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A NOVEL COMPOUND, DEPUDECIN, INDUCES PRODUCTION OF TRANSFORMATION TO THE FLAT PHENOTYPE OF NIH3T3 CELLS TRANSFORMED BY RAS-ONCOGENE

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SUMMARY: A novel compound, depudecin, induced production of the flat phenotype of Ki-ras-transformed NIH3T3 cells at the low concentration of 1 μg/ml. This effect was reversible. Actin stress fiber was detected in these cells after depudecin treatment. Almost complete reversion to the flat phenotype was observed at 6 h after depudecin addition. The synthesis of ras-mRNA did not decrease enough with depudecin treatment at the concentration of 10 μg/ml to reverse the transformed morphology.

ras-Oncogene has been reported to be expressed in many human tumors and tumor cell lines (1). The product of ras-oncogene is a kind of GTP-binding protein, and its GTPase activity can be correlated with the transforming activity (2-5). There have been only a few reports on inhibitors against ras-oncogene (6,7). Oxanosine was found to inhibit the function

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Abbreviations used : TSA, trichostatin A; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline (0.15 M NaCl); D-MEM, Dulbecco's modified minimum essential medium; IC_{50}, concentration of drug required for 50% inhibition of cell growth; DMSO, dimethyl sulfoxide; HMBA, hexamethylene-bis-acetamide; ras/NIH3T3, ras-transformed NIH3T3.

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of ras-oncogene by decreasing the pool of guanine nucleotides inside the cells by inhibiting IMP dehydrogenase (6). Azatyrosine was noted to reverse the phenotype of the ras-transformed cells, giving a flat revertant after long-term culture (7). As there are many GTP-binding proteins which act as normal signal transducers, an inhibitor of ras-oncogene might modulate the normal cell function. To avoid selecting such an inhibitor, we devised a screening system using NIH3T3 cells doubly transformed by ras- and src-oncogenes, based on the hypothesis that there would be a common pathway between the signal transduction after signals of ras and src functions (8). If an agent which reverses the phenotype of such transformed cells can be detected by screening, it should be an inhibitor in the pathway of signal transduction after ras and src functions. After screening numerous samples from a culture broth of fungi, we found a novel compound, depudecin, which was isolated from the fungus Alternaria brassicicola. It could induce transformation into the flat phenotype of cells doubly transformed by ras- and src-oncogenes. We describe here the action of depudecin against ras/NIH3T3 cells.

MATERIALS AND METHODS

Chemicals. Depudecin (Fig. 1) and TSA (9) were prepared in our laboratories. DMSO was purchased from Nakarai Tesque Inc. (Kyoto, Japan), hexamethlene-bis-acetamide (HMBA) and MTT from Wako Pure Chemical Co. (Osaka, Japan), and v-Ki-ras probe from Takara Shuzo Co., Ltd. (Kyoto, Japan).

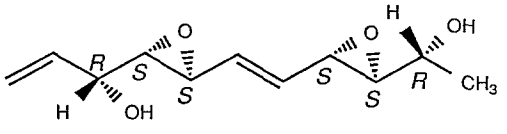

Fig. 1. Structure of depudecin.

Cells. v-Ki-ras-transformed NIH3T3 (ras/NIH3T3) cells were a gift from Dr. K. Yanagihara at the Research Institute for Nuclear Medicine and Biology, Hiroshima University. These cells were grown in Dulbecco's modified minimum essential medium (D-MEM) supplemented with 10% fetal bovine serum.

MTT assay

Inhibition of cell growth by depudecin was determined according to a method described previously using MTT as a dye (10).

Staining of actin stress fiber with rhodamine-phalloizin.

For fluorescence microscopy, the actin stress fiber in cells was observed after staining with rhodamine-phalloizin as reported previously (11).

Northern blotting.

Preparation of total RNA from ras-transformed and normal NIH3T3 cells with or without depudecin treatment, electrophoretic separation of RNA and Northern blot analysis were done as reported previously (12, 13).

RESULTS

Effect of depudecin on the growth and morphology of ras-transformed NIH3T3 cells.

Depudecin at more than 1 μg/ml reversed the transformed phenotype of ras/NIH3T3 cells (Fig. 2). The concentration needed for this activity was much lower than that for inhibition of their growth (the value for IC_{50} was 10 μg/ml, determined by MTT assay) (data not shown). The effect of depudecin was reversible when the cell culture was incubated for more than 24 h at 37°C after its removal (Fig. 3). To find the length of depudecin exposure needed to induce the flat shape, the morphological change was followed after addition of depudecin to ras/NIH3T3 cells. This change was observed after exposure for more than 6 h (Fig. 4). The transformed cells have been reported to lose bundles of actin stress fiber, which are observed in the normal cells (14). Therefore, we examined the appearance of the actin

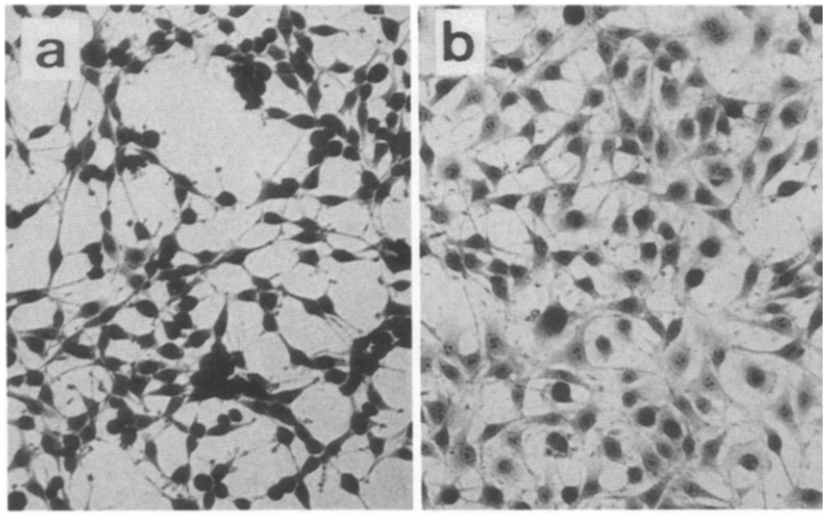

Fig. 2. Effect of depudecin on the morphology of ras/NIH3T3 cells. ras/NIH3T3 cells were inoculated into petri dishes (ϕ 6 cm, Falcon) at 2.5 x 10^4 cells/ml in 5 ml of D-MEM (10% FBS) and cultured overnight in a 5% CO_2 incubator. The cells were incubated for a further 24 h in the absence (a) or the presence (b) of depudecin at 5 μg/ml, and then stained with hematoxylin and eosin, and photographed.

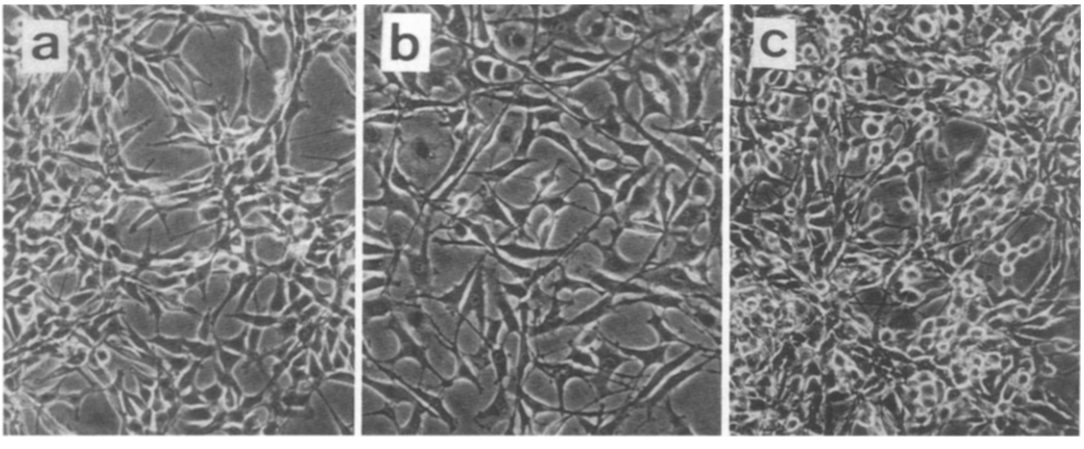

Fig. 3. Reversible effects of depudecin on the morphological change of ras/NIH3T3 cells. ras/NIH3T3 cells were incubated in the absence (a) or the presence (b) of depudecin at 5 μg/ml. After depudecin treatment, the culture was washed and incubated for a further 48 h in the absence of depudecin (c), and photographed.

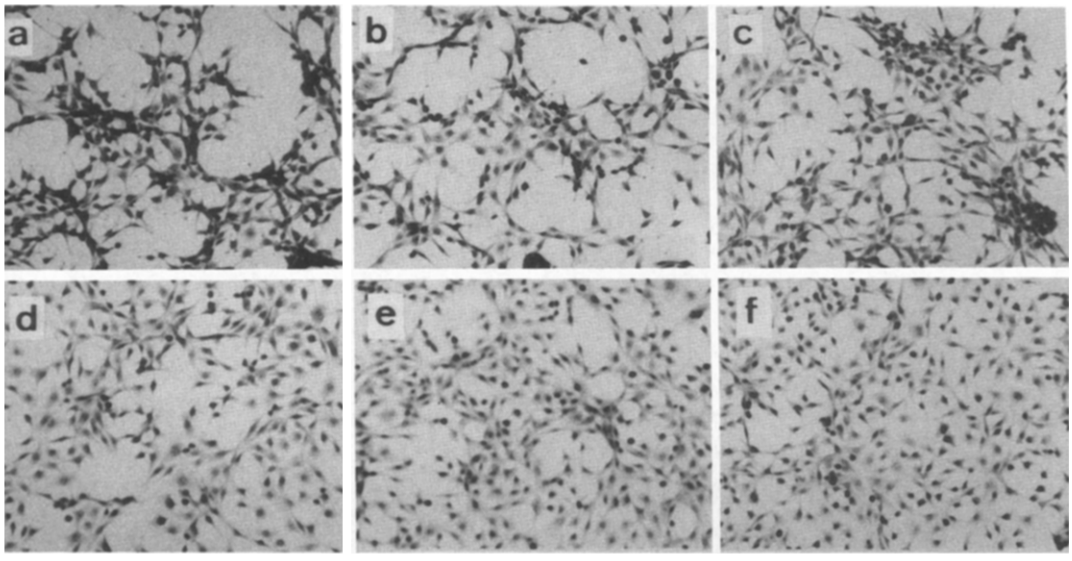

Fig. 4. Kinetics of action of depudecin in reversing the phenotype of ras/NIH3T3 cells. The procedure was the same as that in Fig. 2, except that the morphology of the cells was observed at 0 h (a), 2 h (b), 4 h (c), 6 h (d), 8 h (e), and 10 h (f) after the addition of depudecin, and photographed.

stress fiber in ras/NIH3T3 cells with depudecin treatment. Clear bands were observed by fluoroscence microscopy in ras/NIH3T3 cells after depudecin treatment for 24 h (Fig. 5). To determine whether the morphological reversion was caused by the inhibition of ras-mRNA synthesis, the amount of ras-mRNA was measured by Northern blotting using a ras-oncogene probe in ras/NIH3T3 cells with or without depudecin treatment. Depudecin did not significantly affect the synthesis of ras-mRNA at 10 μg/ml, a concentration at which the phenotype transformation was clearly reversed (Fig. 6).

DISCUSSION

We have described how the novel compound depudecin can reverse the phenotype transformation of ras/NIH3T3 cells at a very low concentration (1 μg/ml) compared to azatyrosine (500 μg/ml). Depudecin induced the transformation of ras/NIH3T3 cells

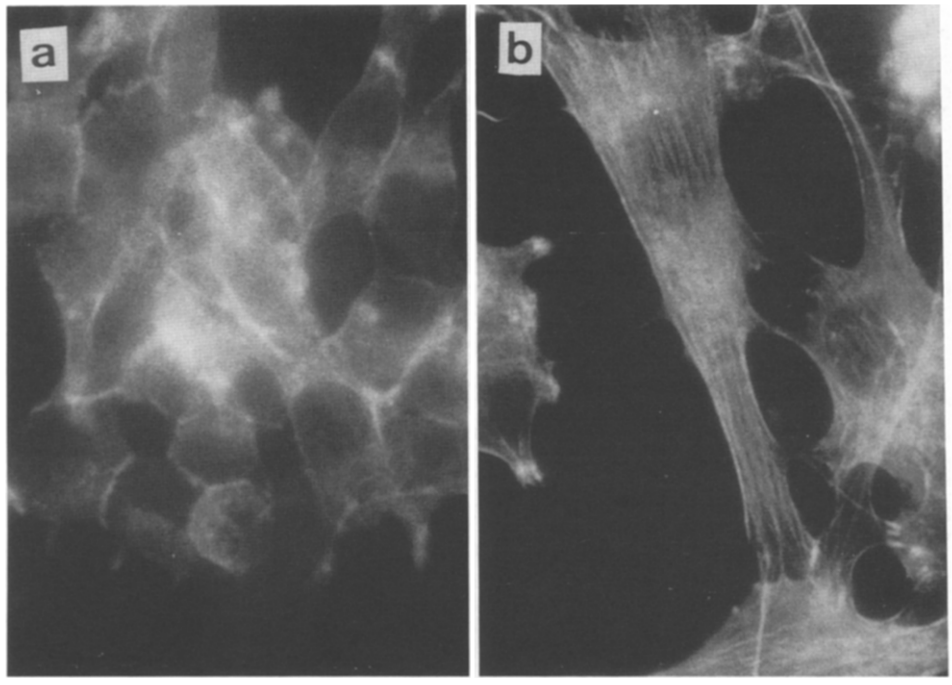

Fig. 5. Restoration of actin stress fiber in ras/NIH3T3 cells with depudecin treatment. Actin stress fiber was stained with rhodamine-phalloizin. (a) Untreated cells, (b) depudecin-treated cells.

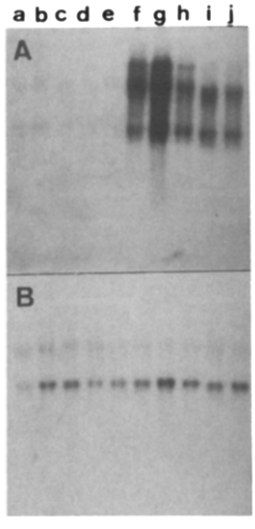

Fig. 6. Effects of depudecin on the synthesis of ras-mRNA. Normal (a, b) and sis-transformed (c, d) NIH3T3 cells were inoculated into a 75 cm^2 bottle (Falcon) at 5 x 10^4 cells/ml in 15 ml of D-MEM (10% FBS) and incubated for 24 h in the absence (a, f) or the presence of depudecin at 1.0 μg/ml (b, g), 2.5 μg/ml (c, h), 5.0 μg/ml (d, i), and 10.0 μg/ml (e, j). (A) v-K-ras probe, (B) actin probe.

to the flat phenotype when the cells had been exposed for more than 6 h (Fig. 4), indicating that the appearance of the flat cells was not due to the selection of depudecin-resistant cells, but to direct action of depudecin on the ras/NIH3T3 cells. This activity of depudecin was reversible, since the flat phenotype of ras/NIH3T3 cells reversed to the transformed one with more than 24 h incubation after removal of depudecin (Fig. 2). Induction of transformation to the flat morphology by depudecin was not caused by inhibition of the synthesis of ras-mRNA (Fig. 6). A preliminary experiment using a monoclonal antibody against $p21^{ras}$ showed that depudecin did not decrease the amount of $p21^{ras}$. These results indicated that the reversion of the transformed phenotype by depudecin was not caused by inhibition of the synthesis of ras-oncogene macromolecules.

Although depudecin has a differentiation-inducing activity on Friend leukemia cells (unpublished data), the induced phenotype by depudecin in ras/NIH3T3 cells was quite different from those induced by TSA, DMSO and HMBA, which have also been reported to have differentiation-inducing activities on Friend leukemia cells (15-17). Our result showed that the mechanism of action of depudecin was different from the other three compounds. Support for this came from the fact that depudecin inhibited the growth of TSA-resistant FM3A mouse mammary gland tumor cells (a gift from Dr. T. Beppu, University of Tokyo) and that depudecin could induce differentiation of the DMSO-resistant variant of a mouse erythroleukemia 745A cell line (a gift from Dr. M. Oishi, University of Tokyo) (unpublished data).

We also examined the effects of depudecin on the morphology of raf-transformed cells (a gift from Dr. K. Yanagihara,

Hiroshima University), as there has been a report that the product of raf-oncogene (Raf-1), which has been shown to be a serine/threonine kinase (18), is a signal transducer after src and ras functions (19). Our results showed that depudecin also reversed the transformed phenotype of these cells (unpublished data). Therefore, the target of depudecin is thought to be involved in the signal transduction after the signal of raf-oncogene.

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