Selective Inhibition of IL-2 Gene Expression by Trichostatin A, a Potent Inhibitor of Mammalian Histone Deacetylase

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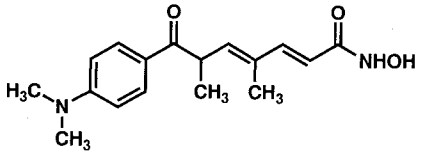
During screening for inhibitors of T cell activation, we have found that trichostatin A (TSA), known as a potent inhibitor of histone deacetylase, showed selective inhibitory activity against IL-2 gene expression. From luciferase reporter experiments on human leukemic Jurkat T cells, TSA was found to inhibit the expression of the luciferase reporter gene directed by the IL-2 enhancer and promoter with a 50% inhibitory concentration value of 0.073 μM. On the other hand, TSA, at the same concentration, enhanced the expression of the luciferase reporter gene directed by the c-*fos* enhancer and promoter. The result of RT-PCR experiments also indicates that TSA has selective inhibitory activity against IL-2 gene expression in Jurkat cells. These results suggest that the change in chromatin structure caused by the hyperacetylation of histone might affect the regulation of IL-2 and c-*fos* gene expression.

Activation of T lymphocytes is a complex process requiring recognition of the antigen by the T cell antigen receptor as well as concomitant costimulatory signals from antigen presenting cells. These events induce the activation of second messenger pathways such as tyrosine kinases and protein kinase C and lead to an increase in intracellular Ca^{2+}. These signals finally give rise to a sequential activation of sets of genes that in turn initiate proliferation, differentiation and immunological functions[1]. Although detailed mechanisms for these events are still unclear, recent studies concerning the molecular actions of cyclosporin A(CsA)[1] and FK506 have provided important new information on the biochemical process which regulates these events. Of special significance has been the realization that these molecules act as "molecular adaptors" which serve to mediate the interaction between their respective intracellular drug-binding proteins (or "immunophilins") and their individual target molecules. Vigorous research on the mode of action of CsA and FK506 has shown that the heterodimeric, Ca^{2+}/calmodulin-regulated phosphatase calcineulin is a major common target of the CsA-cyclophilin A and FK506-FK506 binding protein 12 (FKBP12) drug-immunophilin complex *in vitro* and that drug-immunophilin complex blocks the dephosphorylation by calcineulin of the nuclear factor of activated T cells (NFATc) in cytoplasm, a step that is required for its translocation to the nucleus. The drug-immunophilin complexes with calcineulin and inhibition of its phosphatase activity provide a molecular basis for the inhibitory effect of CsA or FK506 on expression of gene encoding IL-2 and other cytokines[2]. Although several signaling inhibitors have been reported for the effect of the gene expression, as of now no selective inhibitor of IL-2 gene expression is known other than CsA and FK506 (manuscript in preparation).

Trichostatin A (TSA), which had been originally found from its antifungal activity[3], was shown by YOSHIDA *et al.* to induce differentiation of Friend leukemia cell and to inhibit the cell cycle of normal rat fibroblasts in the G1 and G2 phases at low concentration[4,5], and shown by SUGITA *et al.* to induce morphological reversion of *sis*-transformed NIH3T3 cells[6]. YOSHIDA *et al.* also found that the target molecule of TSA was histone deacetylase that is potently and specifically inhibited by

Fig. 1. Structure of trichostatin A.



Trichostatin A

TSA[7]. Since histone proteins have an essential supporting role in the transcriptional machinery for regulating gene expression, the relationship between core histone hyperacetylation caused by the inhibition of the histone deacetylase and several gene expressions is receiving increasing attention[8]. It has been reported that TSA which induces histone hyperacetylation affects the gene expression of gelsolin[9], histone $H1^{0}$ [10], cytokeratin A (*endo* A)[11] and early gene products (c-*jun*, c-*fos* and c-*myc*)[12].

We report here that TSA shows inhibitory activity against the IL-2 gene expression and enhancing activity against the c-*fos* gene expression, and that TSA has immunosuppressive activity in a mouse experimental model.

Materials and Methods

Isolation of Trichostatin A (TSA)

A reporter gene assay, which is luciferase assay described below, has been used to screen for isolation of TSA. The TSA producing organism was isolated from soil collected in Japan and was taxonomically classified as *Streptomyces* sp. GT15. TSA was accumulated in both the mycelium and culture filtrate. After adjustment to pH 4.0 with 6N HCl, the culture filtrate (30 liter) was applied to a column of Diaion HP-20 (2 liter) (Mitsubishi Chemical Industries Limited). The column was washed with deionized water and 30% methanol (MeOH) and then eluted with 100% MeOH. After concentration, the eluate was extracted with normal-butyl alcohol (*n*-BuOH). The extract was concentrated and the residue was subjected to silica gel (Merck Art. No. 7734) column chromatography using the stepwise method of chloroform ($CHCl_3$)-MeOH as eluting solvents. The active fractions were combined and evaporated to dryness. The residue was rechromatographed on silica gel (Merck Lichroprep Si 60) with $CHCl_3$-MeOH, and the active fractions were further purified with HPLC using a packed column (YMC-ODS SH-365-5, 65% MeOH) to yield 63 mg of TSA.

Cell Lines and Culture

Jurkat cells were maintained in complete RPMI1640 (Gibco) supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml) in a 5% CO_2 air atmosphere. Jurkat/pIL2luc2 #15 and Jurkat/pfosluc22 #39 were periodically cycled in the above media with 0.3 mg/ml hygromycin B.

Plasmid Construction and Luciferase Assays

Plasmid construction, transfection and selection of hygromycin-resistant Jurkat clones were described by MIYAJI *et al.* (manuscript in preparation). Jurkat/pIL2luc2 #15 cells were stimulated in 200 μl fresh culture media with 12-*O*-tetradecanoylphobol-13-acetate (TPA) (5 ng/ml) and phytohemagglutinin (PHA) (1 μg/ml) in a tube and incubated for 6 hours at 37°C. Jurkat/pfosluc22 #39 cells were stimulated in 200 μl fresh culture media with TPA (5 ng/ml) and PHA (1 μg/ml) in a tube and incubated for 3 hours at 37°C. TSA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and used at the indicated concentrations. After incubation, luciferase activities were measured using a luminometer (EG&G Berthold Autolumat LB953).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA samples were reverse transcribed with Superscript RT (Bethesda Research Laboratories, Rockville, MD) and IL-2 or c-*fos* specific primers (STRATAGENE) were used to amplify selected IL-2 or c-*fos* with AmpliTaq DNA polymerase (Cetus corpration).

Mixed Lymphocyte Reaction (MLR)

Mice were obtained from Nihon SLC. MLR were set up as previously described by WEBB *et al.*[13]. 1.5×10^5 Lymph node responder cells from B10.BR mice were cultured with 5×10^5 mitomycin C-treated spleen stimulator cells from AKR mice. The culture was pulsed with 1 μCi per well of [^{3}H]thymidine approximately 18 hours before harvesting onto fiber filter-mats, and then counted using a scintillation counter.

Antiproliferation Activity

For determination of the antiproliferative activity of TSA, Jurkat/pIL2luc2 #15 was cultured in RPMI1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. Cells were plated into 96-well microtiter plate (10^4 cells/well) in the presence of various concentrations of TSA and incubated for 72 hours. Culture was pulsed with 1 μCi per well of [^{3}H]thymidine 18 hours before harvesting onto filter-mats, and counted in a scintillation counter. The results were expressed as an IC_{50}, the drug concentration required for 50% inhibition of cell growth. HeLa S3 cells were cultured in DULBECCO's modified EAGLE's medium containing 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). HeLa S3 cells were preincubated for 24 hours at 37°C in 96-well dishes and then treated with different dilutions of TSA for 3 days. Thereafter, the concentration of TSA required for 50 % inhibition of cell growth was determined by Gimsa staining[14].

Delayed-type Hypersensitivity (DTH)[15]

To induce DTH to trinitrophenyl (TNP), 0.1 ml of 10 mM trinitrobenzene sulfonic acid (TNBS) solution (pH 7.4) was subcutaneously injected into two separate sites on the dorsal flanks of the Balb/c mice. The compound dissolved in 10% DMSO was injected intraperitoneal (i.p.) once a day through day 0 to day 4. Ten percent DMSO was used as the control. Challenge was performed

5 days later by injecting 0.05 ml of 10 mM TNBS solution (pH 7.4) into the right footpad. Twenty-four hours after the footpad challenge, DTH reactivity was assessed by measuring the swelling of the footpad. The magnitude of the DTH was expressed as the increment of the thickness of the challenged right footpad as compared with the untreated left footpad. Each group consisted of five mice. TSA was dissolved in DMSO and administered by i.p. injection for 5 consecutive days, beginning on the day of sensitization.

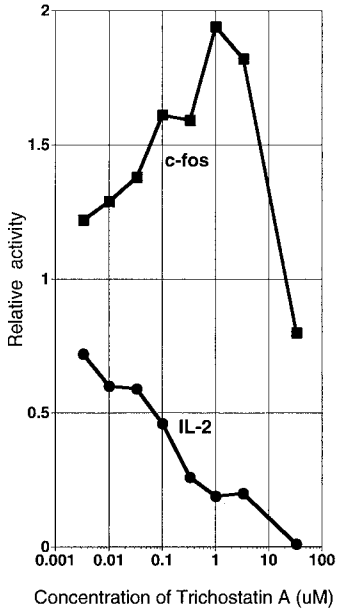
Results and Discussion

In the course of screening for inhibitors of the T cell signal transduction pathway leading to IL-2 gene expression, we have found that a strain of *Streptomyces* sp. GT15 produced a compound which showed a potent inhibitory activity against the expression of the luciferase reporter gene directed by the IL-2 enhancer and promoter. The compound was isolated from the culture broth by the combination of column chromatographies, and identified, based on physico-chemical properties and NMR analysis, as trichostatin A (TSA) previously known as a potent inhibitor of histone deacetylase (Fig. 1).

From the luciferase reporter experiments on Jurkat cells, TSA inhibited the expression of the luciferase reporter gene directed by the IL-2 enhancer and promoter with the 50% inhibitory concentration (IC_{50}) value of 0.073 μM. On the other hand, interestingly, TSA enhanced the reporter gene expression directed by the c-*fos* enhancer and promoter at the same concentration as shown in Fig. 2. These potent contrasting activities of TSA were reduced by glycosylation at the position of hydroxamate, trichostatin C[16] (data not shown), indicating that the hydroxamate moiety of TSA is essential for the effects on the reporter gene expression. These results are showing that contrasting activities of TSA on gene expression of IL-2 and c-*fos* were ascribed to the inhibition of histone deacetylase. Because the inhibitory activity of TSA on present gene expression was dependent on the hydoroxamate moiety which is parallel with the effect on histone deacetylase[7] and also there was no common characteristics in the promoter-enhancer regions of the genes irrespective of different sensitivities to TSA.

Fig. 2. Effect of trichostatin A on IL-2 and c-*fos* reporter expression.

IL-2 (●) and c-*fos* (■) reporter activities in the presence of various concentrations of TSA.



It has been reported that TSA affects the gene expression of gelsolin[9], histone H1[0 10], cytokeratin A (*endo* A)[11] and early gene products (c-*jun*, c-*fos* and c-*myc*)[12], all of these reported gene expressions, except of c-*jun*, have been significantly enhanced by TSA. Therefore, it is noteworthy that TSA selectively inhibits the IL-2 gene expression.

Before the discovery that TSA is an inhibitor of histone deacetylase, sodium *n*-butyrate was used to inhibit histone deacetylase, although its inhibitory activity is weak[17~20]. We have tried to determine whether sodium *n*-butyrate also shows selective inhibitory activity against the IL-2 gene expression and enhancement activity against the c-*fos* gene expression. Although the concentration of the drug is very high (IC_{50} = 1.0 mM), sodium *n*-butyrate inhibited the IL-2 reporter gene expression and enhanced the c-*fos* reporter gene expression like TSA (Fig. 3). While the observations shown here for sodium *n*-butyrate have not been reported, a rapid alteration of the c-*myc* and c-*jun* gene expression caused by sodium *n*-butylate has been reported[21].

These results indicate that core histone hyperacetylation caused by the inhibition of histone deacetylase may be involved in the alteration of IL-2 and c-*fos* gene expression.

To confirm the selective inhibitory activity against the IL-2 gene expression, the effect of TSA on the endogenous IL-2 mRNA expression on Jurkat cells was investigated using a RT-PCR experiment. As shown in Fig. 4, TSA as well as cyclosporin A (CsA) inhibited induction of endogenous IL-2 mRNA at the concentration of 1 μM, not that of β-actin and G3PDH mRNA as controls. Similarly, induction of endogenous c-*fos* mRNA was

Fig. 3. Effect of sodium *n*-butylate on IL-2 and c-*fos* reporter expression.

IL-2 (●) and c-*fos* (■) reporter activities in the presence of various concentrations of sodium *n*-butyrate.

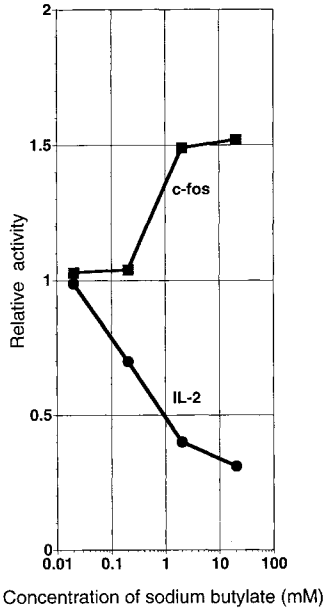
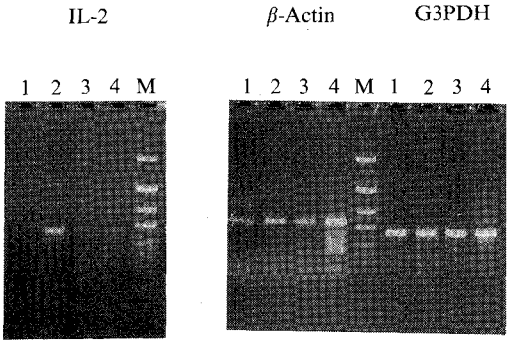


Fig. 4. Effect of trichostatin A on internal IL-2 mRNA expression by RT-PCR.

lane 1: no addition, lane 2: +TPA (5 ng/ml)+PHA (1 μg/ml), lane 3: +TPA (5 ng/ml)+PHA (1 μg/ml)+TSA (1 μM), lane 4: +TPA (5 ng/ml)+PHA (1 μg/ml)+cyclosporin A (1 μM), M: marker.



enhanced by TSA but not by CsA (data not shown). These results indicate the following. First, since the endogenous IL-2 mRNA expression as well as the IL-2 reporter expression were affected by TSA, the activities of TSA against the reporter expression do not appear to be due to the positional effect of a stable transformant of Jurkat cells. Second, the mode of action of TSA is quite different from that of CsA because of its different effect on the c-*fos* gene expression.

IL-2 production on Jurkat cells stimulated by PHA and TPA was also inhibited by TSA using enzyme linked immunosorbent assay (ELISA) (data not shown).

Table 1. Effect of trichostatin A on delayed type hypersensitivity (DTH).

Compound	Dose (mg/kg)			Inhibition(%)
Trichostatin A	100	x 5	ip	64.4 (4/5Toxic)
	50	x 5	ip	47.1
	25	x 5	ip	17.2
	12.5	x 5	ip	8.7
Cyclosporin A	30	x 5	po	94.5

In order to investigate immunosuppressive activity, TSA was first assessed *in vitro* using the mixed lymphocyte reaction (MLR). As results, TSA showed inhibitory activity against the MLR with an IC_{50} value of 0.032 μM and showed antiproliferative activity against the Jurkat cells with an IC_{50} value of 0.052 μM, while the IC_{50} value of antiproliferative activity against HeLa S3, a nonlymphoidic cell line, was 1.1 μM. Secondly, TSA was assessed *in vivo* using the mouse delayed type hypersensitivity (DTH) experiment. TSA showed 47.1% inhibition by i.p. injection of 50 mg/kg × 5 as shown in Table 1, although the inhibition of TSA against DTH is less effective than that of CsA (30 mg/kg × 5 p.o.: 94.5%).

TSA has a variety of biological activities such as antifungal, induction of cell differentiation, cell cycle arrest, morphological change and effect on gene expression[3~6,9~12]. These biological activities of TSA are considered to be a result of the inhibition of histone deacetylase[7]. However, there is little information on the relation between these biological activities and inhibition of histone deacetylase on gene expression.

The level of histone acetylation is mainly controlled by the acetyltransferase-deacetylase equilibrium[22]. Reversible histone acetylation, which occurs at the ε-amino group of specific internal lysine residues located at the concerned domains of the core histones, is supposed to play an important role in the regulation of the chromatin structure and function specificially in DNA replication and transcription[23]. TSA inhibits histone deacetylase resulting in the hyperacetylation of histone[7]. The hyperacetylation of histone is generally considered to provoke relaxation of the chromatin structure to make various transcriptional factors accessible to DNA. It seems that the transient increase in the site specific or phase dependent histone acetylation may be essential for the early stage of gene expression.

Although it is not clear why the inhibition of histone deacetylase causes the inhibition of IL-2 gene expression and activation of the c-*fos* gene expression, it is of great

interest to know the mechanisms that TSA possesses for contrasting activities against immediate-early (c-*fos*) and early gene (IL-2) expression caused by external stimuli on Jurkat cells. The dramatic change in chromatin structure by the hyperacetylation of histone might affect the DNA binding capability of various transcriptional factors or their regulatory molecules. This might result in the difference between the IL-2 gene expression induced only by specific stimuli from the T cell receptor and the c-*fos* gene expression induced by non-specific stimuli. Future experiments must address how a set of transcriptional factors and/or their regulatory molecules recognize the acetylated form of the chromatin structure and control the IL-2 and/or c-*fos* gene expression to coordinate the complex organization of T cell signal transductional events.

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