Histone Deacetylase Inhibitors Up-regulate the Expression of Cell Surface MHC Class-I Molecules in B16/BL6 Cells

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In a previous report, we showed that anthracyclines up-regulate the cell surface expression of MHC class-I molecules in B16/BL6 cells¹⁾. By using the same assay system, we found an other type of MHC class-I up-regulator in the culture broth of a newly isolated soil bacterium, *Bacillus megaterium* 6N38. Here we report the isolation and characterization of this up-regulator, and its possible molecular mechanism.

Starting from 1 liter of culture supernatant of *Bacillus megaterium* 6N38, the MHC class-I up-regulator was purified. After the pH of the supernatant had been adjusted to 13 with NaOH, solvent extraction was performed with 1 liter of dichloromethane. The water fraction was adjusted to pH 2 with HCl, and extracted 3 times with 500 ml of dichloromethane each time. The organic fraction was condensed *in vacuo* to give 2.81 g of dark brown oil. The extract was applied onto a silica gel column (bed volume=147 ml) equilibrated with dichloromethane, and eluted with 1 liter of the same solvent. The eluate was collected and evaporated to give 1.97 g of a yellowish oil. The oil was applied to the second silica gel column (bed volume=100 ml) equilibrated with cyclohexane, and then eluted stepwisely with 250 ml of cyclohexane, 500 ml of cyclohexane:dichloromethane=8:2, and 700 ml of cyclohexane:dichloromethane=6:4. The last fraction was evaporated to give 880 mg of pale yellowish oil. The sample was finally purified by reverse-phase HPLC using a TOSO HPLC system (SC-8010, UV-8010, CCMP) equipped with a semi-preparative column (Nacalai Tesque Cosmosil 5C18-AR, i.d.=22 mm, length=25 cm). The elution pattern was monitored by measuring the absorbance at 210 nm. The elution conditions were as follows: solvent A, 0.1 M sodium phosphate (pH=2); solvent B, equal mixture of 0.05 M sodium phosphate (pH=2) and acetonitrile; linear gradient from B=0% to 100% over 100 minutes; flow rate, 5 ml/minute. The active peak was collected, extracted by dichloromethane,

and evaporated *in vacuo* to afford 20.1 mg of a colorless oil (compound **1**).

The molecular ion peak of **1** detected by FAB-MS was 101 ($[M-H]^-$ ion), indicating the molecular weight of **1** to be 102. The ^1H and ^{13}C NMR spectra of **1** could be explained and assigned consistently, assuming compound **1** was isovaleric acid (data not shown). When the ^1H and ^{13}C NMR spectra of authentic isovaleric acid and those of compound **1** were compared, spectra of both were identical. These results taken together indicate compound **1** is isovaleric acid. In Fig. 1, the structures of isovaleric acid and related compounds are indicated.

Since *n*-butyric acid, a low-molecular-weight fatty acid structurally similar to isovaleric acid (Fig. 1), has been reported to induce cellular differentiation *via* histone hyperacetylation²⁾ caused by the inhibition of histone deacetylase, we examined whether isovaleric acid could hyperacetylate core histones in B16/BL6 cells. Histones isolated from B16/BL6 cells were treated with 15 mM *n*-butyric acid, 15 mM isovaleric acid, or 1 μM trichostatin A for 6 hours, and analyzed by Acid-Urea-Triton gel electrophoresis. As shown in Fig. 2, isovaleric acid elicited histone hyperacetylation in B16/BL6 cells as did known inhibitors of histone deacetylase, *n*-butyric acid²⁾ and trichostatin A³⁾, suggesting that isovaleric acid also inhibits histone deacetylase.

Next, we examined whether histone deacetylase inhibitors would be able to induce the expression of MHC class-I molecules. As shown in Fig. 3 the potent and specific inhibitor of histone deacetylase, trichostatin A³⁾, stimulated the expression of MHC class-I molecules at concentrations markedly lower than those at which *n*-butyric acid or isovaleric acid was effective. These results strongly suggest that histone deacetylase inhibi-

Fig. 1. Structures of isovaleric acid and related compounds.

Fig. 2. Effects of *n*-butyric acid, isovaleric acid, and trichostatin A on histone acetylation in B16/BL6 cells.

After incubation for 6 hours with or without 15 mM *n*-butyric acid, 15 mM isovaleric acid, or 1 μ M trichostatin A, histones of B16/BL6 cells were isolated and analyzed by Acid-Urea-Triton gel electrophoresis as described in "Experimental". The numbers beside the gel denote the positions of histone H4 acetylated by the indicated numbers of acetyl residues.

Fig. 3. Effects of isovaleric acid, *n*-butyric acid, and trichostatin A on the expression of MHC class-I molecules in B16/BL6 cells.

● *n*-Butyric acid, ○ isovaleric acid, ■ trichostatin A.

After B16/BL6 cells had been incubated for 72 h with various concentrations of *n*-butyric acid (closed circles), isovaleric acid (open circles), or trichostatin A (closed squares), the expression level of cellular MHC class-I molecules was determined by the cell ELISA method described in "Experimental". The data are expressed as relative values, assuming the level without any additive to be unity.

diseases.

Experimental

Organisms and Cultivation

Bacillus megaterium 6N38 was isolated from soil collected in Noda-shi, Chiba, Japan, and its taxonomic identification was performed by NCIMB (The National Collection of Industrial and Marine Bacteria Limited, Scotland, UK). This strain has been deposited in the Fermentation Research Institute of The Agency of Industrial Science & Technology, Japan (accession number: FERM BP-3760). The bacteria were grown in a fermentation medium containing (/liter) Na₂HPO₄, 6 g; KH₂PO₄, 3 g; NaCl, 0.5 g; NH₄Cl, 1 g; MgSO₄, 2 mmole; glucose, 4 g; CaCl₂, 0.1 mmole; casamino acid, 5 g; peptone, 40 g; and yeast extract, 5 g. The culture was agitated in a reciprocal shaker (135 rpm) for 48 hours at 45°C. The culture broth was centrifuged, and the supernatant (1 liter) was used as a starting material for the purification of MHC class-I up-regulator.

The established mouse melanoma line B16/BL6 was obtained from the National Cancer Institute, USA. The cells were grown in modified EAGLE's medium supple-

tion leads to the expression of MHC class-I molecule in B16/BL6.

Here we showed that variety of histone deacetylase inhibitors, including isovaleric acid, *n*-butyric acid²⁾, and trichostatin A³⁾, induced MHC class-I expression in B16/BL6 cells. Trichostatin A did not induce the expression of MHC class-II and ICAM-1 molecules in the same cell line (data not shown), indicating there exists some specificity among cell surface molecules. Although recent reports suggest that histone deacetylase is important as a transcriptional corepressor^{4~10)}, details of the control of histone acetylation-deacetylation and its link to specific gene expression remains to be elucidated. Since MHC class-I molecules present intracellular antigens after their processing to be recognized mainly by cytotoxic T cells¹¹⁾, if MHC class-I molecules are up-regulated on cancer cells or virus-infected cells, these cells may be recognized by the host defense system more easily, and thus be eliminated more efficiently. So it may be possible to use histone deacetylase inhibitors as immunotherapeutic drugs against cancer and viral

mented with 10% fetal calf serum (Irvine Scientific, California, USA) at 37°C in a 5% CO₂ humidified atmosphere.

Measurement of MHC Class-I Molecule Up-regulating Activity

The expression of MHC class-I molecules on the surface of B16/BL6 cells was determined as reported previously by an enzyme-linked immunosorbent assay (ELISA)¹⁾.

Acid-Urea-Triton (AUT) Gel Electrophoresis for Determining Histone Hyperacetylation

Cellular histones were extracted as reported previously³⁾. Hyperacetylation of core histone of B16/BL6 cells was determined by the Acid-Urea-Triton gel electrophoresis technique, originally reported by COHEN *et al.*¹²⁾ and modified by YOSHIDA *et al.*³⁾ with further small modification, in which gels were stained with silver instead of Coomassie Brilliant Blue.

Physico-chemical Data

FAB-MS spectrum was obtained with a Finnigan MAT TSQ7000 triple quadrupole mass spectrometer. NMR spectra were recorded on JEOL GSX-270 NMR Spectrometer with ¹H NMR at 270.05 MHz and ¹³C NMR at 67.80 MHz.

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