Trichostatin A Cooperates with Fas-Mediated Signal to Induce Apoptosis in Rheumatoid Arthritis Synovial Fibroblasts

AKIO MORINOBU, BIAO WANG, JUN LIU, SHINICHI YOSHIYA, MASAHIRO KUROSAKA, and SHUNICHI KUMAGAI

***ABSTRACT*.** ***Objective*.** To clarify the effects of trichostatin A (TSA), a histone deacetylase inhibitor, on the growth and survival of rheumatoid arthritis synovial fibroblasts (RA-SF).

Methods. Cell viability was assessed using a WST-8 assay and direct cell counting. Apoptosis was detected by annexin V staining on a flow cytometer. Protein and mRNA expression was determined by Western blotting, flow cytometry, and RT-PCR.

Results. TSA suppressed cell growth of RA-SF in a dose-dependent manner, as determined by WST-8 assay and direct cell counting. Other histone deacetylase inhibitors also showed inhibitory effects on RA-SF proliferation. TSA upregulated $p21^{WAF1/CIP1}$ cell cycle inhibitor, suggesting that cell cycle arrest is involved in the reduction of cell numbers. In addition, TSA cooperated with Fas-induced pathway to induce cell death, determined by WST-8 assay and annexin V staining. TSA reduced FLICE inhibitory protein (FLIP) expression but not Bcl-2, Bcl-X_L, and Fas expression, indicating that the synergistic effect may be through downregulation of FLIP.

Conclusion. TSA has antirheumatic effects on RA-SF and might be a potential therapeutic tool for the treatment of RA. (J Rheumatol 2006;33:1052–60)

Key Indexing Terms:
RHEUMATOID ARTHRITIS — SYNOVIAL FIBROBLAST — APOPTOSIS
FAS — HISTONE DEACETYLASE INHIBITOR

Rheumatoid arthritis (RA) is the most common form of chronic inflammatory arthritis, leading to the progressive destruction of the joints. Synovitis plays a central role in the pathophysiology of the disease[1,2]. Recent evidence shows that RA synovial fibroblasts (RA-SF) play a major role in initiating and driving RA[3]. RA-SF not only respond to cytokine stimuli, but also proliferate and actively contribute to joint destruction. RA-SF have anchorage-independent proliferation and loss of contact inhibition *in vitro* and can attach to and invade the articular cartilage[3,4]. It is well established that RA-SF, especially those in the lining layer, produce matrix metalloproteinase (MMP) and thus contribute to joint destruction[5]. Moreover, RA-SF affect other types of cells. RA-SF promote osteoclast differentiation by activating T cells and/or by expressing receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) on their surface[6]. RA-SF also prevent T and B cells from apoptosis through direct cellular interactions[7,8]. Thus RA-SF, as well as immune cells, are key components of the joint destruction of RA.

One of the most important properties of RA-SF is defective apoptosis, which may explain the massive hyperplasia of RA synovium. Thus induction of apoptosis seems to be a promising therapeutic tool for the treatment of RA[9]. Although some RA-SF are susceptible to Fas-induced apoptosis, the majority are thought to be resistant to apoptosis through this pathway[3]. Several reports indicated that stable activation and/or impaired apoptosis of RA-SF may be explained by altered expression and specific activation of disease-relevant genes. For example, both AP-1 and NF-κB proteins are activated in RA-SF and are probably involved in MMP gene expression[10,11]. Increased proliferation and/or defective apoptosis of RA-SF could be due to enhanced expression of Bcl-2, activation of NF-κB, overexpression of c-myc, somatic mutations of p53, overexpression of small ubiquitin-related modifier (SUMO)-1, and lack of phosphatase with tensin homology expression[12-17]. Exactly how and to what extent each molecule is involved in the altered functions of RA-SF still remains to be elucidated.

Modifications of histone tails have been shown to affect regulation of gene expression[18]. One of those modifications is histone acetylation, which is balanced by histone acetyl transferases and histone deacetylases (HDAC). HDAC inhibitors

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(HDACi) induce histone acetylation by suppressing HDAC. The drugs have been found to induce cell cycle arrest, apoptosis, and differentiation of many tumor cell lines. In contrast, normal cells survive, although their histones are acetylated to the same extent. Thus HDACi are among the most promising anti-tumor drugs and have been evaluated in clinical trials[19,20]. HDACi induce cyclin E and cell cycle inhibitors such as p21$^{WAF1/CIP1}$, p27^{KIP1}, and p16^{INK4}, and reduce cyclins A and D. They also have been reported to affect apoptosis-related molecules, inducing Fas and FasL and repressing Bcl-2 and Bcl-X_L[20]. These alterations of gene expression profile explain the action of HDACi to repress tumor cell growth *in vitro* and *in vivo*. Also, upregulation of MHC and downregulation of hypoxia-inducible factor-α (HIF-α) genes may contribute to tumor suppression by altering immunogenicity and angiogenesis, indicating diverse actions of HDACi to repress tumor growth *in vivo*[21,22].

Recently, *in vivo* effects of HDACi in the treatment of autoimmune disease models have been reported. Phenylbutylate and trichostatin A (TSA) upregulated p21$^{WAF1/CIP1}$ and p16^{INK4}, downregulated tumor necrosis factor-α (TNF-α), and reduced cell infiltration and hyperplasia, resulting in no cartilage and bone destruction in animal models of arthritis[23,24]. To further understand the effects of HDACi on RA-SF, we investigated the mechanisms of action of TSA *in vitro*. We report that TSA induces apoptosis and augments Fas-mediated apoptosis in RA-SF.

MATERIALS and METHODS

Reagents. TSA was purchased from Sigma (St. Louis, MO, USA) and dissolved in ethanol. Sodium butylate, valproic acid, and suberoylanilide hydroxamic acid (SAHA) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Platelet derived growth factor (PDGF) was from R&D. Caspase inhibitors VAD-FMK and IETD-FMK were purchased from MBL Co. Ltd. (Nagoya, Japan). Anti-p21 antibody was purchased from BD Biosciences (Lexington, KY, USA). Anti-p27, anti-p53, anti-Bcl-2, anti-Bcl-X_L, and anti-FLIP (FLICE inhibitory protein) antibody were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-acetyl-p53 was from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-ß-actin antibody was from Sigma. Horseradish peroxidase (HRP) conjugated secondary antibodies were from Zymed Laboratory (San Francisco, CA, USA).

Cell culture. Synovial tissue samples were obtained from patients with RA who received joint replacement surgery. All patients fulfilled the American College of Rheumatology 1987 criteria[25]. The collected tissues were minced and incubated with 4 mg/ml collagenase, and then with 0.05% trypsin (Difco, Detroit, MI, USA) as described[26]. The isolated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, streptomycin/penicillin (Gibco), and non-essential amino acid (Gibco). Adherent cells were used after 2–5 passages of RA-SF for the experiments.

Cell viability assay. Cell viability was assessed using WST-8 (Dojindo, Kumamoto, Japan), a reagent similar to XTT and MTT with higher assay sensitivity. Cells were plated in 96-well plates (3×10^5 cells/well) 2 days prior to the experiments and incubated in the presence of indicated doses of reagents. The wells were pulsed with WST-8 for 3–4 h incubation, and optical density was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA) to determine cell viability. The results were expressed as mean ± SD of 4 wells.

Viable cell counting. Cells were also counted under a light microscope. Cells (3×10^4 cells/500 µl/well) were cultured in 24-well plates in the presence of indicated concentrations of TSA. Nonadherent cells were removed, and adherent cells were washed once with phosphate buffered saline (PBS) and treated with Trypsin-EDTA (Gibco) for counting.

Apoptosis assay. For the double-staining with Annexin V and PI, cells were harvested, washed once with PBS, and then incubated with Annexin V-FITC and PI (MBL) in darkness for 30 min. Samples were then analyzed on a FACScan.

Flow cytometry. Harvested cells were stained with FITC-conjugated anti-Fas (clone UB2, MBL) or control antibodies in PBS containing 2% fetal calf serum for 30 min, washed twice, and suspended in PBS. Surface expression of Fas molecule was analyzed on a FACScan.

Western blotting. Cells were collected and lysed in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM PMSF, 1 mM NaF, 1 mM $NaVO_4$, and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)]. Protein concentration was measured using BCA protein assay reagents (Pierce, Rockford, IL, USA). Equal amounts of cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to PVDF membrane (Millipore, Natick, MA, USA). The membrane was blocked with blocking buffer [5% skim milk in 1% Tween 20 in Tris-based saline (TBS-T)] at room temperature, incubated with primary antibody in blocking buffer overnight at 4°C, washed twice with blocking buffer, and then incubated with HRP-conjugated secondary antibody for 2 h. After washing twice with blocking buffer and twice with TBS-T, immunoreactive bands were visualized using SuperSignal West Dura Extended Duration Substrate kit (Pierce) under CCD camera (ATTO, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR). Real-time RT-PCR for FLIP mRNA expression was performed to quantify levels of FLIP mRNA. Total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA, USA), and 1 µg of RNA was reverse transcribed into cDNA using a Quantitekt reverse transcription kit (Qiagen). Primer pairs for FLIP and G3PD were purchased from Qiagen. Real-time RT-PCR was performed on an ABI Prism 7700 using a QuantiTect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's instructions. The levels of FLIP mRNA expression were expressed as a ratio to those of G3PD.

RESULTS

TSA treatment reduced cell proliferation of RA-SF in a dose-dependent manner. TSA has been shown to have properties to induce growth arrest of various tumor lines *in vitro*[20]. To determine whether TSA also had the same effects on RA-SF, we counted viable cell numbers under microscopy (Figure 1a). In both experiments, RA-SF proliferated well *in vitro,* and the addition of TSA suppressed their growth and/or inhibited their survival in a dose-dependent manner. A quantity of 1 to 5 µM TSA completely inhibited cell proliferation and decreased cell numbers. We then examined the growth inhibitory effects of TSA on RA-SF from 4 different patients on Day 3, using WST-8 assay (Figure 1b). TSA treatment reduced cell viability of RA-SF in a dose-dependent manner, compatible with the results above. TSA was effective in all cases, although one case needed a higher concentration.

TSA treatment induced p21$^{WAF1/CIP1}$ in RA-SF. It has been shown that TSA regulates cell cycles by regulating cell cycle-related molecules. Among them, p21$^{WAF1/CIP1}$ appears to be a key molecule to cause cell cycle arrest[27,28]. Therefore we tested the effects of TSA on p21$^{WAF1/CIP1}$ levels in RA-SF by Western blotting (Figure 1c). TSA treatment upregulated

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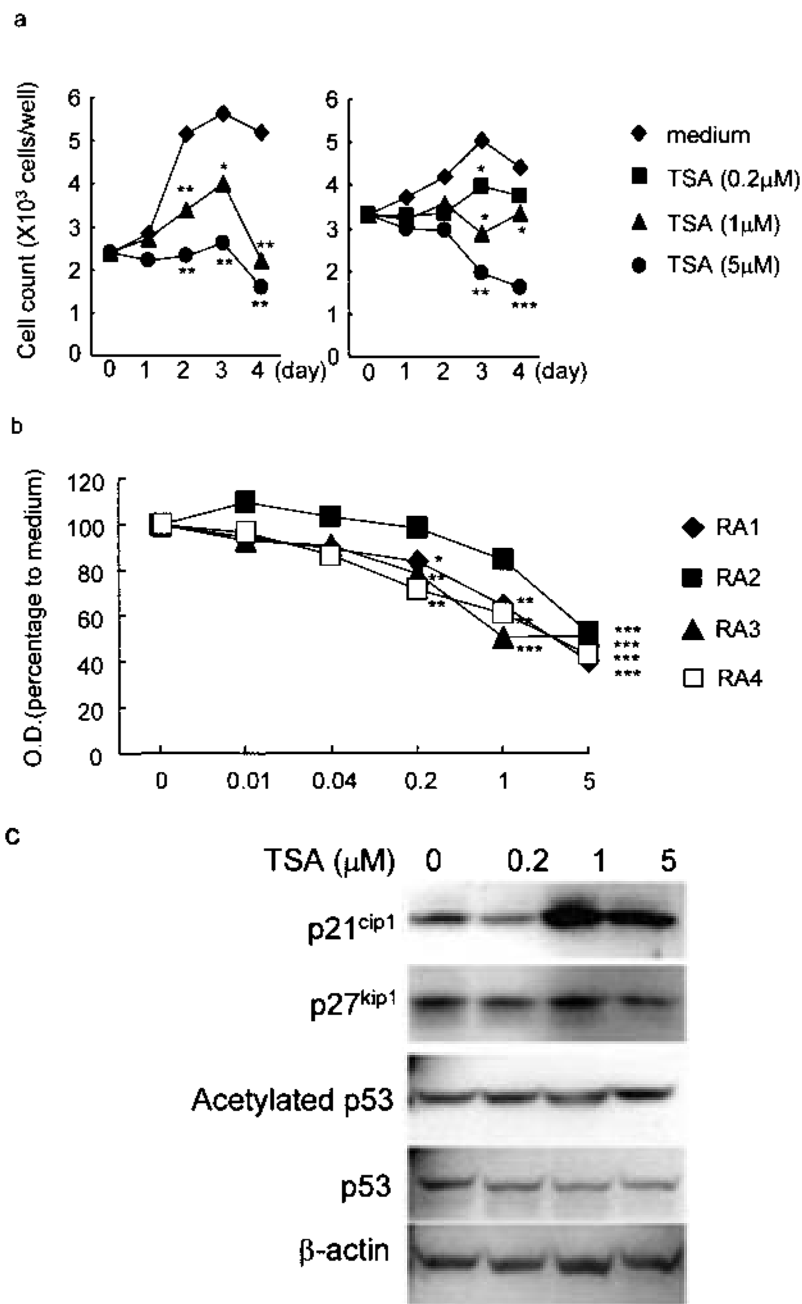

Figure 1. Effects of trichostatin A (TSA) on cell viability and p21 levels in RA-SF. a. RA-SF (3×10^4 cells/500 μl/well) were cultured in 24-well plates in the presence of indicated concentrations of TSA. On Day 0, 1, 2, 3, and 4, adherent cells were harvested, and viable cell numbers were counted under microscopy. Data were expressed as the mean of triplicate cultures. SD bars were less than 10% and were omitted. Results of 2 experiments are shown. b. RA-SF from 4 different donors were incubated in the presence of indicated concentrations of TSA, and the cell viability was determined by WST-8 assay on Day 3. Results are shown as mean of 4 wells. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. c. RA-SF (2×10^6 cell/10 ml) were incubated in the presence of TSA for 40 h. Cell lysates were prepared, and Western blotting was carried out as described in Materials and Methods. Representative results from 3 similar experiments are shown.

expression of $p21^{WAF1/CIP1}$ protein at 1 μM. The other p21 family member, $p27^{KIP1}$, was not upregulated by TSA, in agreement with previous reports[28,29]. It has been shown that p53, one of the most potent pro-apoptotic molecules, upregulates $p21^{WAF1/CIP1}$ expression[22,30]. Also, p53 can be acetylated and deacetylated by p300 and HDAC, respectively, resulting in altered gene regulation[31]. Thus we examined the effects of TSA on p53 expression and acetylation in RA-SF, and found that TSA neither upregulated p53 expression nor induced acetylation of this molecule (Figure 1c). The results suggested that $p21^{WAF1/CIP1}$, but not $p27^{KIP1}$ or p53, may be involved in TSA's effects on growth inhibition of RA-SF.

Other HDACi also reduced cell growth of RA-SF. We next examined whether other HDACi could inhibit cell prolifera-

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tion of RA-SF. We also tested whether these drugs would exert their effects in the presence of PDGF, a potent growth factor for RA-SF (Figure 2). All the HDACi tested, SAHA, sodium butylate, and valproic acid suppressed cell growth of RA-SF in a dose-dependent manner. The effects of the HDACi were also observed in the presence of PDGF. These results indicate that TSA reduced cell growth through its HDAC inhibitory effects.

TSA synergizes with anti-Fas antibody to induce RA-SF into apoptosis. Next we tried to determine whether TSA has synergistic effects with Fas-mediated cell death. In our experiment, RA-SF were cultured in the presence of TSA (1 μM) for 24 h, and then pulsed with anti-Fas antibody (500 ng/ml) for 18 h. WST-assay showed that preincubation with TSA potently augmented Fas-induced apoptosis, demonstrating synergistic effects of TSA and anti-Fas stimulation (Figure 3a). We then asked whether caspase inhibitors suppress Fas-induced apoptosis to determine whether the synergistic effects were dependent on the caspase pathway. We carried out the cell viability assay in the presence of caspase inhibitors VAD-FMK and IETD-FMK, which inhibit global caspase and caspase-8 activity, respectively (Figure 3b). After TSA treatment for 24 h, RA-SF were incubated with anti-Fas antibody as described. Caspase inhibitors were added just prior to the addition of

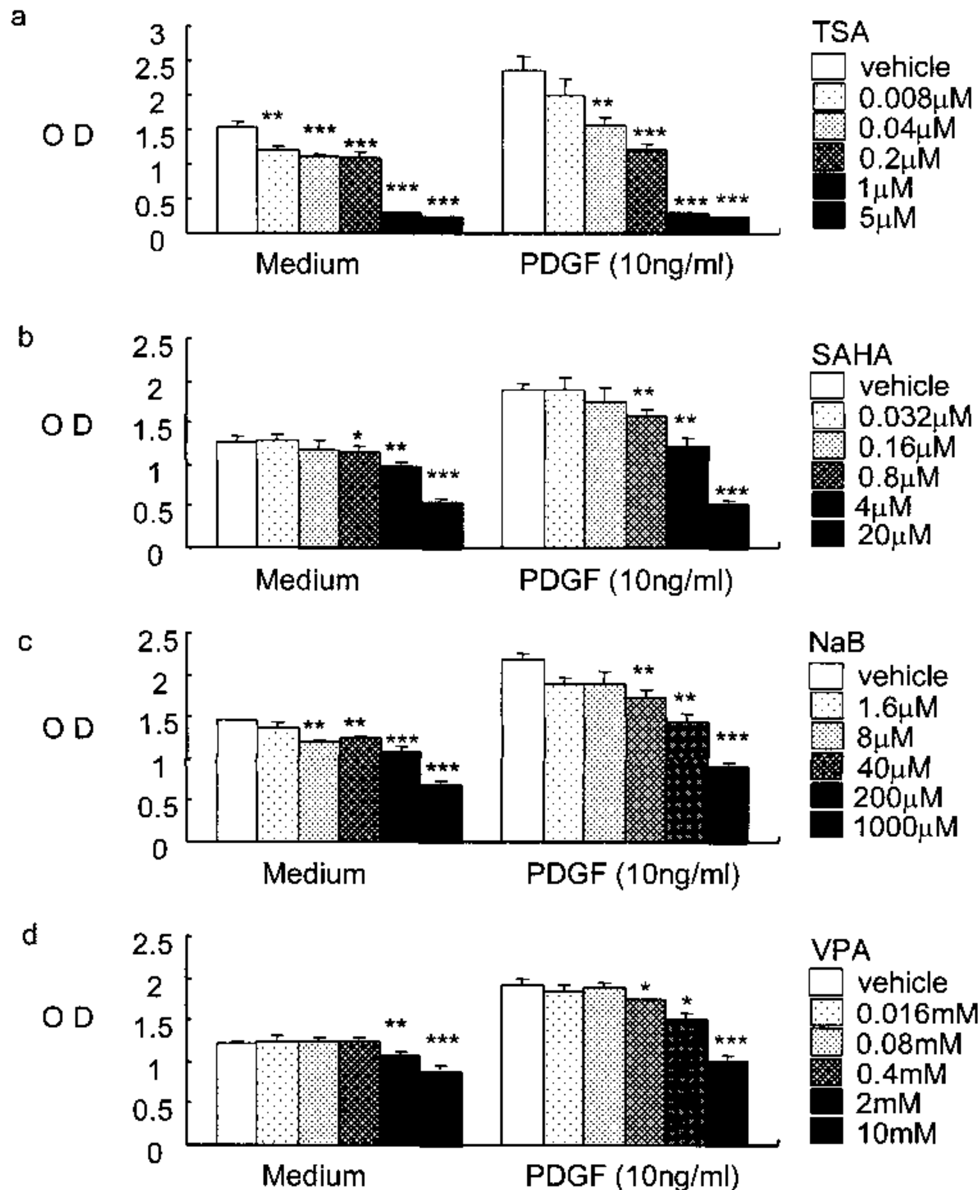

Figure 2. Effects of various HDACi on cell proliferation of RA-SF. RA-SF (5×10^3 cells/100 μl/well) were incubated in the absence or presence of PDGF (10 ng/ml), and indicated doses of TSA (a), SAHA (b), NaB (c), and VPA (d) were added for 3 days. Cell viability was determined by WST-8 assay. Results are shown as mean ± SD of 4 wells. *p < 0.05. **p < 0.01. ***p < 0.001.

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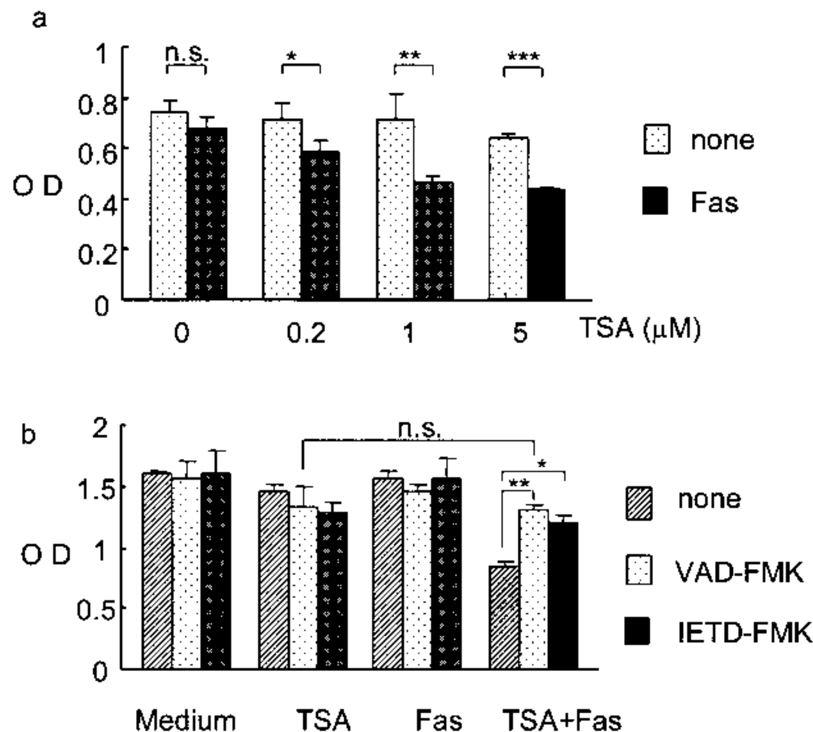

Figure 3. Synergistic effects of TSA and anti-Fas stimulation on cell viability of RA-SF. a. RA-SF (5×10^3 cells/100 μl/well) were incubated in a 96-well plate in the presence of TSA for 24 h. Anti-Fas antibodies (500 ng/ml) were added to the wells for additional 18 h incubation. Cell viability was determined using WST-8 assay. Data were expressed as mean ± SD of 4 wells. Representative results of 3 experiments are shown. b. RA-SF were incubated with TSA (1 μM) and anti-Fas as above. VAD-FMK (4 μM) or IETD-FMK (4 μM) was added just prior to addition of anti-Fas antibody. Data were expressed as mean ± SD of 4 wells. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

anti-Fas antibody. Both VAD-FMK and IETD-FMK reversed the viability of TSA + Fas-treated cells to the level of TSA-treated cells, demonstrating that all the synergistic effects were mediated through the caspase pathway.

Finally, we used annexin V and PI staining to confirm that the synergistic effects were mediated through apoptosis (Figure 4). RA-SF were incubated with medium, TSA, anti-Fas antibody, or TSA + anti-Fas antibody, in the presence or absence of VAD-FMK. Anti-Fas antibody (panel e) induced dead (PI-positive) cells and dying (Annexin V-positive, PI-negative) cells. TSA and anti-Fas stimulation (panel g) potently induced dead and dying cells, consistent with the data obtained by WST-8 assay. VAD-FMK reversed the synergistic effects of TSA and Fas stimulation, confirming the data from the WST-8 assay. These results indicated that TSA treatment sensitized RA-SF to Fas-mediated apoptosis.

TSA did not affect surface Fas expression and Bcl-2 and Bcl-X_L expression. We investigated the mechanism behind the synergistic effects of TSA and anti-Fas stimulation. First, we examined the effect of TSA on surface expression of Fas on FACScan, but did not find any change after 44 h (Figure 5a). Next we examined the effects of TSA on expression of Bcl-2 and Bcl-X_L, which are known as general anti-apoptotic molecules. However, TSA at a concentration even as high as 5 μM did not suppress protein levels of these 2 molecules (Figure 5b). Thus it seemed that neither Fas nor Bcl-2 family proteins were involved in TSA sensitization of Fas-induced cell death.

TSA reduced FLIP expression in RA-SF. Finally, we examined the effect of TSA on FLIP expression because the effect is dependent on the caspase pathway. FLIP is a naturally occurring caspase-8 homolog, which inhibits Fas-induced death signal by preventing procaspase-8 from binding with FADD and thereby inhibiting its activation[32]. FLIP is expressed in RA synovium and appears to be involved in altered apoptosis of synovial cells[33,34]. Two forms of FLIP proteins, FLIPS and FLIPL, are generated through splice variants. TSA treatment for 24 h and 44 h reduced FLIPL protein expression, while FLIPS protein was not detected in RA-SF (Figure 5c). We also found that TSA reduced FLIP mRNA expression. Time-course analysis using real-time RT-PCR showed that FLIP mRNA expression was downregulated as early as 3 h after 1 μM TSA treatment. These results were consistent with a report that showed HDACi suppression of FLIP expression in human myeloma cell lines[29]. Thus we speculate that TSA downregu-

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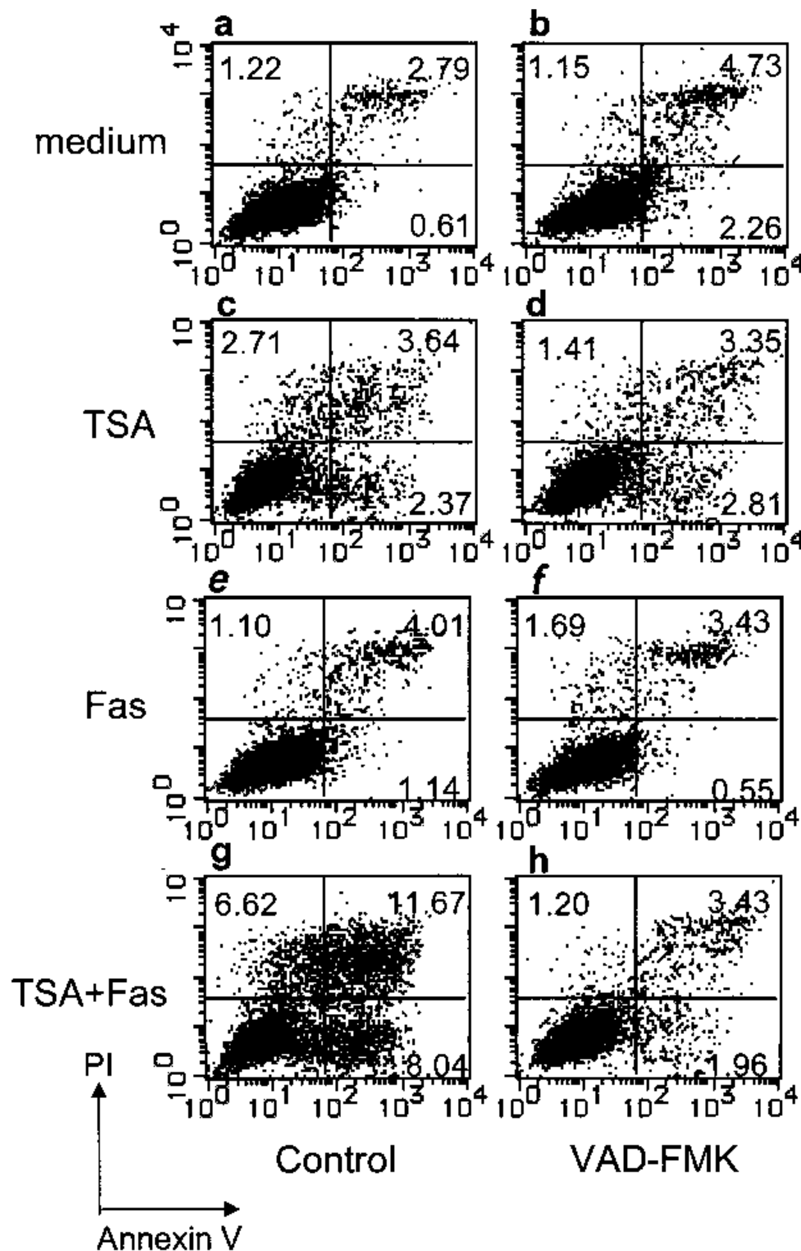

Figure 4. Synergistic effects of TSA and anti-Fas stimulation on apoptosis of RA-SF. RA-SF (3×10^5 cell/2 ml) were incubated as described for Figure 3. Cells were treated with medium (a, b), TSA (1 μM; c, d), anti-Fas antibody (500 ng/ml; e, f), and TSA + anti-Fas antibody (g, h). As well, cells were incubated in the presence (b, d, f, h) or absence (a, c, e, g) of VAD-FMK (4 μM). Cells were harvested and stained with PI and Annexin V for flow cytometer analysis.

lates FLIP expression, thus sensitizing RA-SF to Fas-induced caspase activation.

DISCUSSION

HDACi induce cell cycle arrest, differentiation, and apoptosis in tumor lines. We found that TSA also reduces cell proliferation and induces apoptosis in RA-SF. It has been reported that HDACi cause G1 arrest and G2 checkpoint in transformed and normal cells. While we observed TSA induction of $p21^{WAF1/CIP1}$, we failed to observe cell cycle arrest by PI staining, probably because cell populations in S, M, and G2 phases were not large enough to reveal the differences[20]. HDACi may mediate apoptosis through various pathways depending on cell types. Upregulation of Fas or Fas-L and Bax family proteins and production of reactive oxygen species have been reported[20,35-37]. Thus the molecular mechanism behind TSA's induction of apoptosis in RA-SF should be clarified. It would be interesting to ask how the selective

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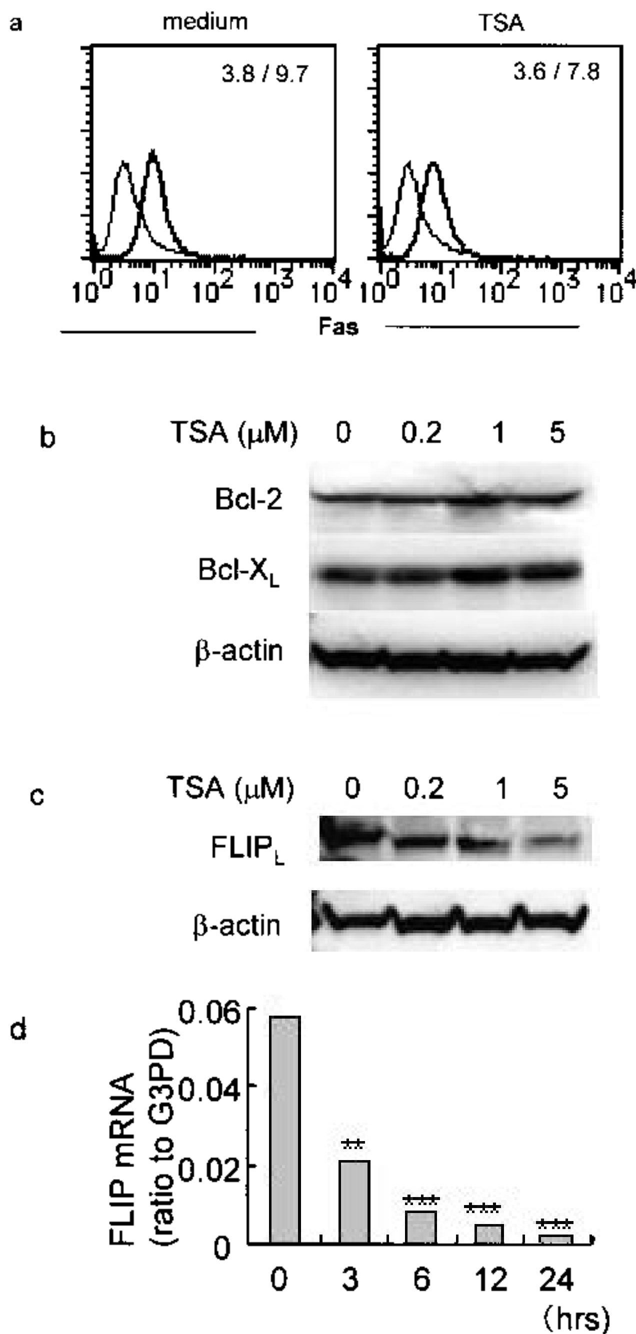

Figure 5. Effects of TSA on the expression of Fas, Bcl-2, Bcl-X_L, and FLIP. RA-SF were incubated in the presence of TSA for 44 h, and harvested. Cell surface Fas expression (a) and protein levels of Bcl-2, Bcl-X_L, and ß-actin (b) were determined by flow cytometry and Western blotting, respectively. c. RA-SF were incubated in the presence of TSA for 24 h. FLIP expression was determined by Western blotting. d. RA-SF were incubated in the presence of 1 μM TSA for indicated hours and FLIP mRNA expression was determined by real-time RT-PCR. SD bars were less than 5% and omitted. **$p < 0.01$. ***$p < 0.001$

genes are regulated and which genes are affected by HDACi in RA-SF. Understanding the molecular mechanisms of action of HDACi will lead to more specific molecular target therapy in the treatment of RA.

The most important result in our work is TSA sensitization of RA-SF to Fas-induced apoptosis. RA-SF express Fas antigen, but apoptosis of these cells has been infrequently detected *in vivo* despite the presence around them of Fas-L-expressing cells or TNF-α. The resistance of RA-SF to death receptor-mediated cell death has been ascribed to increased expression of Bcl-2, FLIP, or SUMO-1[12,16,33,34,38]. Thus downregulation of these molecules seems to be a rational strategy to induce apoptosis of RA-SF. HDACi have been reported to sensitize tumor cells to Fas-mediated cell death, although the mechanisms are unknown[37]. We first showed that TSA downregulates FLIP expression in RA-SF, suggesting one of the molecular mechanisms of the sensitization to Fas signaling. Similar effects have recently been reported in other cell lines[39]. Administration of anti-Fas antibody or Fas-L is effective in abrogating arthritis in animal models, but therapeutic use in humans is limited because of liver toxicity[40,41]. Our results imply that HDACi may be therapeutically of use to induce apoptosis by sensitizing cells to Fas stimulation.

Immunosuppressive effects of HDACi have recently been reported. HDACi can alter T cell function, such as CD154 expression and cytokine production profile, and osteoclast differentiation from bone marrow cells[42-46]. T cells and osteoclasts are involved in the adaptive immune and destruction phases, respectively, in the pathogenesis of RA. Moreover, *in vivo* effects of HDACi in the treatment of autoimmune disease models have been reported. HDACi downregulated cytokine expression, reduced mesangial cell proliferation, and ameliorated renal disease in MRL-lpr/lpr mice[47,48]. Phenylbutylate and TSA upregulated $p21^{WAF1/CIP1}$, and p16INK4 downregulated TNF-α and reduced cell infiltration and hyperplasia, resulting in no destruction to cartilage or bone in a rat model of adjuvant arthritis[23,24]. We speculate that HDACi are effective in the animal models of RA or systemic lupus erythematosus because RA-SF, lymphocytes, and mesangial cells from MRL-lpr/lpr mice are sensitive to HDACi due to the altered growth and cell death of these cells. These *in vivo* results support the application of HDACi for autoimmune conditions.

Some HDACi are in clinical trial for the treatment of leukemia/lymphoma[19,20]. The drugs are well tolerated because they are relatively nontoxic to normal cells. Although it remains unclear how HDACi affect neoplastic cells, proliferating cells seem to be susceptible to HDACi because they are not cytotoxic to cells that are arrested in the GI phase. To date, various kinds of HDACi, such as short-chain fatty acid, hydroxamic acid, and tetrapeptide, have been described[20]. We found that various HDACi suppress RA-SF growth *in vitro*. It is important from the clinical point of view to determine which drugs would have potent effects on RA-SF *in vivo*.

We demonstrated that trichostatin A induces apoptosis and sensitizes to Fas-mediated apoptosis in RA synovial fibroblasts. Our work provides a new strategy to induce apoptosis in RA-SF, and thus may lead to a new option for the treatment of RA.

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