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SELECTIVE INHIBITORY EFFECT OF BENZALDEHYDE ON THE GROWTH OF SIMIAN VIRUS 40-TRANSFORMED CELLS

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Summary: Normal and Simian virus 40-transformed cells were treated with benzaldehyde which has been found in figs as a potent carcinostatic element. Benzaldehyde notably inhibited the growth of transformed cells when cells were cultivated in the presence of 25 μg/ml of benzaldehyde. No significant effect was observed at this concentration on the growth of normal cells. In the presence of 50 μg/ml of benzaldehyde the growth of transformed cells was completely inhibited. The growth inhibition by 50 μg/ml of benzaldehyde was reversible and cells resumed growth after removal of the chemical from the media. A labeling experiment of cells with [^{14}C]-benzaldehyde indicated that the reaction of benzaldehyde with cellular proteins was limited mainly to exposed membrane proteins.

Various aldehydes have been reported to have anti-tumor activity including citral, citronellal (1-3), kethoxal, glyoxal and other α-ketoaldehydes (4), methylglyoxal (5) and others (6-11), which commonly possess a carbon-carbon double bond(s) or a ketone(s) conjugated with the terminal aldehyde. Kochi et al. reported that the extract of figs is effective for the therapy of cancer patients (12). They further identified the active principle as benzaldehyde (13,14). This substance was effective against naturally occurring and certain implanted tumors in mice (13). It was also clinically useful in the treatment of patients with various types of cancer (12). To investigate the mechanism of action of benzaldehyde, we tested the effect of this compound in a simpler in vitro system. In this paper we show selective inhibition of the growth of virus-transformed cells by benzaldehyde added in

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the culture medium and also a preliminary result indicating preferential interaction of benzaldehyde with cell surface components.***

MATERIALS AND METHODS

Cell lines and cell culture. The 3Y1-B clone 1-6, a cell line of Fisher rat embryo fibroblast and a derivative (W-3Y-23) transformed by Simian virus 40 (SV40) were kindly donated by Dr. Yamaguchi of the Institute of Medical Science, University of Tokyo (15). C3H2K-C4, a clonal line of C3H2K derived from C3/He mouse kidney and W-2K-11, a clonal line of SV40 transformed C3H2K-C4 were also obtained from Dr. Yamaguchi. Cells were grown in plastic Petri dishes (Falcon) in Eagle's minimum essential medium containing 10% new born calf serum and antibiotics (100 unit penicillin, 100 µg streptomycin and 60 µg kanamycin per ml of medium) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For growth curve experiments cells were plated at a density of 8×10^4 cells per 60 mm diameter dish and the benzaldehyde treatment started 20 hours later (that is after the cells have attached). The number of viable cells was determined daily. Cells were detached by trypsinization (0.25% trypsin, Gibco) for 15 to 20 minutes at room temperature and viable cells were counted in a hemacytometer in the presence of trypan blue.

Radioactive labeling of cells. For labeling of cells with [^{14}C]-benzaldehyde, cells grown in monolayer in 60 mm dishes (80% confluent) were washed three times in Eagle's minimum essential medium containing 10% new born calf serum. The labeling was performed in 5 ml of above medium containing 25 µCi of [^{14}C]-benzaldehyde for 1 hour at 37°C. Cells were washed three times on monolayer with Dulbecco's phosphate buffered saline (PBS) and incubated for 10 minutes at 37°C in PBS containing 1 mM $NaBH_4$. Cells were then washed three times with PBS and solubilized by boiling for 1 minute in 0.5 ml of sample buffer containing 62.5 mM Tris-HCl 3% SDS, 5% β-mercaptoethanol and 10% sucrose at pH 6.8. Lactoperoxidase catalysed iodination of monolayer cells using carrier free iodine-125 followed the procedure of Hynes (16). Samples for gel electrophoresis were prepared as described above.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The discontinuous system described by Laemmli (17) was used. The 1 mm thick slab gel consisted of 1.0 cm long stacking gel and 9.0 cm long resolving gel. After completion of the gel electrophoresis, the gel was stained with 0.05% Coomassie brilliant blue R250 in isopropanol : acetic acid : water (5:2:13, v/v/v) and destained in methanol : acetic acid : water (1:1:8, v/v/v). Radioactivity in the gel was detected by means of either autoradiography for ^{125}I or fluorography (18) for ^{14}C.

Media, enzymes, chemicals and radioisotopes. Eagle's minimum essential medium was purchased from Nissui Pharmaceutical Co., Tokyo. New born calf serum was prepared by Mitsubishi-Kasei Institute of Life Sciences, Tokyo. Lactoperoxidase and glucose oxidase were purchased from Sigma Chemical Co., and Boehringer Mannheim, respectively. Benzaldehyde was purified through a column of silica gel G to remove oxidized contaminants. Benzaldehyde thus purified gave a single spot on a silica gel G plate developed by hexane : ethyl acetate (17:3, v/v). Stock solutions of benzaldehyde dissolved in degassed water at a concentration of 5 mg/ml and sealed under argon gas were stored frozen. [Carbonyl-^{14}C]-benzaldehyde (specific radioactivity, 3.96 Ci/mole) and carrier free Na ^{125}I were purchased from Amersham.

*** The authors (especially K.S.) propose to call "periactins" the substances which have physiological and possibly therapeutic activity through interaction with the cell membrane and especially with the membrane proteins.

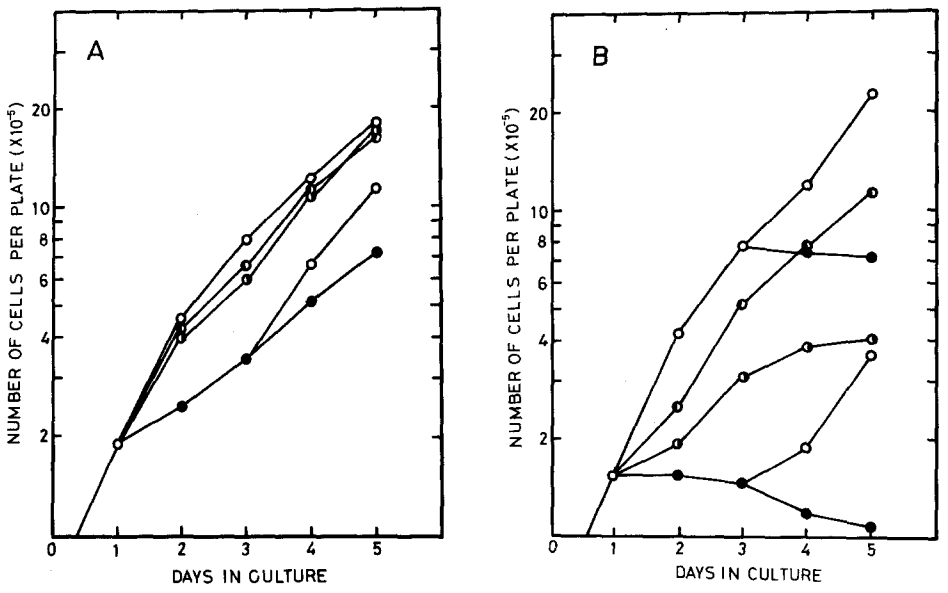

Figure 1. Growth curves of normal (A) and SV 40-transformed cells (B) cultivated in the presence of o—o; 0 µg, ●—●; 10 µg, ◐—◐; 25 µg, ●—●; 50 µg of benzaldehyde per ml of medium.

RESULTS AND DISCUSSION

The effect of benzaldehyde was tested in an *in vitro* system, using transformed and normal cells in culture. Typical growth curves of normal and SV40 transformed rat fibroblast cells (3Y1-B1-6 and W-3Y-23, respectively) growing in monolayer in the presence of benzaldehyde are shown in Fig. 1. The growth of normal cells was little affected by the presence of up to 25 µg of benzaldehyde per ml of medium. Normal cells grew at a slower rate in the presence of 50 µg/ml of the chemical (Fig. 1A). In contrast, the growth of transformed cells was notably inhibited by 25 µg/ml of benzaldehyde. It was completely stopped at the concentration of 50 µg/ml of the chemical. Over ninety percent of cells which remained on the plate after two days of cultivation in the presence of 50 µg/ml of benzaldehyde were still viable as revealed by the exclusion of trypan blue, and the cells resumed growth immediately after changing to the medium free of benzaldehyde (Fig. 1B). In experiments employing normal and transformed mouse fibroblast cells (C3H-2K-

C4 and W-2K-11, respectively) similar results were obtained (data not shown). Benzoic acid, a possible oxidation product of benzaldehyde had no differential effect on the growth of transformed cells when tested under the conditions used for benzaldehyde (19).

Aryl aldehydes are known to react with amino and sulfhydryl groups of proteins forming a Schiff base or thioketal respectively (20). To find the components which react with benzaldehyde, cells growing in monolayer were incubated with [^{14}C]-benzaldehyde in the presence of calf serum, reduced with $NaBH_4$ after removal of unbound benzaldehyde and analyzed by SDS-polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue for proteins. The radioactivity in the gel was detected using a technique of fluorography (18). Fig. 2 shows that the pattern of [^{14}C]-labeled bands was much different from that of total cellular protein bands, indicating a specific interaction of benzaldehyde with only certain species of cellular proteins. Since $NaBH_4$ treatment was not necessary for the labeling (not shown) the exact mechanism of binding of benzaldehyde to proteins is not known. The band most intensely labeled by [^{14}C]-benzaldehyde with a molecular weight of approximately 220,000 dalton (Fig. 2) seems to be the large external transformation sensitive (LETS) protein described by Hynes (16) from the following criteria: 1) the molecular weight; 2) higher intensity of labeling in normal cells than in transformed cells; 3) sensitivity to trypsin treatment (not shown); 4) intense labeling by lactoperoxidase catalysed iodination reaction (see next). To know the location of the proteins labeled by benzaldehyde, the [^{14}C]-labeled protein pattern was compared with the pattern of cellular proteins labeled by lactoperoxidase catalyzed iodination of intact cells, a labeling technique specific to exposed membrane proteins (16). The two patterns are closely similar (Fig. 2), indicating preferential interaction of benzaldehyde with cell surface components under the culture conditions used in the experiments shown in Fig. 1, in spite of the fact that benzaldehyde itself can penetrate through the membrane when cells are incubated in buffer (20).

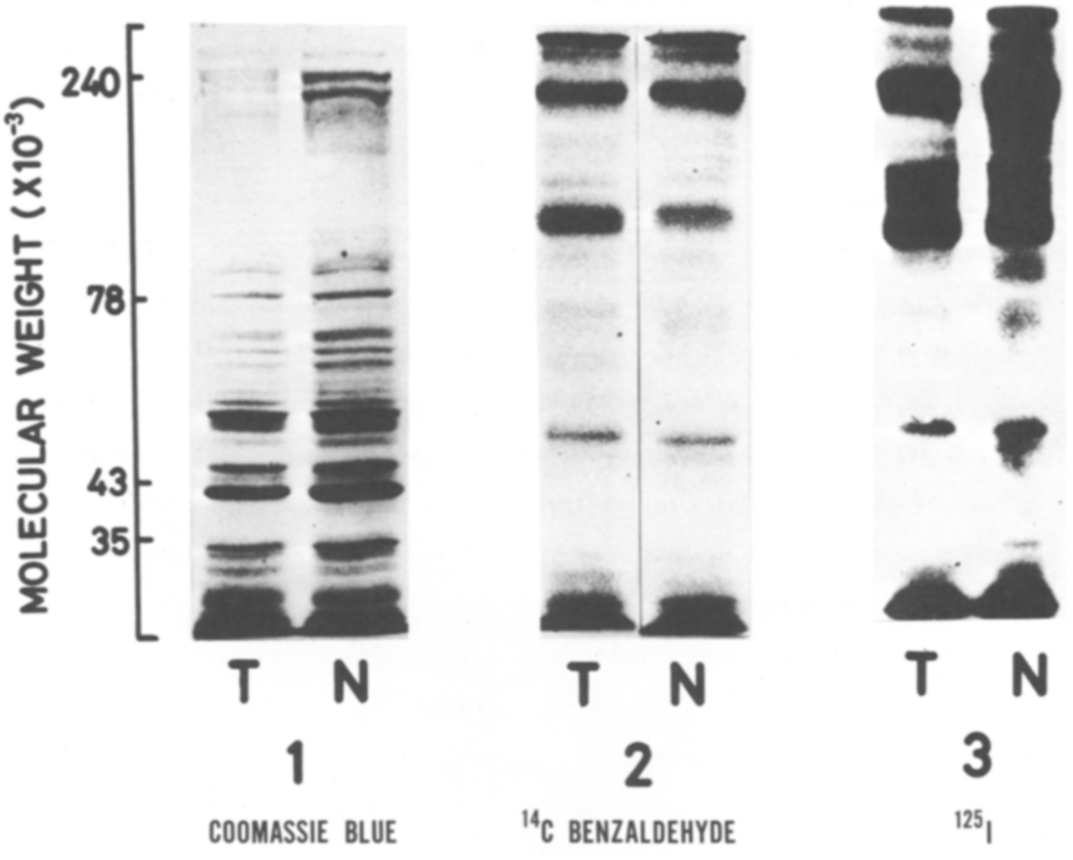

Figure 2. SDS-polyacrylamide gel electrophoretic pattern of normal (N) and SV 40-transformed (T) cells. The bands were detected by staining with Coomassie brilliant blue for total cellular proteins [1], by fluorography for [^{14}C]-benzaldehyde labeled proteins [2] or by autoradiography for [^{125}I]-labeled proteins [3]. The molecular weight was estimated by using erythrocyte ghost protein [8] as standards.

However the apparently specific labeling of exposed surface proteins may also be due to a reaction of ^{14}C-benzaldehyde with a serum protein in the medium, followed by the association of the labeled protein with the membrane.

It is well known that the LETS protein (alias fibronectin) has an important role in cellular adhesion and is usually less abundant in transformed cells than in normal cells (21). If, as in our experiments, the LETS protein is then chemically modified by benzaldehyde, the attachment of transformed cells will be impaired. Support for this interpretation comes

from our observation that transformed cells plated in a medium containing 25 μg/ml benzaldehyde adhere poorly and do not spread on the tissue culture dishes (but normal cells are able to attach and grow).

It is of special interest in these studies that Erwin et al. observed an inhibition of $Na^+ + K^+$-activated adenosine triphosphatase activity of mouse brain by various biogenic aldehydes including benzaldehyde (22). Another example of an alteration of biological activity of proteins by Schiff base formation was reported by Zaugg et al. (20). They found that Schiff base adducts of sickle cell hemoglobin with various aromatic aldehydes have an increased affinity for oxygen, thus decreasing the extent of sickling.

Our results may be interpreted in the light of these findings. If cell surface protein(s) are modified by benzaldehyde, one would expect the properties of these protein(s) to be altered. These changes may explain the selective cytotoxicity to the transformed cells since significant differences exist between normal and transformed cells (23). The strikingly selective inhibition of uptake of thymidine by benzaldehyde into transformed cells only is consistent with this hypothesis (Watanuki and Sakaguchi, in preparation).

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