 50 nM TSA. Cell-cycle analysis by flow cytometry demonstrated (Fig. 4) that TSA increased the proportion of cells in the $G_0/G_1$ phase of the cell cycle compared with control cells without TSA (Fig. 4, B vs. A). The effect did not depend on the culture’s cell density and was the same at 75 nM (data not shown). The results suggest that TSA interferes with the MC cell-cycle progression by blocking the $G_1/S$ transition.

**Effect of TSA on CDKIs in MCs.** It was demonstrated in different models that TSA inhibits cell-cycle progression through the elevation of either p21/Waf1 or p27/Kip1 CDKIs (30). In the next experiment, we investigated whether these CDKIs are involved in TSA-induced MC growth arrest. Quiescent subconfluent MC cultures were incubated with 10% FCS alone or in the presence of 75 nM TSA; total protein was extracted at different time points and analyzed using Western blot analysis. p21/Waf1 protein synthesis was increased 1 h following TSA treatment and continued to be elevated after 24 h of treatment (Fig. 5). Synthesis of p27/Kip1 was also increased, although to a lesser degree than p21/Waf1; this effect of TSA on p27/Kip1 was less pronounced at higher densities of MCs (data not shown).

**Effect of TSA on TGF-β1 production by MCs.** It was previously shown that MCs growing in a medium with serum produce substantial quantities of TGF-β1 (17). Hence, the question of whether TSA affects TGF-β1 production by MCs was addressed. Quiescent low-density MC cultures were incubated for 2 days in a medium containing serum with or without TSA (75 nM). Real-time PCR analyses showed that after 1 day of treatment by TSA, the synthesis of the TGF-β1 mRNA in MCs decreased ($P \leq 0.014$) (Fig. 6). The effect of TSA on protein synthesis is illustrated in Fig. 7. The amount of TGF-β1 protein in the medium after 24 h was similar in the control and the TSA-treated cell cultures (Fig. 7A). After 48 h, the amount of protein in the medium of the TSA-treated cells was significantly reduced (Fig. 7A, $P = 0.05$). It is of note that TSA led to a lower cell count than that of the control after 48 h (36,813 ± 533 cells/cm² vs. 17,038 ± 738 cells/cm², respectively). The amount of the TGF-β1 protein per cell was not different in the TSA-treated cultures compared with the control cultures (Fig. 7B).

TSA’s influence on the morphology of rat MCs. It is known that starvation influences MC viability and changes the expression of such a specific marker of activated MCs as α-SMA (4). When quiescent MCs were incubated in the medium containing 10% FCS, they developed typical elongated bipolar myofibroblast-like morphology of activated MCs and expressed α-SMA (Fig. 8, A–C, left). In the presence of 50 nM TSA, MCs acquired a flattenedstellate-shaped morphology (Fig. 8, A–C, right) and expressed much less α-SMA ($P = 0.01$, Fig. 8D), features that are typical of nonactivated native MCs. In addition, cell content was more homogenous, and fewer cells detached from the growth surface. The morphological differences between TSA-treated and nontreated cells were best observed in chemically fixed cells (Fig. 8B).

**Effect of TSA on α-SMA synthesis by rat MCs.** It is known that changes in morphological cell organization may frequently reflect changes in cell cytoskeleton. We examined whether TSA might affect the α-SMA synthesis. Quiescent low-density cultures of MCs on the gelatin-coated eight-well microscopy slides were stimulated by FCS alone or in combination with 50 nM TSA for 24 h. The cells were then stained by α-SMA-specific immunoperoxidase reaction using an anti-α-SMA antibody (Fig. 8B); the nuclei were counterstained by hematoxylin (Fig. 8C). Staining revealed that TSA-treated MCs synthesized substantially less α-SMA than nontreated cells (Fig. 8D, $P = 0.01$). The effect was the same in two examined TSA concentrations (50 and 100 nM) (data not shown).

**Effect of TSA on collagen protein synthesis by rat MCs.** It was previously shown that MCs growing in vitro in the presence of serum produce substantial quantities of collagen proteins (12). These proteins are important components of extracellular matrix whose compositional change is an impor-

![Fig. 2. TSA increases histone H3 acetylation. MCs were treated as described in Fig. 1A and histone H3 acetylation at different time points was determined using Western blot analysis. One representative experiment out of 3 is depicted. The bar graph is a summary of the results obtained by densitometric quantitation of acetylated H3 that were corrected for total H3. The highest value was considered as 100% and the others were compared with it. The results are means ± SD, *$P = 0.05$ vs. control without TSA at the same time point.](http://ajprenal.physiology.org/)
tant indicator of a glomerular disease. We estimated effects of TSA and valproic acid on total collagen synthesis by MCs by measurement of $[3H]$-proline incorporation. Both TSA (75 nM) and valproic acid (4 mM) substantially reduced collagen proteins synthesis (Fig. 9, $P < 0.01$ in both conditions). The effect was qualitatively the same in low-density and subconfluent cell cultures (data not shown).

Synthesis of the TSA on the collagen IV synthesis was also assessed using immunofluorescent analysis. As shown in Fig. 10, TSA treatment led to a substantial reduction in collagen IV staining after 24 h of incubation ($P < 0.01$). Real-time PCR analysis of collagen IV gene transcription revealed no difference between TSA-treated and control groups of cells ($P = 0.32$) (Fig. 6), suggesting that collagen IV synthesis is influenced by TSA on a posttranscriptional level.

Effects of treatment by HDIs on the experimental mesangial proliferative glomerulonephritis. The acute model of mesangial proliferative glomerulonephritis, known as anti-Thy1.1 glomerulonephritis, was used. As illustrated in Fig. 11, before any treatment (day 0) the 24-h protein excretion in all groups was < 10 mg/24 h. Within 24 h after injection of anti-Thy1.1 antibodies (day 1), the rats developed a significant proteinuria of 44.5 ± 12.2 mg/24 h ($P = 0.05$ compared with day 0). The proteinuria increased progressively to 87.9 ± 20.4 mg/24 h ($P = 0.01$) on day 2, 161.1 ± 29.1 mg/24 h ($P = 0.01$) on day 3, and 164.8 ± 35.7 mg/24 h ($P = 0.01$) on day 4. Thereafter, the proteinuria decreased gradually, but was still significantly elevated compared with day 0. Treatment with TSA led to a significant reduction of 25–51% in the proteinuria on days 3 to 6 compared with the disease group (Fig. 11). Valproic acid led to an immediate reduction in the proteinuria (Fig. 11, day 1). Proteinuria was reduced by 39–68% throughout the study (Fig. 11).

Rats that had not been injected with anti-Thy1.1 antibodies but had been treated with either TSA or valproic acid had protein excretion rates undistinguishable from the vehicle control group.

DISCUSSION

This study presents the first evidence that both HDIs, TSA and valproic acid, inhibit MC proliferation in vitro. This...
inhibition was concentration dependent and was obtained at low nontoxic concentrations. TSA was shown to induce synthesis of p21/Waf1, and to some extent p27/Kip1, and increased the fractions of MCs at G0/G1 phases of the cell cycle. This effect of TSA was associated with rapid histone acetylation in MCs. This result, together with the finding that a different HDI, i.e., valproic acid, has a similar effect on MC proliferation, suggested a cause and effect relationship between histone acetylation and inhibition of MC proliferation. In addition to its antiproliferative effect, TSA interfered in vitro with MC activation: it suppressed α-SMA expression, collagen IV synthesis, and morphologic changes seen in activated MCs.

A number of factors, such as antibodies, IGF, high glucose, advanced glycation end products, angiotensin II, and changed hydrostatic conditions, may trigger physiological changes throughout the nephron. Under such conditions, some types of kidney cells, such as MCs and epithelial tubular cells, may acquire myofibroblast-like properties. These cells express α-SMA and produce a number of extracellular matrix molecules, such as collagens I and IV and laminin (7, 32, 35).

MC activation is the hallmark of many glomerulopathies. In some glomerulopathies, MC activation is the major trigger for disease development; in others (e.g., diabetes) it is a necessary step in the development of glomerulosclerosis (23). In any case, MC activation is a needed step in the pathway toward proliferation and extracellular matrix synthesis (7, 23).

In the present study, the effect of HDIs on MCs growing in a medium containing serum was evaluated. In this medium, MCs express α-SMA, actively proliferate, synthesize extracellular matrix molecules, and may serve as a model of glomerulonephritis (41). Temporal starvation may additionally increase this phenotype of activated MCs (4, 44). On this in vitro model we demonstrated that both the HDIs used (TSA and valproic acid) effectively inhibited MCs. This effect was independent of cell density and serum concentration.

Inhibition of histone deacetylases activity by HDIs causes transcriptional activation of certain genes, such as CDKIs. Papeleu et al. (29) demonstrated that TSA may induce G1-, G2-, or S-phase arrest in primary rat hepatocytes, depending on the physiological conditions of the cells in the experiment. The specific roles of p21/Waf1 and p27/Kip1 CDKIs in MCs have been shown (23). In the present study, an increased fraction of G0/G1-arrested MCs accumulated in the presence of TSA. We observed an increased protein level of p21/Waf1 following

Fig. 7. Effect of TSA on TGF-β production by MCs. Quiescent low-density cultures of MCs were stimulated by FCS alone or in combination with 75 nM TSA for 24 h. A: TGF-β content in the medium was determined after 24 and 48 h of incubation. B: relative amount of synthesized TGF-β per 1,000 cells was calculated. The results are means ± SE of 3 different experiments. *P = 0.05 vs. control without TSA.

Fig. 8. TSA affects expression of α-smooth muscle actin (α-SMA) in MCs. Quiescent low-density cultures of MCs on the gelatin-coated, 8-well, microscopy slides were stimulated by FCS alone or in combination with 50 nM TSA for 24 h. MCs were stained using specific anti-α-SMA immunocytological reaction; the nucleus was counterstained by Mayer’s hematoxylin. Representative results of 3 independent experiments are shown. A: phase-contrast microscopy of live MCs. B: costaining of α-SMA (brown) and nucleus (blue). C: as in B, but without primary anti-α-SMA antibody. D: bar graph summary of results obtained by densitometric quantitation of α-SMA. The results are means ± SE. #P = 0.01 vs. control without TSA.

Fig. 9. Effects of TSA and valproic acids on serum-induced collagen proteins accumulation. Quiescent low-density cultures of MCs were stimulated with 10% FCS for 24 h in the presence of either 75 nM TSA or 4 mM valproic acid. Pepsin-resistant, salt-precipitated, extracellular/cell surface-associated collagen was quantified with a liquid-scintillation counter and corrected for cell number. The results are means ± SE of 4 different experiments. *P = 0.01 vs. control.
renal epithelial cells. In human proximal tubular epithelial cells
(H9252) by TSA were independent of the effect on TGF-
1. Consequently, suggesting that the morphological changes induced
by TSA, substantially within the first 24 h of incubation with TSA.

In particular, by the serum-activated MCs also decreased
blast-like elongated morphology of nontreated cells. Syn-
thesis is characteristic of both in vivo- and
in vitro-activated MCs (17, 18, 20, 31, 50). TSA reduced
accumulation reflects the difference in total cell count between
nontreated MCs as well.

TSA treatment; p27/Kip1 was also increased, although to a
lesser degree and only at low cell densities. CDKIs may affect
cell-cycle progression through the inhibition of cyclin/CDK
complexes, which results in the arrest at G1/S transition and/or
G2 phase (2). The role of this mechanism in TSA-induced
growth arrest of vascular smooth muscle cells was recently
elucidated (27). It is conceivable that this mechanism may take
place in TSA-treated MCs as well.

TGF-β1 synthesis is characteristic of both in vivo- and
in vitro-activated MCs (17, 18, 20, 31, 50). TSA reduced
substantially the accumulation of the total TGF-β1 in the
medium after 48 h (but not after 24 h) of treatment. TSA
decreased DNA synthesis in MCs ([3H]-thymidine incorpora-
tion) already after 24 h. However, a lower cell count was
observed after 48 h only. Thus, the difference in TGF-β1
accumulation reflects the difference in total cell count between
the control and the TSA-treated cells as a consequence of the
antiproliferative action of TSA.

When MCs were treated by TSA for 24 h, they acquired
a flattened stellate shape morphology resembling that of
native nonactivated cells, which is different from the fibro-
blast-like elongated morphology of nontreated cells. Syn-
thesis of α-SMA, total collagens proteins, and collagen IV,
in particular, by the serum-activated MCs also decreased
substantially within the first 24 h of incubation with TSA.
However, treatment of MCs with TSA for the first 24 h did
not change TGF-β1 accumulation in the medium signifi-
cantly, suggesting that the morphological changes induced
by TSA were independent of the effect on TGF-β1.

The effect of TSA on MCs is similar to that described in
renal epithelial cells. In human proximal tubular epithelial cells
(52) and normal rat kidney epithelial cells (19), TSA efficiently
prevented TGF-β1-induced collagen I and α-SMA upregulation.
The effect of TSA on epithelial cells was associated with the
induction of Id2 and bone-morphogenetic protein-7 mRNA, in-
hbitors of TGF-β1 signals.

In epithelial cells, TGF-β1 is the major mediator inducing
epithelial-to-mesenchymal transition (48). In our study, mor-
phological changes caused by TSA in MCs, which appeared
within the first 24 h of treatment, preceded the changes in
TGF-β1 accumulation in the medium. Nevertheless, linkage
between MCs proliferation and matrix accumulation in renal
diseases was described (5, 14).

The in vivo study of experimental mesangial proliferative
glomerulonephritis supported the in vitro novel finding. We
used 24-h urinary protein excretion as a marker for the severity
of the experimental nephritis. The relatively low doses of TSA
and valproic acid (24, 25, 36, 37) significantly suppressed
proteinuria in this study.

Reduction of glomerulonephritis severity by HDIs was re-
cently described in the SLE-like mice models (24, 36). In these
studies, HDIs suppressed manifestation of autoimmunity in the
SLE-like mice, which led to decreased immune complex pre-
cipitation in the glomeruli and thereby reduced manifestation
of nephritis. In contrast, in the anti-Thy1.1 nephritis used in this
study, antibodies damage MCs directly, causing mesangiolysis,
which is followed by mesangial cell proliferation and activation,
leading to nephritis. Treatment with HDIs interfered with MCs
directly, regardless of the immunologic insult, and thus attenu-
dated disease severity as expressed by decreased proteinuria.
Moreover, in the in vitro studies in SLE mice, 100 nM TSA
had no effect on cytokines, such as IFNγ, IL-10, and IL-12
production. The effective dose started at 300 nM and above
(24). In contrast, in the present study, TSA at a concentration
of 50–75 nM, which does not affect T cells, decreased MCs
proliferation and activation significantly. It is of note that the
in vivo dose in the present study was substantially less than that
in the murine SLE studies (24, 36), suggesting that its effect
was on MCs and not immune cells.

In conclusion, this study demonstrates for the first time that
HDIs’ treatment of rat MCs in vitro suppresses MCs prolifera-
tion and affects some of their myofibroblasts-like features.
Moreover, treatment with HDIs may attenuate mesangial pro-
liferative glomerulonephritis. This suggests that the use of the
nontoxic TSA, currently undergoing clinical trials,
and of valproic acid at doses comparable to those used in clinical practice may have a potential role in the treatment of human glomerular diseases in which MCs have a prominent role. These diseases include diabetic nephropathy and IgA nephropathy, which are responsible for the majority of chronic kidney diseases, as well as membranoproliferative glomerulonephritis and lupus nephritis.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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