Morphological Reversion of sis-transformed NIH3T3 Cells by Trichostatin A

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ABSTRACT

Trichostatin A (TSA) induced the normal and flat phenotype of sis-transformed NIH3T3 cells at quite a low concentration of 1 ng/ml. Although morphological changes were found in other oncogene-transformed cells, they were not the same as those seen for the sis-transformed cells. Almost complete reversion into the flat phenotype was seen at 6 h after administration of the compound, suggesting that the morphological change was caused not merely by selection of TSA-resistant cells of the flat phenotype. The effect of TSA was reversible when the cell culture was incubated after its removal. Synthesis of sis-mRNA did not decrease with the treatment of TSA at a concentration sufficient to reverse the transformed morphology. Cycloheximide abolished the activity of TSA, showing that TSA required new protein synthesis to express its activity.

INTRODUCTION

As oncogenes have been shown to be involved in carcinogenesis (1), specific inhibitor(s) against oncogene function would be a good tool for identifying oncogene function and might yield specific therapeutic drugs against cancers expressing identical oncogenes.

TSA was found at first as an antifungi agent in 1976 (2). In 1987, Yoshida et al. reported new functions of TSA, the differentiation-inducing activity of Friend leukemia cells (3), and inhibitory activity of the cell cycle of rat fibroblast (3Y1) cells at the G1 or G2 stage (4). They also reported that TSA changed the morphology of HeLa cells into a flat type and further that it showed cytodal activity against SV40-transformed 3Y1 cells, although it had only cytostatic activity against nontransformed cells (5). Recently, TSA was reported to inhibit the activity of histone deacetylase, which might lead to the transformation-inducing activity of Friend leukemia cells (3), on oncogene-transformed NIH3T3 cells, and found that TSA in

RESULTS

Flattening of Morphology of sis-transformed NIH3T3 Cells

To determine whether the effect of TSA is specific to sis-transformed cells, TSA was added to various oncogene-transformed NIH3T3 cells (Fig. 4). TSA did not induce the normal change to the original transformed phenotype when incubated for more than 6 h (Fig. 2). Next, the inhibitory effect of TSA on the growth of sis-transformed cells was examined (Fig. 3). TSA (25–200 ng/ml) was added to the sis-transformed cells for more than 6 h (Fig. 2). Next, the inhibitory effect of TSA on the growth of sis-transformed cells was examined (Fig. 3). TSA did not induce a significant change in the morphology of normal NIH3T3 cells even at the concentration of 100 ng/ml. To find how long the cells had to be exposed to TSA to make their shape flat, the morphological change was followed after addition to TSA to sis-transformed cells. This change was observed in almost all cells after exposure for more than 6 h. The inhibitory effect of TSA on the growth of sis-transformed cells was examined (Fig. 3). TSA (25–200 ng/ml) was added to the sis-transformed cells with the 1-day culture after the cell inoculation, and viable cells were counted at 24 and 48 h after TSA addition. TSA inhibited the cell growth of sis-transformed cells in a dose-dependent manner, and induced the flat phenotype at all concentrations tested. The effect of TSA was cytostatic up to 100 ng/ml, because the flat cells began to grow with the morphological change to the original transformed phenotype when incubated after the removal of TSA (data not shown). However, TSA became cytodal at 200 ng/ml, since the number of viable cells decreased and dead and round cells were observed.

MATERIALS AND METHODS

Chromos. TSA, a gift from Dr. K. Matsumoto in our laboratories, has been prepared as described previously (2). DMSO was purchased from Nakarai Tesque, Inc. (Kyoto, Japan), HMBA and n-butryate were from Sigma Chemical Co. (St. Louis, MO), 0.225 M phosphate buffer solution with 0.15 M NaCl powder was from Nissui Pharmaceutical Co., Ltd., and v-sis probe (1.23 kilo base pairs) was from Takara Shuzo Co., Ltd. (Kyoto).

Cells. v-sis-, v-src-, v-fgr-, v-abl-, and v-fos-transformed NIH3T3 cells were gifts from Dr. K. C. Robbins of NIH. v-Ha-ras-transformed NIH3T3 cells were a gift from Dr. K. Yanagihara of Research Institute for Nuclear Medicine and Biology, Hiroshima University. These cells were cultured in Dulbecco's modified minimum essential medium supplemented with 10% fetal bovine serum.

Cell Growth Inhibition Assay. Sis-transformed cells were inoculated at 1.0 x 10⁵ cells/ml in 2 ml into 12-well plates (Rinbro), and incubated at 37°C in a 5% CO2 incubator. After 24 h, the adequate concentrations of TSA were applied to the wells, and cultures were incubated at 37°C, harvested at 24 or 48 h after TSA addition, and the cell number was counted.

Northern Blotting. Preparation of total RNA from sis-transformed and normal NIH3T3 cells with or without the treatment of TSA and electrophoretic separation of RNA and Northern blot followed basically the method reported previously (13, 14).
REVERSION OF sis-TRANSFORMED CELLS BY TSA

Fig. 1. Effect of TSA on the morphology of sis-transformed and normal NIH3T3 cells. Normal (a, b) and sis-transformed (c, d) NIH3T3 cells were inoculated into Petri dishes (diameter, 6 cm, Falcon) at 2.5 x 10^6 cells/ml in 5 ml of Dulbecco's modified minimum essential medium (10% fetal bovine serum) and cultured overnight in a 5% CO2 incubator. Next, TSA was added to the cultures (b, d) at 8 ng/ml, and the morphology of the cells was observed after 24 h of incubation and photographed.

Fig. 2. Kinetics of TSA activity to reverse the phenotype of sis-transformed NIH3T3 cells. The procedure was the same as that in Fig. 1, except that the morphology of the cells was observed at 0 h (a), 2 h (b), 4 h (c), 6 h (d), 8 h (e), and 10 h (f) after the addition of TSA.

Fig. 3. Inhibitory effect of TSA on the growth of sis-transformed cells. sis-transformed cells were inoculated in a 12-well plate (Rinbro) in 2 ml of Dulbecco's modified minimum essential medium at 1.0 x 10^6 cells/ml and incubated for 24 h. TSA was added at the concentration of 0 ng/ml (C); 25 ng/ml (O); 50 ng/ml (△); 100 ng/ml (△); and 200 ng/ml (A). After 24 or 48 h of incubation, the cells were harvested and the number of viable cells was counted.

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Since TSA was found to be a differentiation inducer of Friend leukemia cells (3), the action of TSA in sis-transformed cells was compared with those of other compounds (DMSO, HMBA, n-butyrate) which were reported to have inducing activities of differentiation (16-18). The flat morphology induced by n-butyrate was similar to that by TSA (data not shown), suggesting that these compounds have similar activities. Actually, there is evidence that these two compounds have inhibitory activity against histone deacetylase (6, 19), although this has not yet been shown to be correlated with the inducing activity of the flat phenotype. Polar agents, DMSO and HMBA, also induced the flat morphology, but the shape was rather round, not similar to that induced by TSA (data not shown).

Effect of TSA on Synthesis of sis-mRNA. Yoshida et al. reported that TSA did not inhibit the total RNA synthesis of Friend leukemia cells (3). To determine whether TSA inhibits the synthesis of sis-mRNA in sis-transformed cells, the Northern blotting method using v-sis oncogene probe was performed to measure the amount of sis-mRNA. As shown in Fig. 5, TSA did not decrease the synthesis of sis-mRNA at 10 ng/ml, at which the transformed phenotype was clearly reversed.

This result showed that the induction of the flat phenotype of sis-transformed cells.

Type of sis-transformed cells.

flat morphology of any other oncogene-transformed cells, although the morphological changes of ras-, src, and fos-transformed cells were observed. No morphological change was seen in fgr- and abl-transformed cells with TSA treatment. Thus, TSA seemed to be a specific inducer of the normal flat phenotype.
Fig. 4. Effect of TSA on the morphology of NIH3T3 cells transformed by various oncogenes. The procedure was the same as that in Fig. 1, except that v-Ha-ras- (a, b), v-src- (c, d) and v-fgr- (e, f) transformed NIH3T3 cells were used instead of sis-transformed cells.

by TSA was not due to a decrease in the amount of the transcript of sis-oncogene.

Effect of Cycloheximide on TSA Activity. To find whether TSA changed the cell morphology directly or required the synthesis of a protein causing reversion of the cell morphology, we examined the effect of cycloheximide, an inhibitor of protein synthesis, on the action of TSA. Simultaneous addition of cycloheximide abolished the activity of TSA to reverse the morphology (Fig. 6), suggesting that TSA required new protein synthesis to induce the flat morphology of the transformed cells. Thus, TSA might promote the synthesis of a putative protein with a function correlated with the reversion of the transformed phenotype.

DISCUSSION

Agents which induce the flat phenotype of various oncogene-transformed cells have been described in several reports (20–28). Against the sis-transformed cells, suramin is the representative inhibitor and blocks the binding of PDGF (the counterpart of the v-sis product in normal cells) to the receptor on the cell membrane (11). This inhibition is not specific to PDGF, as this compound also inhibits the binding of basic fibroblast growth factor to the specific receptor (29).

This paper describes the reversed phenotype of sis-transformed cells induced by TSA. This phenomenon was specific to sis-transformed cells, because TSA did not induce the normal flat phenotype of other oncogene-transformed ones (Fig. 4). The flat phenotype of sis-transformed cells was induced by exposure of the cells to TSA for more than 6 h (Fig. 2), showing that this phenomenon was not due to the selection of flat cells which were rather insensitive to TSA, but to the direct action of TSA on the transformed cells. The induction of the flat phenotype by TSA was not caused by inhibition of the synthesis of sis-mRNA (Fig. 5). A preliminary experiment which used the Western blotting method by using the monoclonal antibody against PDGF (a kind gift of Dr. S. Aaronson, NIH, Bethesda, MD) indicated that TSA did not inhibit the synthesis of p28\textsuperscript{a}, the product of sis-oncogene (data not shown). These results showed that TSA reversed the cell morphology without inhibiting the transcription or translation of sis-oncogene. The action of TSA was abolished by the addition of cycloheximide, an inhibitor of protein synthesis (Fig. 6), showing that TSA required new protein synthesis to reverse the transformed phenotype. Yoshida et al. (6) reported that TSA inhibited the activity of histone deacetylase, which might lead to expression of the gene(s) (30–32). Thus, the sis-transformed cells treated with TSA might promote the expression of new gene(s) whose product(s) could reverse the transformed phenotype. Contente et al. (33) described the expression of the ras recision gene (r7g) in the flat revertant of Ha-ras-transformed NIH3T3 cells induced by interferon treatment, while the expression of this gene was not observed in the original transformed cells. It might be important to see whether the similar gene is expressed in sis-transformed cells whose phenotype changed to the flat type after TSA treatment.

This phenotypic change by TSA closely resembled that in-
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REFERENCES


duced by n-butyrate, which was also reported to inhibit histone deacetylase (18). However, the induced phenotype by TSA or n-butyrate was quite different from those induced by the other compounds (DMSO and HMBA), which were reported to have the same differentiating activity of Friend leukemia cells as TSA (15, 16). This showed that the ability to induce the flat phenotype should be distinguished from the ability to induce the differentiation. However, both phenomena induced by TSA required new protein synthesis (Fig. 6; Ref. 5). Oishi et al. (34, 35) reported that there were differentiation-inducing factors which were produced during the differentiation of Friend leukemia cells. It would be interesting to know whether TSA would induce these same factors during the differentiation; this may lead to an understanding of the common mechanism of the induction of differentiation.

ACKNOWLEDGMENTS

The authors thank Dr. T. Yoshida for his support during this work.

Fig. 6. Blocking of action of TSA by cycloheximide. The procedure was the same as shown in Fig. 1, except that cycloheximide was added simultaneously to one dish which had already received TSA. (a) control; (b) TSA, 8 ng/ml; (c) TSA, 8 ng/ml, and cycloheximide, 0.1 μg/ml.

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