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INHIBITION OF RET TYROSINE KINASE ACTIVITY BY HERBIMYCIN A

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Summary: We examined the effect of herbimycin A, a potent inhibitor of tyrosine kinases, on NIH(ret) cells and TPC-1 papillary thyroid carcinoma cells, both of which express the active *ret* genes. Herbimycin A reversed the morphology of NIH(ret) cells to flat cells with a concomitant reassembly of microfilament bundles. On the other hand, it did not induce a significant change in cell shape of TPC-1 cells. When tyrosine kinase activities of the active *ret* gene products in herbimycin A-treated NIH(ret) and TPC-1 cells were examined in immunocomplex kinase assays, they drastically decreased in both cells as compared with untreated cells. In addition, herbimycin A strongly inhibited tyrosine phosphorylation of 40 kDa and 31 kDa proteins present in the immunoprecipitates of both cells, suggesting that these proteins could associate with the Ret proteins.

The *ret* proto-oncogene (proto-*ret*) encodes a receptor-type tyrosine kinase (1,2) and is activated as oncogenes by DNA rearrangement with other cellular sequences (3-6). In particular, high frequency of its activation (10-25%) was found in papillary thyroid carcinomas of Caucasian people (6,7) while the frequency was very low (<3%) in Japanese patients (8). In most cases of this tumor, the proto-*ret* gene rearranged with D10S170 sequence, a locus formerly designated H4, leading to the generation of the transforming sequence *ret*/PTC (6). Recently, it was shown that this fusion gene was generated by a paracentric inversion of the long arm of chromosome 10, inv(10)(q11.2q21) (9). In addition,

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activation of the proto-*ret* gene was often detected by transfection of NIH3T3 cells with various tumor DNAs although rearrangements were not somatic (3-5).

Herbimycin A, a benzoquinonoid ansamycin antibiotic, is known to reverse the morphology of various cells transformed by tyrosine kinase oncogenes including *src*, *fps* and *abl* genes (10). It inhibited the tyrosine kinase activities of oncogene products and reduced tyrosine phosphorylation of cellular proteins. In the present study, we investigated the effect of herbimycin A on NIH(ret) and TPC-1 (human papillary thyroid carcinoma cell line) cells which express the distinct forms of the active *ret* genes (11,12).

Materials and Methods

Cell lines

NIH(ret) and TPC-1 cells were previously described (11,12).

Antibody

A synthetic peptide corresponding to the carboxy-terminal 20 amino acids of the human proto-Ret protein of 1072 amino acids was prepared by a solid phase method (13) and purified by high performance liquid chromatography. Rabbits were immunized with 100 μg of the synthetic peptide coupled to 500 μg of thyroglobulin in complete Freund's adjuvants.

Immunoprecipitation and *in vitro* protein kinase assay

Cells were lysed in RIPA buffer (20 mM Tris-HCl, pH7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40) containing 0.5 mM sodium orthovanadate and 1 mM phenylmethyl-sulfonyl fluoride (PMSF) and clarified by centrifugation (200,000 x g) for 45 min. The resulting supernatant was incubated with antiserum for 60 min at 4 °C and antigen-antibody complexes were collected using protein A-sepharose (Sigma, U.S.A.). The immunoprecipitates were incubated with [γ-^{32}P]ATP (10 μCi, 6000 Ci/mmol, Amersham, England) in 50 μl of kinase buffer containing 20 mM Tris-HCl, pH7.5, 10 mM $MnCl_2$, 1 mM PMSF, 10 μg/ml Futhan and 0.05% NP-40 for 20 min at 30 °C. The reaction was terminated by addition of SDS sample buffer (20 mM Tris-HCl, pH6.8, 2 mM EDTA, 2% SDS, 10% sucrose, 20 μg/ml bromophenol blue (BPB) and 80 mM dithiothreitol (DTT)) and boiled for 3 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis and [^{32}P]-labelled proteins were detected by exposure of dried gels to films.

Phosphoamino acid analysis

Phosphorylated bands were cut out from the gels and eluted in 1.5 ml of 50 mM NH_4HCO_3. The proteins were digested with 50 μg/ml proteinase K at 37 °C overnight. After lyophilization, the samples were subjected to acid hydrolysis in 50 μl of 6N HCl for 2 hrs at 110 °C. Then, they were suspended in 1 ml of distilled water and lyophilized three times. The phosphoamino acids were resolved by two-dimensional electrophoresis at pH 1.9 and 3.5 on cellulose thin layer plates (14).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton-X for 3 min at room temperature. They were stained with rhodamine-conjugated phalloidin.

Results and Discussion

Tyrosine kinase activity of active *ret* gene products

We first examined the tyrosine kinase activities of the active Ret proteins expressing in NIH(ret) and TPC-1 cells by *in vitro* immunocomplex kinase assays. The Ret proteins were shown to be expressed as 100 kDa and 96 kDa membrane-bound glycoproteins in NIH(ret) cells (11) and as 64 kDa and 56(-59) kDa cytoplasmic proteins (designated *ret*/PTC) in TPC-1 cells (12,15). Lysates from NIH3T3, NIH(ret) and TPC-1 cells were immunoprecipitated with anti-Ret antibodies and incubated with [γ-^{32}P]ATP. Phosphorylated bands corresponding to the Ret proteins were detected in the immunoprecipitates of NIH(ret) and TPC-1 cells (Fig. 1). As shown in Fig. 2, the Ret proteins were phosphorylated predominantly on tyrosine residues, indicating their autokinase activities.

Since the molecular weight of 56 kDa is consistent with that of the immunoglobulin heavy chain, a 56 kDa band detected in the immunoprecipitates

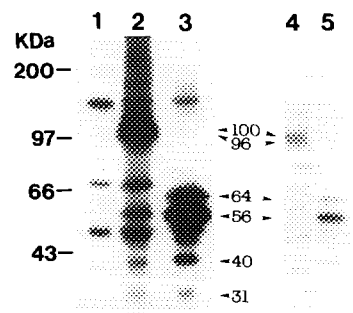

Fig. 1. Phosphorylation of the *ret* oncogene products in *in vitro* immunocomplex kinase assay. NIH3T3 (lane 1), NIH(ret) (lanes 2 and 4) and TPC-1 (lanes 3 and 5) cells were lysed in RIPA buffer and immunoprecipitated with anti-Ret antibodies. The immunoprecipitates were incubated with [γ-^{32}P]ATP in the kinase buffer. The 100 kDa and 96 kDa Ret proteins in NIH(ret) cells and the 64 kDa and 56 kDa *ret*/PTC proteins in TPC-1 cells are indicated. 40 kDa and 31 kDa phosphorylated bands were also detected in the immunoprecipitates of both cells. A 56 kDa band comigrated with the immunoglobulin heavy chain. Left (lanes 1 to 3) and right (lanes 4 and 5) panels represent 6 hrs and 20 min exposure of films, respectively.

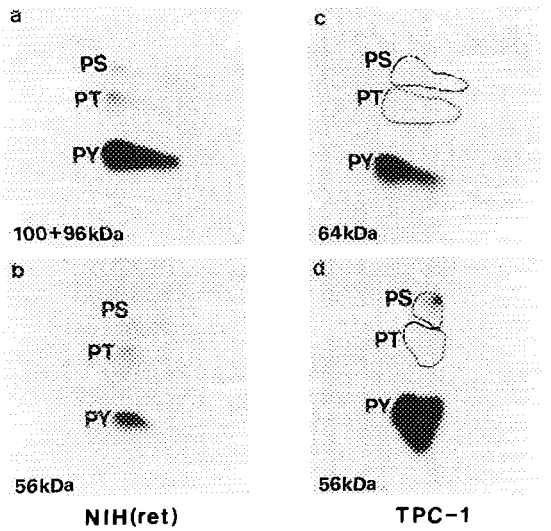

Fig. 2. Phosphoamino acid analysis of the *ret* oncogene products. The phosphorylated bands corresponding to the100 kDa and 96 kDa Ret proteins in NIH(ret) cells (a), the 64 kDa *ret*/PTC protein in TPC-1 cells (c) and the 56 kDa proteins in both cells (b, d) were analyzed by two-dimensional electrophoresis. Since it was impossible to separate the 100 kDa and 96 kDa bands, a mixture of both bands was analyzed (a).

of NIH(3T3) cells seemed to represent its tyrosine phosphorylation. Similarly, it is likely that the 56 kDa phosphorylated bands in TPC-1 cells included both *ret*/PTC protein and immunoglobulin heavy chain. In addition, 40 kDa and 31 kDa phosphorylated bands were present in the immunoprecipitates of both cells (Fig. 1). These two bands may be cellular proteins coprecipitated with the Ret proteins. Phosphoamino acid analysis indicated that these proteins were also phosphorylated on tyrosine residues (data not shown).

Effect of herbimycin A on NIH(ret) and TPC-1 cells

Herbimycin A can convert the morphology of cells transformed by tyrosine kinase oncogenes to a normal phenotype (10). Thus, we estimated its effect on NIH(ret) and TPC-1 cells. NIH(ret) cells were reversed completely to flat cells in the presence of herbimycin A (0.2-0.5 μg/ml). Consistent with the morphological conversion, the reorganization of microfilament bundles occurred in herbimycin-treated NIH(ret) cells (Fig. 3). On the other hand, Herbimycin A did not induce a significant change of the morphology of TPC-1 cells. The *ret*/PTC proteins may affect the growth of TPC-1 cells rather than their morphology.

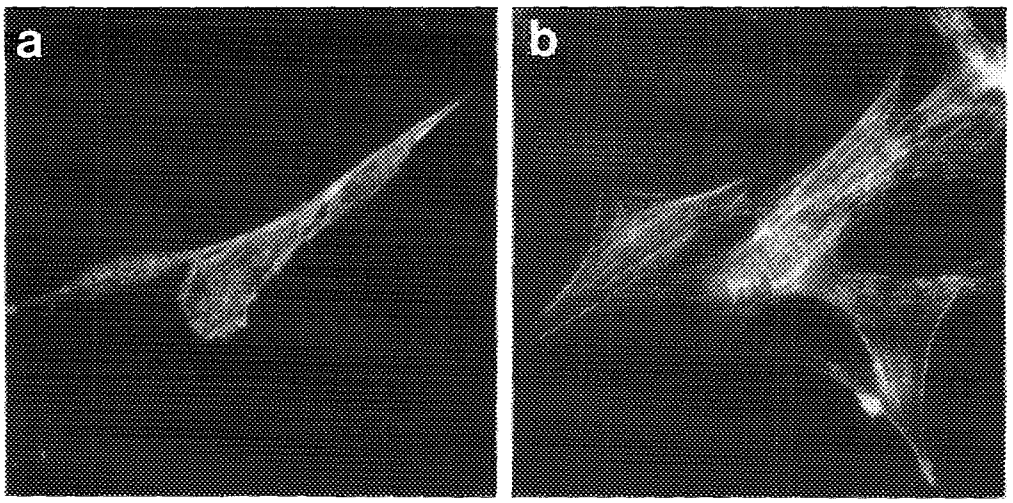

Fig. 3. Immunofluorescence analysis of F actin in herbimycin-treated NIH(ret) cells. NIH(ret) cells treated (b) or untreated (a) with herbimycin A (0.5 μg/ml) for 16 hrs were stained with rhodamine-conjugated phalloidin.

We investigated the tyrosine kinase activities of the Ret proteins in herbimycin-treated and untreated cells. Incubation of the immunoprecipitates of herbimycin-treated cells with [γ-^{32}P]ATP showed the drastic inhibition of the *ret* tyrosine kinase activities (Fig. 4), although the expression levels of the active Ret proteins were almost the same in the herbimycin-treated and untreated cells (data not shown). In addition, even when the immune complex was extensively washed,

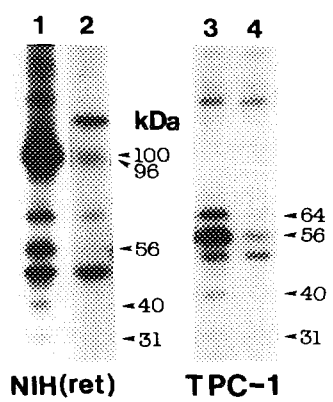

Fig. 4. Inhibition of *ret* tyrosine kinase activity by herbimycin A. NIH(ret) and TPC-1 cells were cultured in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of herbimycin A (0.5 μg/ml) for 16 hrs. The immunoprecipitates were incubated with [γ-^{32}P]ATP.

the *ret* tyrosine kinase activities were not recovered, indicating that the inhibition by herbimycin A was irreversible.

In addition to the Ret proteins, tyrosine phosphorylation of the 56 kDa, 40 kDa and 31 kDa bands also decreased. Thus, it is possible that these proteins were phosphorylated by the Ret protein. Previous studies showed that the active Ret proteins in NIH(ret) and TPC-1 cells are present as membrane-bound and cytoplasmic proteins respectively (11,12). The fact that the same 40 kDa and 31 kDa tyrosine phosphorylated proteins were detected in the immunoprecipitates of both cells suggests the possibility that two distinct forms of the active Ret proteins could transduce their signals through common target proteins irrespective of the difference of the intracytoplasmic localization.

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