Expression of cytokeratin confers multiple drug resistance

(multidrug resistance/cytoskeleton)

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ABSTRACT The cytokeratin network is an extensive filamentous structure in the cytoplasm whose biological function(s) is unknown. Based upon previous data showing the modification of cytokeratin by mitoxantrone, we investigated the ability of cytokeratin networks to influence the survival response of cells to chemotherapeutic agents. We have compared the survival of mouse L fibroblasts lacking cytokeratins with that of L cells transfected with cytokeratins 8 and 18 in the presence of chemotherapeutic drugs. The expression of cytokeratins 8 and 18 conferred a multiple drug resistance phenotype on cells exposed to mitoxantrone, doxorubicin, methotrexate, melphalan, Colcemid, and vincristine. The degree of drug resistance was 5–454 times that of parental cells, depending upon the agent used. Drug resistance could not be attributed to altered growth characteristics, altered drug accumulation, or an altered drug efflux in the transfected cells. Cytokeratin does not confine resistance to ionizing radiation, which damages DNA independently of intracellular transport mechanisms. These data suggest a role for cytokeratin networks in conferring a drug resistance phenotype.

A major obstacle to successful cancer chemotherapy is tumor cell resistance to chemotherapeutic agents. Resistance may develop in response to selection pressure exerted by drug treatment or may be part of an inherent cellular response without prior treatment. In acquired resistance, a deviation from the normal cell phenotype occurs, and that deviation (for example, overexpression of the P-glycoprotein in multidrug resistance; ref. 1) renders cells less sensitive to the antiproliferative effects of the drug. De novo resistance is defined as a phenotypic characteristic in place prior to drug treatment.

We have hypothesized previously that cytokeratin filament networks could alter the survival response of cells to chemotherapeutic agents, based partly on our observations of drug-induced cytokeratin modifications (2, 3). Cytokeratins are members of a diverse group of intermediate-filament proteins that share common structural motifs, including a central nonhelical domain flanked by nonhelical termini (4). They are obligate heteropolymers expressed specifically in epithelial cells, to which they lend structural integrity. Cytokeratins may also function in intracellular or cell-to-cell signaling (reviewed in ref. 5).

Cytokeratin expression is a persistent phenotype which is used in diagnosing cancers, enabling identification of tumor type, metastatic tumor origin, and stage of disease (6). Changes in the pattern of expression of cytokeratin during tumor progression can involve a reversion to the simple epithelial cytokeratins 8 and 18 (7). These particular cytokeratins are also preferentially expressed in many carcinoma cell lines (6, 8).

We utilized a mouse fibroblast cell line and a transfected variant expressing cytokeratins 8 and 18 (9) to test whether cytokeratin expression could alter the cell survival response to six different chemotherapeutic drugs.

MATERIALS AND METHODS

Tumor Cell Lines, Growth Curves, and Cell Cycle Distributions. Parental L cells and transfected LK8+18 cells were obtained from Robert Oshima (La Jolla Cancer Research Foundation, La Jolla, CA) and maintained as described (9). Mock transfectants (LPBMC) were created by simultaneous calcium phosphate transfection of pGEM-3 (Promega) and pSV2neo (10) in a weight ratio of 10:1. LPBMC cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, gentamicin (30 μg/ml), and G418 (400 μg/ml). All cells were kept in a humidified incubator under 95% air/5% CO2 at 37°C for at least 2 days after passage prior to experiments. Growth rates were obtained by seeding 1 × 105 cells in several individual 35-mm dishes and counting them daily.

To prepare cells for flow cytometric cell cycle analysis, cells were trypsinized and resuspended in DMEM. As resuspended cells were being gently vortexed, two volumes of a 3:1 solution of methanol/acetic acid were added to fix the cells prior to analysis. The suspension was then washed free of acid, treated with DNase-free RNase A (0.1 mg/ml) and stained with propidium iodide (100 μg/ml). Stained samples were stored refrigerated in the dark. Cell cycle analysis was performed as described (3).

Drug Resistance Assays. Drug resistance was assayed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] dye assay (11). Briefly, cells were plated in 96-well plates in the presence of various chemotherapeutic agents. After 3 days, MTT dye was added to each well, and plates were incubated an additional 4 hr. After the contents of the wells were aspirated, dimethyl sulfoxide was added to the monolayers and plates were shaken for 5 min. The absorbance at 540 nm of each well was immediately recorded. Absorbance values were normalized to the values obtained from wells incubated with cells but without drug.

Indirect Immunofluorescence Microscopy. Cells were grown on glass coverslips (no. 1.5), fixed for 5 min in ice-cold methanol, washed in phosphate-buffered saline, and incubated with a monoclonal antibody against cytokeratins 8 and 18 (antibody 10.11; ref. 12) at a dilution of 1:800 for 30 min at room temperature. Cytokeratin was visualized with a fluorescein isothiocyanate-conjugated rabbit anti-mouse secondary antibody. Confocal microscopy was performed with a Zeiss confocal laser scanning microscope equipped with an argon laser, utilizing a scan time of 8 sec and a line average

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Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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of 18 times. Images of both L and LK8+18 cells were recorded on ASA 400 film.

Immunoblot Analysis. Cytokeratins were isolated from LK8+18 cells by using 20 mM Tris-HCl, pH 7.4/0.6 M KCl/1% (vol/vol) Triton X-100. The resulting cell lysate was centrifuged at 12,000 × g and the pellet was dissolved for electrophoresis in an SDS/10% polyacrylamide gel according to Laemmli (13). Proteins were then transferred to nitrocellulose and incubated with antibody 10.11 (12). Cytokeratin was visualized with a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody and the ECL chemiluminescence detection kit (Amersham).

Colonies Formation. Cells in logarithmic growth were subjected to γ irradiation (96Co) at doses ranging from 0 to 10 Gy. Immediately after irradiation, cells were trypsinized and counted. Irradiated cells were plated at two different cell densities into 60-mm tissue culture dishes in 5 ml of growth medium. Cells were incubated at 37°C for 14 days. Colonies were fixed in 3:1 methanol/acetic acid, stained with crystal violet, and counted manually. For each dose of radiation, surviving colonies were counted. The mean number of surviving colonies per dose was divided by the mean number of colonies obtained from unirradiated cells to determine survival fractions.

Drug Accumulation and Efflux. One day prior to the study, cells were plated directly into glass scintillation vials at a density of 1 × 10⁶ cells per vial and allowed to adhere overnight. At various times (14C)mitoxantrone was added to triplicate vials at a final concentration of 10 μM and incubated with the cells for 1 hr. Accumulation was determined at the end of the hour by immediately aspirating the drug and medium off the cells. Washing the cells twice with ice-cold phosphate-buffered saline, and digesting the cells in 0.2 M NaOH (efflux time is 0 hr). Digests were neutralized with 1 M HCl before scintillation counting. Doxorubicin accumulation was measured by adding the drug at a final concentration of 10 μM for 1 hr at 37°C to cells which were resuspended in medium lacking phenol red. For efflux at times 5, 15, 30, and 60 min, the drugs were replaced by fresh medium at the end of the initial incubation and left on cells for the designated times. Subsequently, for the mitoxantrone measurements, the medium was removed and the cells were washed and digested, and the digest was neutralized before scintillation counting. For doxorubicin, ice-cold phosphate-buffered saline was added to the cell suspensions to stop efflux and the peak intracellular fluorescence was determined by flow cytometry.

RESULTS

LK8+18 Cells Produce and Assemble Cytokeratin. The expression of cytokeratin proteins in transfected LK8+18 cells was demonstrated by both immunoblot blot analysis and indirect immunofluorescence (Fig. 1). Two bands of apparent molecular mass 50 and 43 kDa from LK8+18 cells reacted with a mouse monoclonal antibody against human cytokeratins 8 and 18 (ref. 12, Fig. 1D). These proteins were absent from L cells.

The LK8+18 cells assembled the cytokeratin monomers into filaments, although network morphology varied slightly from cell to cell (Fig. 1A). The parental L cells contained no detectable cytokeratin (Fig. 1C). The small regions of punctate staining observed in L cells are nonspecific, since the staining was also present when L cells were incubated with secondary antibody only (data not shown).

Cytokeratin Confers Drug Resistance. The MTT dye assay was used to test whether cytokeratin might serve a protective role against chemotherapeutic agents in LK8+18 cells. L, LK8+18, and LPBMC cells were incubated in 96-well plates in the presence of various anticancer drugs. Absorbance values due to mitochondrial metabolism of the dye were used to assess cell viability in the presence and absence of drug. The values presented are the mean drug concentrations that decreased absorbance by 50% (IC50 values, Table 1).

When mitoxantrone was added to L cells, a 50% decrease in absorbance occurred at a mean concentration of 0.07 μM mitoxantrone (Table 1). In contrast, the IC50 of mitoxantrone in LK8+18 cells was 0.54 μM, a concentration 7.6 times higher than the corresponding IC50 in L cells. LPBMC cells resembled L cells, with a mean IC50 of 0.05 μM. Cytokeratins 8 and 18 conferred resistance to mitoxantrone-induced cytotoxicity as measured by the MTT assay.

To test whether this resistance was specific to mitoxantrone, doxorubicin was substituted and the experiments were repeated. LK8+18 cells were about 5 times more resistant to doxorubicin than L cells, with a mean IC50 of 0.68 μM compared with an IC50 of 0.13 μM in the parental cells.

Mitoxantrone and doxorubicin are drug analogs. Both bind DNA and inhibit topoisomerase II (14). Both probably cause DNA damage via formation of drug-dependent free radicals.

Table 1. Cytokeratin expression confers drug resistance

<table>
<thead>
<tr>
<th>Cells</th>
<th>Mitoxantrone</th>
<th>Doxorubicin</th>
<th>Methotrexate</th>
<th>Melphalan</th>
<th>Vincristine</th>
<th>Colcemid</th>
<th>Sf,* % (fold resistance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>0.07 (1)</td>
<td>0.13 (1)</td>
<td>0.0013 (1)</td>
<td>6.5 (1)</td>
<td>0.20 (1)</td>
<td>179.8 (1)</td>
<td>75.6 (1)</td>
</tr>
<tr>
<td>LPBMC</td>
<td>0.05 (0.7)</td>
<td>0.03 (0.2)</td>
<td>0.0013 (1)</td>
<td>11.2 (1.7)</td>
<td>0.03 (0.2)</td>
<td>119.9 (0.7)</td>
<td>69.7 (0.9)</td>
</tr>
<tr>
<td>LK8+18</td>
<td>0.54 (7.6)</td>
<td>0.68 (5.2)</td>
<td>0.0065 (4.9)</td>
<td>42.0 (6.5)</td>
<td>90.8 (454)</td>
<td>3084 (17.2)</td>
<td>57.4 (0.8)</td>
</tr>
</tbody>
</table>

*Surviving fraction × 100 at 2 Gy.
(15, 16). To test whether or not LK8+18 cells were resistant only to mechanistically related drugs, experiments were performed with methotrexate, an inhibitor of dihydrofolate reductase; melphalan, a DNA-alkylating agent; and vincristine and Colcemid, both of which bind and destabilize microtubules. With the exception of the antitubulin drugs, LK8+18 cells were 5–8 times more resistant to the drugs tested than L and LPBMOC cells.

The highest IC50 of any of the drugs tested was 3 mM Colcemid. At this dose, transfected LK8+18 cells were about 17 times more resistant to the drug than parental or mock-transfected cells. In the case of vincristine, the IC50 was lower, but the LK8+18 cells were 454 times more resistant than the L cells. The LPBMOC cells appeared somewhat more sensitive to vincristine than the parental cells.

To determine whether cytokinin conferred resistance to other DNA-damaging agents, both L and LK8+18 cells were irradiated with 60Co, which directly damages DNA without requiring specific uptake, transport, and distribution processes. Radiation damage was scored by colony formation on plastic tissue culture plates. The fraction of LK8+18 cells surviving a dose of 2 Gy was lower than the corresponding surviving fraction of L cells. Cytokeratin did not confer resistance to radiation-induced cytotoxicity.

Drug Resistance Is Not Due to Altered Growth Characteristics in LK8+18 Cells. Neither the growth rate nor the distribution of LK8+18 cells within the cell cycle was different from that of the parental cells (Fig. 2). The transfected cells appeared to double at a slightly slower rate than the L cells, but a statistical comparison of their logarithmic-phase slopes yielded no statistical difference between the cell types.

Drug Resistance Is Not Due to Altered Drug Accumulation or Efflux. Because drug resistance phenotypes are frequently associated with aberrant expression of transport proteins that function to decrease cellular drug accumulation or increase the rate of drug efflux, we characterized the 1-hr accumulation (0-hr efflux) and efflux of [14C]mitoxantrone and doxorubicin in L and LK8+18 cells (Fig. 3). Intracellular drug accumulation was determined immediately after a 1-hr exposure as described. The mean amount of intracellular [14C]mitoxantrone in L cells was determined to be 3.2 μmol.

**Fig. 2.** Growth and cell cycle analysis of L and LK8+18 cells. (A) Increase in number of L (○) and LK8+18 (△) cells. (B and C) DNA content of L and LK8+18 cells, respectively. The cells were fixed with 3:1 methanol/acetic acid and treated with propidium iodide for analysis by flow cytometry. Cell cycle distributions were as follows: for L cells, 48.5% G1, 36.7% S, and 14.8% G2/M; for LK8+18 cells, 44.0% G1, 35.9% S, and 20.1% G2/M.

**Fig. 3.** Drug accumulation and efflux of L and LK8+18 cells. L (○) and LK8+18 (△) cells were exposed to 10 μM [14C]mitoxantrone (A) or 10 μM doxorubicin (B) for 1 hr. Cells were then washed, and remaining drug was measured either by radioactivity (mitoxantrone) or flow cytofluorimetry (doxorubicin) after drug removal. Intracellular concentrations of drug were normalized to the highest amount of intracellular drug accumulated. The range of radioactivity was 4.3 × 10^6 to 1.5 × 10^7 dpm. Data are mean amounts of drug accumulated ± standard error.
versus a mean intracellular accumulation of 3.3 μmol in LK8+18 cells for two experiments. The two cell types therefore accumulated equivalent amounts of mitoxantrone. Likewise, the intracellular drug levels at various times after initiation of efflux measurements were similar, as were drug efflux rates for both mitoxantrone and doxorubicin (Fig. 3).

**DISCUSSION**

The *de novo* expression of cytokeratin confers multiple drug resistance on the LK8+18 cells as measured by the MTT assay. LK8+18 cells are resistant to the mechanistically unrelated drugs mitoxantrone, doxorubicin, methotrexate, melphanal, vincristine, and Colcemid. The greatest degree of resistance observed was to the antitubulin drugs Colcemid and vincristine. Physical links between the intermediate-filament and microtubule networks have been proposed to explain how the chemical collapse of microtubules and microfilaments results in a concomitant collapse of intermediate filaments (17). It may be that the introduction of an additional cytoskeletal network to L cells stabilizes the microtubule network and inhibits its depolymerization by vincristine and Colcemid.

Our data indicate that drug resistance in LK8+18 cells cannot be attributed to altered growth characteristics, drug accumulation, or drug efflux. Resistance in LK8+18 cells is therefore not likely to be due to an overexpressed or activated drug-transport protein. Nor can resistance be attributed to an altered response to DNA damage, since the LK8+18 cells are not resistant to γ-irradiation. At this time, it is unknown whether the *de novo* drug resistance is conferred by cytokeratins or requires the coexpression of cytokeratin and the endogenous vimentin intermediate-filament proteins in the transfected cells. Further, it is unknown whether the mechanism(s) involved in the cytokeratin-dependent drug resistance phenotype are similar for all the drugs tested. Our current working hypothesis is that the cytokeratin networks may influence the intracellular drug distribution so that particular nuclear targets are spared. Alternatively, since cytokeratin networks interact with the nucleus, the processing and repair of bulky nuclear damage may be influenced by cytoplasmic structure (18, 19).

In addition to elucidating the mechanism responsible for the *de novo* drug resistance, it will be of interest to test whether the disruption of cytokeratin networks will sensitize human breast cancer cells to chemotherapeutic agents. Although mitoxantrone is effective in the treatment of hematologic malignancies, *de novo* drug resistance is a major factor limiting the treatment of epithelial-type malignancies such as breast cancer. The disruption of the cytokeratin networks which are present in breast cancer cells may remove the *de novo* drug resistance phenotype.

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