Distinct Effects of Clinically Used Anthracycline Antibiotics on ras Oncogene-Expressed Cells

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Doxorubicin, pirarubicin, and FAD-104, but not aclacinomycin or MX 2, flattened the morphology of NIH3T3 cells that had been transformed by human H-ras and K-ras. The effect appeared on almost all cells, as early as 2 d following exposure to the antibiotics at concentrations inhibiting cell growth by 50% or more. The morphological alteration accompanied other normal cell phenotypes, such as the restoration of actin stress fibers, anchorage dependence of cell growth and an increase in nucleoside diphosphate (NDP) kinase activity. NIH3T3 cells transformed by src and other tumor cell lines responded less prominently, if at all.

Keywords ras; anthracycline; morphology; actin fiber; anchorage independent growth; NDP kinase

Clinical studies have identified activated ras oncogenes in a wide variety of human neoplasms; hence, inhibitors of ras-gene functions have been widely sought for a possible new type of antitumor chemotherapy targeting the signaling pathways where ras p21 is involved, i.e., ras-specific chemotherapy. We found some new antibiotics in such trials, but so far we have not been able to test their usefulness as antitumor drugs because of limited production. In addition to the efforts to find new antibiotics, we examined some clinically used antitumor drugs to determine whether they had any selective activity against ras oncogene-expressed cells (referred to as anti-ras activity). The study was based on the expectation that, if the target tumors were diagnosed to have activated ras oncogenes, better chemotherapy could be achieved with drugs having anti-ras activity.

Materials and Methods

Cell Lines NIH3T3 cell lines transformed with a human c-K-ras gene carrying a point mutation at codon 12 (K-ras 3T3) or with a human c-H-ras gene carrying a point mutation at codon 61 (H-ras 3T3) were provided by Dr. T. Sekiya, National Cancer Center Research Institute, Tokyo. An NIH3T3 cell line infected with Rous sarcoma virus SR-D (src 3T3) was provided by Dr. S. Kawai, the Institute of Medical Science, University of Tokyo, Tokyo.

Cell Culture in Liquid Medium Cells were grown under the conditions described below in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated calf serum (DMEM-CS), in 5% CO2-containing humidified air at 37°C. To assay growth inhibition by drugs, the cells were seeded at 1–1.5 x 10⁶ cells/ml DMEM-CS/2% well of Coster 24-well tissue culture clusters (day 0). Drugs were added to the wells on day 1, and incubation continued until day 3. Cells were photographed during the culture period, if necessary. Cell growth was quantified by cell counting or by a colorimetric MTT (tetrazolium) assay, which was conducted as follows. A culture was combined with 100μl of 5mg MTT/ml, incubated for 4h at 37°C, combined with 500μl of 20% sodium dodecyl sulfate (SDS) and incubated at 37°C for 16h in humidified air to solubilize the MTT-formazan product. The content in a well was mixed by pipetting, and the optical density at 540 nm was read against a background which had been run with 1 ml DMEM-CS/well (without cells). The results of the colorimetry closely paralleled cell counting. Cell growth of a test run was expressed as a relative value calculated using the following equation:

\[
\frac{\text{cell number on test run on day } 3 - \text{cell number on day } 1}{\text{cell number on control run on day } 3 - \text{cell number on day } 1} \times 100 (%)\]

Cell Culture in Semisolid Medium A semisolid medium was made up of DMEM-CS and 0.6% agarose (GIBCO), and 3 ml portions therefrom were added to 21 cm² dishes (Falcon 3022) at 40°C and left at room temperature to gel (bottom layer). Two ml portions of another semisolid medium consisting of the same components, but with a suspension of either H-ras 3T3 at 2 x 10⁶ cells/ml or src 3T3 at 4 x 10⁶ cells/ml, were layered over the bottom layer at 40°C and allowed to gel as above (top layer). The dishes received 1 ml aliquots of DMEM-CS dissolving drugs and were incubated for 14 d for H-ras 3T3 or 12 d for src 3T3. Colonies larger than 200μm in diameter were counted in each dish. Runs were triplicated.

Staining Actin Stress Fiber K-ras 3T3 cells were suspended in DMEM-CS at 5 x 10⁶ cells/ml and 0.15 ml portions therefrom were placed on cover glasses, which had been set in 8 cm² dishes (Falcon 3001) and incubated for 20 h. The medium was removed, fresh medium (1 ml/dish) dissolving a drug was added, and incubation was continued for another 48 h. F-actin was stained as reported.64 Cells adhering to each cover glass were freed of the medium by rinsing with 1 ml of phosphate-buffered saline (PBS)-II twice, and each cover glass was then immersed in 1 ml of 3% formaldehyde for 20 min at room temperature. The fixed cells were washed with 1 ml of PBS-II three times, permeabilized with 1 ml of 0.2% Triton-X-100 in PBS-II for 20 s, rinsed three times with PBS, and covered with 250 μl of 10 units of rhodamine-phalloidin/ml of PBS for 20 min at room temperature to stain F-actin. After being washed with 1 ml of PBS eight times, the stained cells were covered with 50% (v/v) glycerol/PBS (one drop on a cover glass) and inspected under a fluorescence microscope.

Measurement of NDP Kinase Cells were seeded at 9 x 10⁴ cells (H-ras 3T3, K-ras 3T3) or 4.5 x 10⁵ cells (src 3T3)/10 ml DMEM-CS/5%2 dish (Corning 25200), cultured for 20 h, treated with drugs and cultured for another 48 h. Each cell layer was rinsed with 5 ml of PBS three times, and 10 ml of PBS, dispersed, and then centrifuged at 150 x g for 3 min. The cells were suspended in 0.5 ml of PBS and disrupted with a Branson sonifier at 10% power for 5 s. The sonicate was centrifuged at 15000 x g for 10 min and the supernatant (cell extract) was submitted to NDP kinase activity determination using a reaction coupled with an excess amount of pyruvate kinase,6 as follows. A reaction mixture contained, in 0.5 ml, 50 mm Tris-HCl, pH 7.4, 80 mm KCl, 5 mm MgCl₂, 1 mm 1-phospho-D-fructose, 1 mm ATP, 0.2 mm Na-2HPO₄, 0.5 mm MgCl₂, 0.5 mm Na-2HPO₄, 0.5 mm MgCl₂, 1 mm Na bicarbonate, 1 mm 1-phospho-D-fructose, 0.5 mm ATP, 0.2 mm Na-2HPO₄, 0.5 mm MgCl₂, and 1 mm 1-phospho-D-fructose. The reaction was started with 5 μl of cell extract (last added). The reaction proceeded at 37°C for 20 min and was terminated by mixing with 0.08 ml of 0.1% 5,5'-dinitro-2-hydroxydisulfonic acid, 0.05 ml of 50 mm Tris-HCl, 50 mm NaCl, 50 mm KCl, 1 mm 1-phospho-D-fructose, 0.5 mm ATP, 0.2 mm Na-2HPO₄, 0.5 mm MgCl₂, 0.5 mm Na-2HPO₄, 0.5 mm MgCl₂, and 1 mm 1-phospho-D-fructose. The reaction was stopped, and 5 μl of 100-fold diluted test extract (last added). The reaction proceeded at 37°C for 20 min and was terminated by mixing with 0.08 ml of 0.1% 5,5'-dinitro-2-hydroxydisulfonic acid, 0.05 ml of 50 mm Tris-HCl, 50 mm NaCl, 50 mm KCl, 1 mm 1-phospho-D-fructose.

Results and Discussion

Alteration of Cell Morphology In a previous paper we reported that a new anthracycline, 2-demethyl stefycin D, altered the morphology of NRK cells expressing ras
oncogene (K-ras NRK, CRL-1569, ATCC) into that of NRK-49F (CRL-1570, ATCC), the normal counterpart. We later found that some other anthracyclines shared this activity, although to a lesser extent. As Fig. 1 shows, doxorubicin flattened the morphology of K-ras 3T3 at concentrations higher than IC$_{50}$. This cell-flattening effect appeared on almost all cells, as early as 2d following exposure to the antibiotic. This effect was considered irreversible; the flattened cells did not return into the original morphology on further culture in the drug-free medium and they eventually died. Pirarubicin and FAD-104$^{45}$ also changed the cell morphology in a similar manner. By contrast, aclacinomycin and MX 2$^{29}$ did not show such activity at any concentration. The experiment was extended to other cell lines, such as K-ras NRK H-ras 3T3, src 3T3, raf 3T3 and abl 3T3. Here again, only the first three compounds mentioned showed similar cell-flattening effects on the cell lines expressing any of the ras oncogenes, but less prominently with src 3T3 and the others.

**Restoration of Actin Stress Fiber**  The alteration of cell morphology of K-ras 3T3 by doxorubicin accompanied the restoration of actin stress fiber, another characteristic of a normal cell phenotype (Fig. 2). The same was true with pirarubicin and FAD-104, but not with aclacinomycin and MX 2. These results led us to test whether doxorubicin, pirarubicin and FAD-104, but not aclacinomycin and MX-2, could revert other tumorous phenotypes, such as anchorage-independent cell growth (growth in a soft agar medium).

**Inhibition of Anchorage-Independent Cell Growth** An antibiotic capable of inducing a normal cell phenotype should inhibit cell growth more strongly in a soft agar medium than in the usual liquid medium. The results proved this to be the case; doxorubicin, pirarubicin and FAD-104 were about 30 times as active as aclacinomycin in inhibiting the growth of H-ras 3T3 cells in a soft agar medium, whereas there was no such difference in the liquid medium (Fig. 3, Table I). The same type of experiment conducted with src 3T3 showed less difference among the compounds (Table I). K-ras 3T3 was not tested in this experiment because the cells grew only 1/50 as fast as those of H-ras 3T3 in the semisolid medium. The low level of anchorage-independence of K-ras 3T3 may not be inherent to the K-ras gene, but due rather to some artifactual factors, such as gene dosage, state of the exogenous gene, etc.
**TABLE I. Effect of Anthracyclines on Growth of H-ras 3T3 Cells and src 3T3 Cells**

<table>
<thead>
<tr>
<th>Drug</th>
<th>H-ras 3T3 cells</th>
<th>src 3T3 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (ng/ml)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Growth in agar medium (a)</td>
<td>Growth in liquid medium (b)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.5</td>
<td>46</td>
</tr>
<tr>
<td>Pirurubicin</td>
<td>1.0</td>
<td>23</td>
</tr>
<tr>
<td>FAD-104</td>
<td>4.9</td>
<td>100</td>
</tr>
<tr>
<td>Aclarubicin</td>
<td>52.0</td>
<td>72</td>
</tr>
<tr>
<td>MX2</td>
<td>42.0</td>
<td>110</td>
</tr>
</tbody>
</table>

Cloning efficiencies in the agar medium of H-ras 3T3 cells and src 3T3 cells are 10.8% and 3.3%, respectively.

Fig. 3. Effect of Doxorubicin and Aclarubicin on the Growth of H-ras 3T3 Cells

Cells were incubated with doxorubicin (○) or aclarubicin (△) in an agar medium (-----) or liquid medium (———).

**Activation of NDP Kinase Metastasis** is the most harmful trait of tumor cells and it has been known that metastatic potential is inversely correlated with the expression of NM23 (NDP kinase gene) in some cell lines transformed with activated H-ras.$^{10}$ An interesting finding in this connection was that doxorubicin, pirurubicin and FAD-104 elevated the NDP kinase level in H-ras 3T3 as high as 160% of the control, while aclarubicin lowered the enzyme level (Table II). However, in K-ras 3T3, NDP kinase was lowered by doxorubicin.

**Anthracyclines versus Oncogenes** Induction of normal cell phenotypes in ras-expressing cells by doxorubicin, but not by aclarubicin, as demonstrated above, is just the opposite of the ability of the two antibiotics in inducing the maturation of promyelocytic leukemia cells into phagocytes; aclarubicin, but not doxorubicin, induced the differentiation of HL-60. $^{11}$ It has been suspected that myc oncogene has a role in phagocytic maturation. $^{12}$ The clinical effectiveness of aclarubicin to a leukemic patient who did not respond to doxorubicin was reported, $^{13}$ although the molecular mechanism was not known. Now that the bedside diagnosis of oncogenes and tumor suppressor genes is becoming practical, $^{14}$ better chemotherapy could be conducted by using drugs which have specificity to the genetic alteration of target tumors. Studies are in progress to elucidate how doxorubicin and the other two interfere with the signaling pathway via p21$^{*}$$. A preliminary experiment indicated that K-ras 3T3 cells which had been exposed to doxorubicin still retained a control level of p21$^{*}$$. The target point, therefore, must be a step downstream of the signaling pathway. DNA topoisomerase II is considered a major target of anthracyclines. Since doxorubicin and aclarubicin almost equally inhibit this enzyme (Setsuko Kunimoto, personal communication), it is unlikely that the inhibition of this enzyme is involved.

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**References and Notes**


