Cytokeratin Expression Results in a Drug-resistant Phenotype to Six Different Chemotherapeutic Agents

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ABSTRACT

The cytokeratin network is an abundant cytoplasmic system whose function is largely unknown. Recently, we have found that the introduction of a cytokeratin network into eukaryotic cells results in a drug resistance phenotype. The current study was undertaken to determine the universal nature of this phenomenon by investigating the survival response of two different cell lines to six different DNA-damaging agents using two different assays of cell survival. To correlate our in vitro assays of survival with known in vivo responses to DNA damage, we compared the apoptotic response of cytokeratin-positive and cytokeratin-negative cell lines. The results show that the introduction of a cytoskeletal network confers a resistant phenotype to mitoxantrone, doxorubicin, melphalan, bleomycin, and mitomycin C in the different cytokeratin-positive cell lines. No survival advantage was noted when damage was conferred by cisplatin or UV irradiation. We found the cytokeratin-positive cell lines were protected from apoptosis, while the cell lines without cytokeratins showed apoptosis in response to mitoxantrone exposure. Cytokeratin-dependent drug resistance is observed in different cell lines but is not observed with all DNA-damaging agents. The data suggest that the mechanism of this drug resistance may be attributed, in part, to a cytokeratin-conferred protection against apoptosis.

INTRODUCTION

The treatment of epithelial tumors continues to be a major medical challenge due in part to both cellular and noncellular mechanisms of drug resistance (1). Noncellular mechanisms of resistance, including vascular perfusion and blood tissue barriers, can be bypassed but cellular mechanisms (acquired and innate) of resistance remain. Examples of the biological mechanism of acquired resistance include the down-regulation of key targets (2), increase in free radical scavengers (3), alteration in cellular pH (4), altered drug activation (5), and increased drug inactivation (3). In contrast, mechanisms of innate resistance have been attributed primarily to the P-glycoprotein MDR pump (3) and drug efflux unrelated to the MDR pump (6).

Previously we had shown that a chemotherapeutic agent, mitoxantrone, was associated with the cytokeratin network in human colon carcinoma cells. The association of mitoxantrone to cytokeratin was persistent and suggested that the interaction may either represent a new cellular target (i.e., increased damage) or a mechanism for an intracellular redistribution of the drug (i.e., increased survival; Ref. 7). Recently, work from our laboratory has shown that the addition of a cytokeratin network into a mouse fibroblast cell line conferred a MDR pattern (8). In studies of P-glycoprotein and non-P-glycoprotein multidrug resistance, it has been found that resistant cell lines lose their sensitivity to ionizing radiation and cytotoxic drug-induced apoptosis (9, 10). The purpose of the present study was to determine the universal nature of the cytoskeletal-dependent drug resistance using two different cell lines, six different DNA-damaging agents, and two different assays for cell killing. To investigate the possible cellular mechanism of drug resistance, we compared the apoptotic response of cytokeratin-positive cell lines with cytokeratin-negative cell lines.

MATERIALS AND METHODS

Murine L parental and cytokeratin 8/18-transfected cell lines were obtained from Dr. Oshima (La Jolla Cancer Research Foundation, La Jolla, CA) and maintained as previously described (11). NIH 3T3 parental and 8/18-transfected cell lines were courtesy of Dr. B. Lane (University of Dundee, Scotland; Ref. 12). Cytokeratin-positive cells were maintained in DMEM (GIBCO), 10% FBS (Gemini), penicillin, streptomycin (GIBCO), 1 mg/ml G418 (GIBCO), and 200 mg/ml hygromycin (Calbiochem). Parental cells were cultured in DMEM supplemented with 10% FBS, penicillin, and streptomycin. All cell lines were maintained at 37°C in a humidified incubator containing 5% CO2. Two subclones of the NIH 3T3 cytokeratin-
positive cells were obtained via serial dilution. In brief, 100 cells/100 ml selection medium were diluted with an equal volume of conditioned medium. Two hundred μl cell suspension were added to each well of a 96-well microtitre plate (Falcon). After 3 weeks of growth, cells from those wells containing single colonies were resuspended with trypsin and maintained in growth medium containing the double selection agents (G418 and hygromycin).

For indirect immunofluorescence microscopy studies, cells were grown on 12-mm round coverslips within a 35-mm dish containing the appropriate growth medium for at least 24 h. The medium was removed, and the cells were washed with PBS, and fixed on ice with methanol (−20°C) for 5 min. Coverslips were washed with 5% BSA/PBS and incubated with a mouse monoclonal antibody directed against cytokeratins 8 and 18 (antibody 10.11; Ref. 13) at a 1:50 dilution for 30 min at room temperature. A rabbit antimouse rhodamine-conjugated secondary antibody was utilized to demonstrate the cytokeratin filaments. Visualization of filaments was performed using a Zeiss LSM 10 confocal microscope. Images were collected using a HeNe laser and a scan time of 8 s. Images were transferred onto a Quadra 800 computer and recorded using a film recorder using Kodak TMX 135 film ASA 100.

Cell survival was measured using the MTT assay (14) and a colony-forming assay (8). For the MTT assay, 1000–5000 cells in 180 μl appropriate growth medium were plated in each well of a 96-well microtiter plate (Falcon) on day 1. The following day, 20 μl of a 10× concentration of the specified drug were added to the appropriate well. After 3 days of incubation, 50 μl of 2 mg/ml MTT were added to each well and incubated for 4 h at 37°C. The medium was subsequently removed and replaced with 100 μl DMSO. The plates were shaken, and the optical absorbance was determined at 540 nm. Absorbance values were normalized to the values obtained for the vehicle-treated cells to determine the value for percentage of survival. For determination of cell survival after UV irradiation, cells were plated as described above for the MTT assay. The following day, cells were irradiated with a predominantly UVc germicidal lamp (Sylvania). Prior to irradiation, the energy output (W/cm²) of the lamp was determined at a source to surface distance of 16 cm with a UV meter. The final UV dose (J/m²) was achieved for each group of cells by adjusting the total time of exposure. The final doses achieved in creating the dose-response curve were 2, 6, 20, 60, and 200 J/m². The remainder of the MTT assay was carried out as described above. The colony-forming assay was performed with the L cells suspended in their appropriate growth medium with or without 0.03 or 0.3 μM mitoxantrone. Cells were plated in 100-mm dishes at cell concentrations of 25, 50, 100, 200, 400, 800, or 1600 cells/dish. After 13 days of undisturbed incubation, the cells were fixed with methanol:acetic acid (3:1), stained with crystal violet.

Fig. 1 Immunofluorescent detection of cytokeratin networks. NIH 3T3 cytokeratin 8 + 18 transfectants (clone 1 and clone 2) synthesize an elaborate cytokeratin network extending from the perinuclear zone to the cell surface. LK 8 + 18-transfected cells synthesize cytokeratins but create a less elaborate network.
Fig. 2 Mitoxantrone and doxorubicin inhibition of cell survival. NIH 3T3 cells were exposed to a series of increasing doses of mitoxantrone and doxorubicin for 3 days, and then cell survival was determined via the MTT assay. There is a dose-dependent decrease in cell survival, and the addition of cytokeratins 8 + 18 confers a survival advantage. C, clone. Bars, SD.

Fig. 3 Dose-response curves for L cells and LK 8 + 18-transfected cells exposed to mitoxantrone and doxorubicin. There is a dose-dependent decrease in cell survival, and the addition of cytokeratins 8 + 18 confers a survival advantage compared to parental cells after a 3-day exposure to mitoxantrone and doxorubicin. Cell survival was determined via the MTT assay. Bars, SD.

rinsed, and air dried. Colonies arising from untreated cells were counted to determine the plating efficiency (colony count/number of cells plated) and were adjusted to 100% survival. For each mitoxantrone-treated dish, the colony count/number of cells plated was determined. This value was divided by the control plating efficiency to give the percentage of survival.

To evaluate the apoptotic response, we performed agarose gel analysis of DNA fragmentation and FACS analysis (9, 15). Murine L parental and cytokeratin 8/18-transfected cell lines were treated for 24 h at 37°C with 1 μM doxorubicin and, a second set, with 1 μM mitoxantrone. A third set of both cell lines was treated with 10 Gy of γ radiation using a 60Co source with a dose rate of 61.3 cGy/min. The cells (5 × 10⁶) were harvested, washed, and then lysed with a solution of 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% sarcosine, and 300 μg/ml proteinase K (GIBCO-BRL) for 2 h at 37°C. The cell lysate was then extracted sequentially with phenol, phenol/chloroform, and chloroform. The nucleic acids were then precipitated with the addition of 0.2 volume of 11 M ammonium acetate and 2.5 volumes of 95% ethanol, resuspended in water, and treated with RNase A (100 μg/ml) for 30 min at 37°C. RNase A was removed by phenol/chloroform and chloroform extractions. The DNA was then precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol. The DNA was then washed in 70% ethanol, resuspended in water, and quantitated by absorbance measurements at 260 nm (16). The DNA (20 μg) was then analyzed using a 2% agarose gel, stained with ethidium bromide, and the DNA was visualized using UV illumination (9). For the FACS analysis, the murine L and cytokeratin 8/18 cell lines were exposed to 1 μM mitoxantrone for 24 h at 37°C. The cells (1−2 × 10⁶/ml) were harvested, washed, and fixed in 70% ethanol at 4°C for 1 h. The cells were then washed and incubated with RNase A (500 μg/ml) and propidium iodide (50 μg/ml) for 15 min at room temperature in the dark. Propidium iodide fluorescence of nuclei was then determined using FACScan (Becton Dickinson; Ref. 15).
RESULTS

NIH 3T3 cells and the L cells were assessed for the presence of a cytokeratin network by indirect immunofluorescence confocal microscopy. In the NIH 3T3 cells, 13 subclones were subsequently isolated to avoid the variability in cytokeratin expression inherent in the transfected cell populations. Two clones (designated C1 and C2) displayed an elaborate intracellular cytokeratin array (Fig. 1) and were studied further. The L cells, containing the cytokeratins 8 and 18 (designated LK 8/18) also contained cytokeratin networks, although not as elaborate as the NIH 3T3 transfectants (Fig. 1). The parental NIH 3T3 cells did not contain cytokeratin networks (Fig. 1) as well as the parental L cells (8).

Cells were incubated in 96-well microtiter plates in the presence of six different DNA-damaging agents (mitoxantrone, melphalan, adriamycin, mitomycin C, cisplatin, and bleomycin). The MTT dye assay was utilized to determine cell survival based on the ability of the viable cells to convert MTT to a purple formazan dye product (14). Cell survival was normalized to the control (vehicle), and the cell survival curves were generated. Anthracyclines (doxorubicin) and anthracycline-related compounds (mitoxantrone) can generate free radicals and cause topoisomerase II-induced DNA fragmentation (14). Both doxorubicin and mitoxantrone resulted in a dose-dependent decrease in cell survival that is altered by the presence of cytokeratins 8 and 18 (Fig. 5). In contrast to the NIH 3T3 cells, cytokeratin-positive L cells had a survival advantage in the presence of cisplatin (Fig. 7).

In our previous report it was demonstrated that murine L cells transfected with cytokeratins 8 and 18 (LK 8 + 18) gained a survival advantage with a shift in the IC_{50} compared to parental cells. The original report is expanded to include mitomycin C, cisplatin, and bleomycin along with the cell survival curves for melphalan, adriamycin, and mitoxantrone (Figs. 3, 5, and 7). Both mitomycin C and bleomycin induce a dose-dependent decrease in cell survival that is altered by the presence of cytokeratins 8 and 18 (Fig. 5). In contrast to the NIH 3T3 cells, cytokeratin-positive L cells had a survival advantage in the presence of cisplatin (Fig. 7).

To determine whether the conferred resistance in the NIH 3T3 cells is a consequence of drug entry and transport, we evaluated the effects of UV irradiation on cell survival. UV irradiation will cause the formation of various bulky photoproducts, bypassing transport systems. UV irradiation caused a dose-dependent decrease in cell survival from doses of 2 J/m^2 to 60 J/m^2. The presence of cytokeratins 8 and 18 conferred no survival advantage (Table 1). A colony-forming assay was performed with murine L fibroblasts transfected with cytokeratins to corroborate the MTT assay data. Murine L fibroblasts with or without cytokeratins 8 and 18 were grown for 13 days in the presence of 0.3 and 0.03 μM mitoxantrone. There was a dose-dependent decrease in cell survival, and the cytokeratin positive cells demonstrated a 25-fold increase in survival compared to controls in the presence of 0.03 μM mitoxantrone.

The agarose gel analysis of DNA fragmentation showed laddering characteristic of physiological cell death in the murine L cell line (negative for cytokeratins) when exposed to 1 μM mitoxantrone (Fig. 8) or 1 μM doxorubicin (data not shown). In contrast, the cytokeratin 8/18-positive murine cell line did not respond to mitoxantrone or doxorubicin by undergoing apoptosis. Since the cytokeratin-positive 8/18 cell lines did not show resistance to ionizing radiation, we tested whether any alteration of apoptosis occurred. Both the cytokeratin-positive and -negative cell lines are capable of undergoing apoptosis using this type of damage (Fig. 8). Since nucleosomal ladders can be an underestimate of apoptosis (18), the DNA fragmentation studies were corroborated by cell cycle analysis. Murine L cell lines with and without cytokeratin 8/18 were exposed to 1 μM mitoxantrone for 24 h and then harvested. The cells were stained with propidium iodide and FACS analysis was performed. The murine parental L cell line negative for cytokeratins showed a DNA peak to the left of G_1-G_2 in the hypodiploid region. The

Table 1  Cell survival response to DNA-damaging agents (IC_{50})

<table>
<thead>
<tr>
<th>Cell</th>
<th>Doxorubicin (nM)</th>
<th>Mitoxantrone (nM)</th>
<th>Bleomycin (μM)</th>
<th>Mitomycin C (μM)</th>
<th>Cisplatin (μM)</th>
<th>UV Irradiation (J/m^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>6.92 ± 1.95</td>
<td>3.67 ± 1.55</td>
<td>0.260 ± 0.102</td>
<td>0.118 ± 0.055</td>
<td>3.932 ± 0.996</td>
<td>35.87 ± 5.39</td>
</tr>
<tr>
<td>Clone 1</td>
<td>54.16 ± 9.13</td>
<td>97.5 ± 56.57</td>
<td>8.794 ± 4.624</td>
<td>1.858 ± 0.640</td>
<td>9.253 ± 2.224</td>
<td>35.22 ± 2.54</td>
</tr>
<tr>
<td>Clone 2</td>
<td>64.29 ± 11.9</td>
<td>255.9 ± 62.4</td>
<td>6.018 ± 2.285</td>
<td>1.243 ± 0.187</td>
<td>5.657 ± 0.796</td>
<td></td>
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</table>

a IC_{50} is the concentration of drug required to reduce cell survival to 50%. Each value represents the mean ± SE from a minimum of two independent experiments.

b Colony-forming assay performed with 0.03 μM mitoxantrone showed a 25-fold increase in survival in cytokeratin-positive cell lines.
cytokeratin-positive murine L cell line showed no shift from the diploid G0-G1 peak (Fig. 9).

DISCUSSION

The data presented here confirm our initial report indicating that the addition of a cytokeratin 8 and 18 network to cells normally lacking cytokeratin expression confers a survival advantage in the presence of different classes of drugs. In our previous report the major indicator of cell survival was the MTT assay. In the MTT assay, cell survival is based on the ability of cellular mitochondria to convert the MTT into a purple formazan product (14). Thus, the cell survival curves are not a direct measure of cell growth, although several research groups have demonstrated a close correlation between the results of cell growth and the MTT assay (14). In the case of the L cells, the MTT cell survival data were corroborated by a colony-forming assay. Although the MTT assay underestimated the cytotoxic effects of a 13-day exposure to mitoxantrone (Figs. 2 and 3 and Table 1), the cytokeratin-positive cells still had a 25-fold increase in survival as compared to the cytokeratin-negative parental cells.

The fold resistance we were able to demonstrate compares favorably with resistance levels reported for other known drug resistance mechanisms, namely, the P-glycoprotein MDR pump and the MDR protein. The human KB epidermal carcinoma cell line KB-8-5, known to express mdr, is 3–6-fold resistant to most natural product cytotoxic drugs (19–21). Non-P-glycoprotein mechanisms of drug resistance, demonstrated in a small cell lung cancer cell line, showed the nuclei of resistant cells were approximately 10-fold resistant to the induction of single-strand DNA breaks when compared to the parent cell line (22). HeLa cells transfected with eukaryotic expression vectors containing MDR protein complimentary DNA were found to be 5–15-fold resistant to doxorubicin, daunorubicin, epirubicin, vincristine, and etoposide. These same cell lines demonstrated what was characterized by the authors as a low level of resistance (<3 fold) to Taxol, vinblastine, and cholchicine (23). Higher levels of drug resistance (up to
Fig. 6 Cisplatin and melphalan inhibition of cell survival. NIH 3T3 cells were exposed to increasing concentrations of cisplatin for 3 days, and there was no survival advantage in the cytokeratin 8 + 18-transfected cells. In contrast, cytokeratin 8 and 18 transfection confers a survival advantage compared to parental cells after a 3-day exposure to melphalan. Cell survival was determined via the MTT assay. C, clone. Bars, SD.

400-fold can be induced by selection in tissue culture, but this level of resistance is often unstable (20). In our study, the cell lines are 8–69-fold resistant to agents without selection (Table 1).

It is interesting that the observed resistance is not observed with three agents: cisplatin, UV irradiation in the NIH 3T3 cells, and in 60Co-irradiated L cells (8). The reason for this is not clear but may involve the fact that irradiation and cisplatin can result in an apoptotic response in some cell lines (24, 25).

Apoptosis has been found to be associated with human tumor cell lines treated with cytotoxic drugs or ionizing radiation (26–30). Drug-resistant cell lines have demonstrated a loss of sensitivity to ionizing radiation induced and cytotoxic drug-induced nucleosomal DNA fragmentation (9, 10). We examined the possible role of apoptosis in cytokeratin conferred drug resistance. Although both cytokeratin-positive and cytokeratin-negative cell lines exposed to 10 Gy ionizing radiation showed evidence of physiological cell death, the cytokeratin-positive cell lines did not show evidence of apoptosis when treated with cytotoxic drugs. These data suggest two interesting points. First, the cytokeratin network in some manner protects against apoptosis, resulting in a drug-resistant phenotype. These results imply that the cytotoxic effect of some chemotherapeutic agents may be mediated by the induction of apoptosis and that intrinsic drug resistance may be mediated by the inhibition of apoptosis. Second, different DNA-damaging agents may elicit apoptosis by different pathways. Both cell lines respond to ionizing radiation by induction of apoptosis. In contrast, mitoxantrone-induced DNA damage is only observed in the cytokeratin-negative cell line.

Although the resistance phenotype is observed here with ectopically expressed cytokeratin, it is important to note that cytokeratins are normally expressed in epithelial cells and their associated malignancies (31). Many epithelial tumors (lung, breast, colorectal, and prostate) remain a chemotherapeutic challenge secondary to drug resistance. Chemotherapeutic dose intensification is critical in the treatment of breast cancer (32). Our data would suggest that the dose intensification required may occur as a result of the abundant cytokeratin networks in epithelial cells. It should also be noted that the agent (cisplatin) whose toxicity was not affected by the cytokeratin networks is also one of the most active chemo-

Fig. 7 L and LK 8 + 18 cell survival after a 3-day exposure to cisplatin and melphalan. There is a dose-dependent decrease in cell survival, and the addition of cytokeratins 8 + 18 confers a survival advantage compared to parental cells after exposure to cisplatin and melphalan. Cell survival was determined via the MTT assay. Bars, SD.
therapeutic agents in the treatment of head and neck squamous cell carcinomas (33). Thus, the presence of cytokeratins may play a significant role in intrinsic drug resistance. Future experiments will be directed at using antisense oligonucleotides to diminish cytokeratin expression to reverse the drug-resistant phenotype.

It is possible that cell cycle mechanisms may play a role (i.e., an operative cell surveillance mechanism may exist or operate more effectively in cytokeratin-positive cells). The initial data suggest that a G2-M cell cycle block is not involved since γ and UV irradiation, which induce G2-M cell cycle blocks (34), resulted in the same survival response for L parental and LK transfectants (8) and the NIH 3T3 cells, respectively (Table 1). Furthermore, the initial cell cycle distribution for the L and LK cells was similar, and the growth curves were similar for the L, LK cells (8), and NIH 3T3 cell lines (data not shown). We have noted with interest that the FACS analysis of the treated cell lines did show substantial differences in the number of cells undergoing apoptosis. Whether the differences in FACS analysis between the sensitive and resistant cell lines are attributable singly to the apoptotic response or if other cell cycle alterations (i.e., G0 or S-phase blockage) after treatment are otherwise significant is the subject of further study.

The possible role of cytokeratins in intracellular signaling may be significant in conferring drug resistance. The cytokeratin network surrounds the nucleus and projects into the cytoplasm of the cell. The data which demonstrate no advantage of cytokeratins in protection against UV and ionizing radiation may be due to the fact that these agents damage DNA independent of an interaction with the cytokeratin network. The lack of cytokeratin exposure to these damaging agents may explain why both cytokeratin-positive and -negative cell lines became apoptotic in response to ionizing radiation.

Cytokeratins' established role as cytoskeletal components would appear to hold the most obvious key to the mechanism of their conferred drug resistance. Cytokeratins may play a role in the stabilization of cytoarchitecture. This stabilization may be important in a cell's ability to resist apoptosis. In our previous publication, the LK cytokeratin-positive cells were most resistant to microtubule disrupters (8). Similarly, NIH 3T3 cells transfected with cytokeratins 8 and 18 are also quite resistant to microtubule disrupters (colcemid) and microfilament disrupters.

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**Fig. 8** Induction of apoptosis in murine parental L cells following exposure to 1 μM mitoxantrone for 24 h, and 10 Gy ionizing radiation. Cytokeratin 8/18-positive murine L cells showed induction of apoptosis only with exposure to 10 Gy ionizing radiation. For each sample, 20 μg DNA were analyzed using a 2% agarose gel.

**Fig. 9** FACS analysis of the parental murine L cell line and cytokeratin 8/18-positive murine L cell line after being exposed to 1 μM mitoxantrone for 24 h, harvested, washed, fixed, and stained with propidium iodide. Arrow, apoptotic population.
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Cytokeratin expression results in a drug-resistant phenotype to six different chemotherapeutic agents.


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