A549 subclones demonstrate heterogeneity in toxicological sensitivity and antioxidant profile

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Accepted 18 January 2002; accepted in final form 27 April 2002

THE A549 CELL LINE, ORIGINALLY established from a lung adenocarcinoma of a Caucasian male in 1972 (22), has been widely used as a model of lung type II epithelial cells, as is reflected by almost 2,000 published articles. However, like other carcinoma cell lines, A549 cells are a nonhomogenous cell population that consists of multiple clones with a modal chromosome number of 12 and counts of 64, 65, and 67 chromosomes occurring with high frequency (American Type Culture Collection, http://atcc.org/SearchCatalogs/longview.cfm?view=ce,323118,CCL-185&text=a549). In fact, several subpopulations with various characteristics would be related to differences in their cellular antioxidant levels. To address this possibility, the antioxidant profiles of the subclones were isolated from the parental cell populations (14, 16). Nevertheless, the majority of research with A549 cells continues to be done using the heterogeneous population.

In our initial studies of apoptosis, we also found that A549 cells contain cells that are both susceptible and resistant to cytotoxicity by tumor necrosis factor-α (TNF-α) plus actinomycin D. Therefore, several clones from the parental A549 cells were isolated by limiting dilution, and TNF-α plus actinomycin D-susceptible (G4S) and -resistant (D3R) clones were selected for additional studies. Subsequent study revealed that the different sensitivity was dependent on actinomycin D alone, and that the resistant/sensitive phenotype was also indicative of the response to other macromolecule synthesis inhibitors as well as to some oxidative insults.

Reactive oxygen species (ROS) are associated with the initiation of cell death by a variety of cytotoxic insults such as ultraviolet radiation and chemotherapeutic agents. Depending on the burden of ROS, the mode of cell death can be either apoptosis or necrosis or a combination of the two (12). Nonetheless, a network of antioxidants and antioxidant enzymes [superoxide dismutases (SOD) catalase, glutathione peroxidases (GPx), glutathione (GSH), NADPH generated by the pentose phosphate pathway (PPP), thioredoxin (Trx), peroxiredoxins, and glutathione S-transferases (GST)] protects cells against ROS and cytotoxic products of lipid peroxidation while maintaining cellular thiols in their reduced states.

Overexpression or deletion of various antioxidant enzymes can change the sensitivity to various cytotoxic insults (3, 6, 26, 27, 30, 33, 35, 45) as well as growth rates (5, 27), which substantiates the notion that ROS or their reactive derivatives are critical components of signal transduction pathways. Therefore, our simple original hypothesis was that the differences in the sensitivity of these clones to cytocidal agents, as well as other characteristics, would be related to differences in their cellular antioxidant levels. To address this possibility, the antioxidant profiles of the subclones were...
characterized. Although some antioxidants were higher in the resistant clone than in the susceptible clone, others including the frontline enzymes catalase and GPx were markedly lower. The results also showed that the two clones differ asymmetrically in factors other than antioxidant levels. Thus, even when oxidants are used to initiate cell death, antioxidant profiles alone cannot predict the sensitivity to cell death of asymmetrical clones. Furthermore, although subcloning appears to be advisable for any mechanistic study using the A549 cell line or other unstable cell lines, comparison among such clones is unreliable in determining mechanisms, as there are often unknown variables.

MATERIALS AND METHODS

Reagents. Unless otherwise noted, all chemicals were from Sigma (St. Louis, MO). Acetyl-Asp-Glu-Val-Asp-7-amino-4-(trifluoromethyl)-coumarine (Ac-DEVD-ACF) and acetyl-Leu-Glu-His-Asp-7-amino-4-(trifluoromethyl)-coumarine (Ac-LEHD-ACF) were from Calbiochem (La Jolla, CA); 4-hydroxy-2-nonenal (HNE) was from Cayman Chemical (Ann Arbor, MI); 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) was from Oxis Research (Portland, OR); glycylglycine was from ICN Biomedicals (Aurora, OH); and l-γ-glutamyl 7-amino-4-methylcoumarin (AMC) was purchased from Bachem BioScience (King of Prussia, PA).

A549 cells and isolation of the subclones. A549 cells were purchased from the American Type Culture Collection (ATCC). Parental cells and subclones were cultured in F-12K medium (GIBCO-BRL) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin within a humidified atmosphere containing 5% CO₂ at 37°C. Subcloning of A549 cells was done by limiting dilution using 96-well culture plates. Several wells that contained a single colony were randomly selected and aliquoted. An aliquot from each clone was treated with TNF-α (50 ng/ml) plus actinomycin D (0.5 μg/ml) for 8 h, and one susceptible clone (G4S) and one resistant clone (D3R) were randomly selected. The actinomycin D-susceptible phenotype of G4S cells was not stable above 30 doublings. Therefore, we conducted all the experiments within this doubling number. In the subsequent study, unless otherwise noted, those cells were seeded in culture plates 12–18 h before initiation of experiments at the indicated density.

Cytotoxicity assay by crystal violet staining. Cells in 96-well plates (1.5 × 10⁴ cells/well) were treated with various agents in the culture medium for 8 h. Cells were washed with PBS [that contained (in mM) 10 Na₂HPO₄, 1 KH₂PO₄, 137 NaCl, and 2.7 KCl, pH 7.4] and then fixed and stained with 100 μl of crystal violet solution [0.5% (wt/vol) crystal violet, 1.5% (vol/vol) formaldehyde, and 1% (vol/vol) ethanol] for 30 min. After the wells were washed with water, the stained cells were lysed with 1% (wt/vol) deoxycholate, and the absorbance at 550 nm was read in a microplate reader.

Caspase assays. Cells in 24-well plates (1.5 × 10⁵ cells/ well) were treated with various agents in medium for 2 or 4 h. After the treatment, both attached and detached cells were collected and combined. The cells were lysed in 250 μl of 0.1% Triton X-100/NaPi (0.1 M sodium phosphate buffer, pH 7.4) and centrifuged at 10,000 g for 10 min at 4°C to obtain the supernatant. Caspase assays were carried out in a 96-well assay plate. The final concentrations of each constituent in 200 μl were as follows: cell lysate equivalent to the original 6 × 10⁴ cells, 10 mM dithiothreitol, 0.05% (vol/vol) Triton X-100, and 50 μM either Ac-DEVD-ACF (caspase 3 substrate) or Ac-LEHD-ACF (caspase 9 substrate) in NaPi. After the reaction progressed at 37°C for 60 min, the fluorescence intensity was measured in a fluorescence microplate reader (SpectraMax GeminiXS, Molecular Devices) with excitation and emission wavelengths of 400 and 500 nm, respectively. The values were converted to AFC concentrations using an external AFC standard.

Measurement of cellular antioxidant enzyme activities. Unless otherwise indicated, the measurements were made using aliquots of lysate prepared as follows: cells were cultured in 100-mm culture dishes until attainment of ~70% confluence (D3R, ~3 × 10⁶; G4S, ~1.5 × 10⁶ cells/dish), 100% confluence (D3R, ~6 × 10⁶; G4S, ~3 × 10⁶ cells/dish), or overconfluence (24 h after confluence: D3R, ~10 × 10⁶; G4S, ~4.5 × 10⁶ cells/dish). Cells were washed with PBS and lysed with 0.1% Triton X-100/NaPi. The lysates were centrifuged at 10,000 g for 15 min at 4°C, and the supernatants were collected. Lysates were aliquoted and stored at ~80°C, and each aliquot was used only once. Protein concentrations were determined by the Bradford method (Bio-Rad) with BSA as a standard.

Antioxidant enzyme assays in microplate plate reader. Unless otherwise indicated, all antioxidant enzyme assays described were conducted at 22°C in a 96-well assay plate with a total volume of 200 μl. The reaction kinetics were measured with a SpectraMax Plus microplate reader (Molecular Devices) that was controlled by the dedicated software (SOFTmax Pro, Molecular Devices). Each sample was measured in either duplicate or triplicate.

Total SOD activity was measured by the cytochrome c/xanthine/xanthine oxidase system according to the methods of McCord and Fridovich (32) with modifications. The final concentrations of each constituent were as follows: 100 μg/ml cell lysate, 10 μM ferricytochrome c, 3.32 mM xanthine, 50 μM xanthine, 10 μg/ml catalase, and 100 μM diethylenetriaminepentaacetic acid (DETAPAC) in 0.05% (vol/vol) Triton X-100/NaPi. The rate of ferricytochrome c reduction was monitored at 550 nm. The addition of known amounts of exogenous SOD into the lysate produced the expected increases in SOD activity that indicated the absence of interference from either oxidases (i.e., cytochrome c oxidase) or reductases in the lysate (data not shown). One unit was defined as 50% inhibition of the cytochrome c reduction, and the activity was calculated from the following equation: activity (in U/ml) = Vcontrol/Vlysate − 1, where Vcontrol and Vlysate are the rate of cytochrome c reduction in the absence (100 μg/ml BSA was added instead) or presence of cell lysate, respectively.

NADPH:quinone oxidoreductase (NQO) activity was measured by dicoumarol-inhibitable reduction of 2,6-dichlorophenolindophenol (DCPIP) according to Lind et al. (28) with slight modifications for use with the plate reader. One unit was defined as 1 μmol/min of DCPIP reduction.

Glutathione reductase (GR) activity was assayed by monitoring glutathione disulfide (GSSG)-dependent NADPH oxidation (11) with slight modifications made for use with the plate reader. One unit was defined as 1 μmol/min of NADPH oxidation.

GPX activity was determined by measuring GR-coupled NADPH oxidation according to the methods of Flohe and Gunzler (19) with slight modifications made for use with the plate reader. One unit was defined as 1 μmol/min of NADPH oxidation.

Aldose reductase activity was measured according to the methods of Srivastava et al. (40) with slight modifications made for use with the plate reader. One unit was defined as 1 μmol/min of NADPH oxidation.

AJP-Lung Cell Mol Physiol • VOL 283 • OCTOBER 2012 • www.ajplung.org
Thioredoxin reductase (TrxR) activity was evaluated by NADP(D)-dependent reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) according to the methods of Arner et al. (4) with slight modifications made for use with the plate reader. One unit was defined as the generation of 2 mol/min of 5-thio-2-nitrobenzoic acid (TNB). NADPH-dependent DTNB reduction in a cell lysate can also be achieved by other antioxidant systems, particularly GSH/GR, although most GR activity was suppressed under our assay conditions owing to the presence of 5 mM DTNB. Nonetheless, the contribution of the GSH/GR present in each lysate to the respective apparent TrxR activity was estimated from comparison with authentic GSH and GR amounts equivalent to those present in each clone. The GSH/GR system accounted for no more and probably far less than 20 and 10% of the reported TrxR activity for D3R and G4S, respectively (data not shown).

Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) were determined according to the methods of Tian et al. (42) with slight modifications made for use with the plate reader. Briefly, total PPP activity (G6PD + 6PGD) was measured by the rate of NADPH generation in the presence of both 6-phosphogluconate (6PG) and glucose 6-phosphate (G6P). Activity of 6PGD was measured by the rate of NADPH generation in the presence of 6PG. Finally, G6PD activity was calculated from the rate of total PPP – 6PGD. One unit of each activity was defined as the generation of 1 μmol/min of NADPH.

Lactate dehydrogenase (LDH) assay was performed using the CytoTox kit (Promega) according to the manufacturer’s instructions. The measured absorbance was converted into LDH activity using equine muscle LDH (Sigma) as a standard.

γ-Glutamyl transpeptidase (GGT) activity was measured according to the methods of Forman et al. (20) with slight modifications. Cells in six-well plates were lysed by 10% native PAGS on ice. After centrifugation at 10,000 g for 15 min at 4°C, the supernatant was collected. The protein (50 μg) was resolved by 10% native PAGS at 4°C, and MnSOD activity was measured. The intensity of the MnSOD activity was within a linear range, which was confirmed using serially diluted authentic Cu,ZnSOD.

Intracellular GSH content was determined by HPLC according to the methods of Royall and colleagues (37) with slight modifications. Cells in six-well plates (4.5 × 10^4 cells/well) were incubated with 25 mM aminotriazole (ATZ) in medium for various times. At the end of the incubation, cells were washed with PBS and immediately lysed with 0.3 ml of 0.1% Triton X-100/NaPi on ice, and lysate was prepared as described (see Measurement of cellular antioxidant enzyme activities). Catalase activities were measured as described previously, and the rate constant (k_catalase) for the pseudo-first-order inactivation kinetics was determined. The steady-state H2O2 concentration ([H2O2]) was calculated from the following relationship: [H2O2] = k_catalase/k_1, where k_1 is the second-order rate constant of compound I (catalase + H2O2 → compound I + H2O) formation from catalase and H2O2 (1.7 × 10^3 M^-1 s^-1).

Data and statistics. All values expressed are means ± SD. Statistical analysis was carried out with Student’s t-test or one-way ANOVA followed by Student-Newman-Keuls test.

RESULTS

Isolation of A549 subclones. Our initial studies were designed to investigate the mechanism of cell death caused by the combination of TNF-α and actinomycin D using A549 cells as a model. However, the studies revealed that although some cells (<10%) died in several hours, the rest were resistant to the treatment even for 24 h (data not shown). Therefore, several clones from the parental A549 cells were isolated. On the basis of the sensitivity to actinomycin D plus TNF-α treatment, a highly susceptible clone and a highly resistant clone were selected for detailed study. Subsequently, it was found that the susceptibility of the clone to actinomycin D plus TNF-α treatment was almost completely dependent on actinomycin D alone (data not shown). The growth rate of the actinomycin D-resistant clone was faster than that of the susceptible clone (~1.5 times; see Fig. 2E, control). The susceptible clone was named G4S for its relative susceptibility and slower growth rate and the resistant clone D3R for its relative resistance and rapid growth rate.

Morphologically, the two clones were very different. Figure 1 shows the microscopic views of the parental A549 cells and the two subclones at confluence. D3R cells appeared to be tightly connected to each other and to proliferate as colonies (Fig. 1B), whereas G4S cells were large and had a fibroblast-like appearance and spread on the culture matrix (Fig. 1C). The parental A549 cells consisted of cells of various sizes (Fig. 1A). The morphological characteristics of D3R and G4S cells seemed to bear resemblance to some of the subpopulations of A549 cells reported by Croce et al. (14), who used a Percoll gradient separation technique to isolate subpopulations.
Sensitivity of clones to cytotoxic agents. Figure 2 shows the difference in the sensitivity of D3R and G4S clones to various agents evaluated by crystal violet staining. Cell death by actinomycin D was preferentially induced in G4S cells (Fig. 2A). For example, at an actinomycin D concentration of 0.5 \( \mu \text{g/ml} \), the number of G4S cells declined to 40% of the control at 8 h while 90% of D3R cells remained viable. The apparent slight decrease in the number of D3R cells at the high actinomycin D concentration was due not to cell death but to growth arrest as revealed by microscopic observation (data not shown). The more evident contrast could be seen at 24 h. Figure 2E shows the number of cells present after 24 h of exposure compared with the initial number of cells before exposure. It also shows the relative detachment of cells at 24 h (Figure 2E, inset). Thus both cell death and growth arrest were examined. The number of D3R cells remained the same as the initial value, but no cell death was microscopically observed (Fig. 2E), indicating the cells committed to growth arrest. In contrast, the number of G4S cells evaluated as dead by crystal violet staining was reduced to 15% of the original value, although this value represented some debris from dead cells, and, microscopically, almost all cells were observed as shrunken dead cells (Fig. 2E). Thus G4S cells were susceptible, whereas D3R cells were almost completely resistant to the cytotoxic effects of actinomycin D under our experimental conditions.

To determine whether the difference in sensitivity between the subclones was restricted to the RNA synthesis inhibitor, the response of the clones to the protein synthesis inhibitor anisomycin was investigated. Cell death was also selectively induced in G4S cells (Fig. 2B). After treatment with 10 \( \mu \text{g/ml} \) of anisomycin for 8 h, the number of G4S cells was reduced to 36% of the control level, while the number of D3R cells was 77% of the control level. The apparent decrease in the D3R cell number at this dose was also due to growth arrest as revealed by microscopic observation (data not shown). At 24 h after treatment with 10 \( \mu \text{g/ml} \) anisomycin, almost all G4S cells were microscopically observed as dead cells, and the cell number by crystal violet staining was reduced to <15% (Fig. 2E). On the other hand, all D3R cells were microscopically observed as alive without a decrease in the cell number by crystal violet staining (Fig. 2E), which indicates that D3R cells were growth arrested. Similarly, D3R cells were also completely resistant to another protein synthesis inhibitor, emetine, whereas G4S cells were sensitive to the cytotoxicity caused by this agent (data not shown). Thus, with respect to the macromolecule synthesis inhibitors tested, D3R cells were demonstrated to be growth arrested but completely resistant to cell death, whereas G4S cells were susceptible to these inhibitors under our experimental conditions.

Because ROS generation and cellular oxidation have been suggested to be involved in cell death caused by a wide range of cytocidal agents including actinomycin D (18, 24, 33, 43), we investigated whether the clones also showed differences in sensitivity to some prooxidant treatments. Enzymatic reduction and spontaneous reoxidation of DMNQ generates \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in a continuous redox-cycling manner. When the clones were exposed to DMNQ for 8 h, the toxicity was more severe in G4S cells than in D3R cells (Fig. 2C). At 50 \( \mu \text{M} \) DMNQ, the number of viable G4S cells was reduced to 40% of the control, whereas 70% of D3R cells were viable (Fig. 2C). In contrast to macromolecule synthesis inhibitor-induced cell death, where D3R cells were totally resistant, dead cells were also observed microscopically in D3R cultures at 8 h (data not shown) as well as at 24 h (Fig. 2E), although to a much lesser extent than in G4S cultures. Interestingly, when plotted, the sensitivity of G4S cells to DMNQ yielded a bell-shaped curve with the highest toxicity around 50 \( \mu \text{M} \) DMNQ. The cytotoxicity seen in G4S cells near this concentration could be attenuated by >50% by addition of catalase to the medium (Fig. 2F), which sug-
Fig. 2. Differences in the sensitivity to macromolecule synthesis inhibitors and oxidants of A549 cell subclones. Cells were treated with the indicated concentrations of actinomycin D (A), anisomycin (B), 2,3-dimethoxy-1,4-naphthoquinone (DMNQ, C), or 4-hydroxynonenal (HNE, D) for 8 or 24 h (E), and the viable cells were detected by crystal violet staining. Values are means ± SD from 6 (control) or 3 (experimental) cultures in A–D or all 5 cultures in E in each representative experiment. In A–D, *P < 0.01 and **P < 0.001, D3R vs. G4S cells at each concentration. In E, the number of cells after 24-h exposure is compared with the initial number of cells before exposure. Relative detachment of cells at 24 h is also shown; thus both cell death and growth arrest were examined. *P < 0.01, number of cells at 24 h vs. original number of cells; #P < 0.001, D3R vs. G4S cells in each treatment. Effect of superoxide dismutase (SOD) and catalase on DMNQ-induced cell death in G4S cells is shown (F). G4S cells were treated with DMNQ (67 μM) together with SOD (100 μg/ml) or catalase (100 μg/ml) or a combination of both (100 μg/ml each) for 8 h, and viability was evaluated as in B. Values are means ± SD from 6 (control) or 3 (others) cultures. ***P < 0.001 compared with enzyme controls (−). Act D, actinomycin D; Aniso, anisomycin.
suggests that the site of generation of H$_2$O$_2$, which can freely diffuse through the plasma membrane, is in close proximity to the plasma membrane. The sensitivity to the lipid peroxidation product HNE, which is a potent electrophile that can react with a wide variety of key cellular regulatory molecules such as c-Jun NH$_2$-terminal kinase (36), was also examined. G4S cells were also more sensitive to HNE than D3R cells (Fig. 2D). At 200 $\mu$M HNE, the number of viable cells in G4S culture at 8 h was diminished to 24% of the control, whereas the number of D3R cells was 68% of the control. Thus G4S cells were susceptible to both macromolecule synthesis inhibitors and oxidative insults, whereas D3R cells were totally resistant to macromolecule synthesis inhibitors and relatively resistant to oxidative insults under our experimental conditions.

Identification of mode of cell death. Apoptotic cell death, which is characterized as the activation of caspases, is associated with cell death by various cytotoxic agents (15, 23). Therefore, using the activities of caspase 3 and caspase 9 as indexes of apoptosis, the mode of cell death by the cytotoxic agents was investigated. Both caspase 3 (Fig. 3A) and caspase 9 (Fig. 3B) were activated in G4S cells but not in D3R cells after treatment with actinomycin D, anisomycin, HNE, and DMNQ at doses lethal to G4S, which is consistent with the sensitivity of each clone to these agents as measured by crystal violet staining (see Fig. 2). Thus, in G4S cells, cell death by the cytotoxic agents was at least partially due to apoptosis and involved the mitochondrial/caspase 9 cascade (23). In contrast, the limited cell death in D3R cells, which occurred with HNE and DMNQ but not the macromolecule synthesis inhibitors, appeared to be via some other mechanism.

Antioxidant profile of clones. The difference between the clones in the sensitivity to oxidants suggested that differences in the cellular antioxidant systems might be responsible. Because antioxidant enzyme activity can be affected by various culture conditions including cell confluency (8, 9), antioxidant enzyme profiles were investigated at three different cell densities (Table 1): subconfluent (~70%), confluent (~100%), and overconfluent (24 h after confluence).

Total SOD activity (2O$_2^-$ + 2H$^+$ $\rightarrow$ H$_2$O$_2$ + O$_2$), which reflects mostly Cu,ZnSOD, was similar between the clones and was independent of the degree of cell confluency (Table 1). MnSOD activity was measured by the in-gel assay that separates the two SOD enzymes; however, there was no substantial difference between the clones (Fig. 4 and Table 1). Because there was also no difference in the total SOD activity as measured by the in-gel assay that separates the two SOD enzymes; nevertheless, both clones were found to be several times higher in GSH content than those for several epithelial cells of other origins as well as macrophages and endothelial cells (data not shown). GR reduces GSSG back to GSH and accelerates the nucleophilic reaction of GSH with various electrophiles (GSH + $\text{X}$ $\rightarrow$ GS-$\text{X}$, where $\text{X}$ is an amino acid). GST accelerates the nucleophilic reaction of GSH with various electrophiles (GSH + X $\rightarrow$ GS-X, where X is an electrophile). Both GR and GST activities were also higher in D3R cells; at the confluent state, D3R cells had 3.2- and 1.6-fold higher activities in GR and GST, respectively, compared with G4S cells (Table 1). GSH adducts and a portion of both GSH and GSSG are excreted from cells. GGT degrades extracellular GSH, GSSG, and GSH adducts, thereby providing cysteine for de novo GSH synthesis (GSH + aa $\rightarrow$ Cys-Gly + $\gamma$-Glu-aa, where aa is an amino acid). It has been reported that high GGT activity increases resistance to various oxidative stresses including DMNQ (25, 39). However, there was no difference in the GGT activity between D3R cells and G4S cells (Table 1). Surpris-
Table 1. Antioxidant profile of A549 cell subclones

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>D3R</th>
<th>G4S</th>
<th>D3R</th>
<th>G4S</th>
<th>D3R:G4S</th>
<th>D3R</th>
<th>G4S</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (total), U/mg</td>
<td>10.3 ± 0.8</td>
<td>9.1 ± 1.0</td>
<td>10.0 ± 0.2</td>
<td>10.6 ± 0.5</td>
<td>1:1.06</td>
<td>10.2 ± 1.5</td>
<td>10.2 ± 0.2</td>
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<tr>
<td>MnSOD, arbitrary unit</td>
<td>ND</td>
<td>ND</td>
<td>1.047 ± 0.76</td>
<td>1.214 ± 0.80</td>
<td>1:1.16</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Catalase, U/mg</td>
<td>5.5 ± 0.6</td>
<td>11.85 ± 0.3</td>
<td>5.9 ± 0.3</td>
<td>17.31 ± 0.8</td>
<td>1:2.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.3 ± 2.0</td>
<td>21.7 ± 3.3</td>
</tr>
<tr>
<td>GSH, nmol/mg</td>
<td>ND</td>
<td>ND</td>
<td>110 ± 8</td>
<td>66.8 ± 13.6</td>
<td>1.641&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GR, mU/mg</td>
<td>99.5 ± 1.7</td>
<td>28.5 ± 4.3</td>
<td>108.6 ± 2.6</td>
<td>34.5 ± 1.8</td>
<td>3.151&lt;sup&gt;a&lt;/sup&gt;</td>
<td>135.7 ± 1.1</td>
<td>42.1 ± 3.2</td>
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<tr>
<td>GST (total), mU/mg</td>
<td>83.3 ± 7.7</td>
<td>ND</td>
<td>87.4 ± 3.7</td>
<td>54.4 ± 3.0</td>
<td>1.611&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110.0 ± 0.3</td>
<td>57.0 ± 7.3</td>
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<tr>
<td>GPx, mU/mg</td>
<td>5.9 ± 1.4</td>
<td>ND</td>
<td>9.0 ± 0.9</td>
<td>50.2 ± 0.8</td>
<td>1.558&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.1 ± 0.7</td>
<td>51.9 ± 0.3</td>
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<tr>
<td>GGT, mU/mg</td>
<td>ND</td>
<td>ND</td>
<td>9.29 ± 1.89</td>
<td>8.65 ± 0.64</td>
<td>1.071</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>NQO, mU/mg</td>
<td>ND</td>
<td>ND</td>
<td>1.227 ± 0.14</td>
<td>1.157 ± 0.36</td>
<td>1.061</td>
<td>1.604 ± 0.42</td>
<td>1.502 ± 0.19</td>
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<tr>
<td>TrxR-like, mU/mg</td>
<td>ND</td>
<td>ND</td>
<td>1.99 ± 0.07</td>
<td>2.81 ± 0.26</td>
<td>1.141&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>AR, mU/mg</td>
<td>6.1 ± 0.3</td>
<td>22.3 ± 1.9</td>
<td>6.2 ± 0.1</td>
<td>26.6 ± 1.7</td>
<td>1.429&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>29.3 ± 2.1</td>
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<tr>
<td>6PGD, mU/mg</td>
<td>ND</td>
<td>ND</td>
<td>783 ± 26</td>
<td>650 ± 35</td>
<td>1.201&lt;sup&gt;a&lt;/sup&gt;</td>
<td>943 ± 49</td>
<td>741 ± 15</td>
</tr>
<tr>
<td>LDH, U/mg</td>
<td>7.77 ± 0.02</td>
<td>3.38 ± 0.13</td>
<td>8.23 ± 0.49</td>
<td>3.94 ± 0.35</td>
<td>2.091&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.05 ± 0.59</td>
<td>5.71 ± 1.03</td>
</tr>
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</table>

Values are means ± SD; n = 2 (subconfluent and overconfluent) or 3 (confluent) cultures, except for glutathione (GSH, n = 6) and thioredoxin (Trx) and MnSOD (n = 4). ND, not determined; SOD, superoxide dismutase; GR, glutathione reductase; GST, glutathione S-transferase; GPx, glutathione peroxidases; GGT, γ-glutamyl transpeptidase; NQO, NADPH:quinone oxidoreductase; Trx, thioredoxin; TrxR, Trx reductase; AR, aldose reductase; 6PGD, glucose 6-phosphate dehydrogenase; 6PDG, 6-phosphogluconate dehydrogenase; LDH, lactate dehydrogenase. *P < 0.01, significant difference between clones.

Fig. 4. MnSOD activity in A549 cell subclones. In-gel SOD assay was conducted as described in MATERIALS AND METHODS. On the gel, the presence of SOD yielded a colorless band on an otherwise blue background. Intensities of the SOD spots were then quantitated (see Table 1). R, lysates from D3R cells; S, lysates from G4S cells. For each clone, four different cultures at the confluent state were assayed. Photograph is a representative of three independent experiments.

The clones, the contribution of GSH/GR in each lysate was estimated as no greater than 20% and 10% for G3R and G4S cells, respectively, regardless of the extent of confluence. Taking these maximum corrections into account, there was no substantial difference in TrxR-like activity between D3R and G4S cells. There was also no difference in the activity of NQO (Q + NADPH + H⁺ → QH₂ + NADP⁺), where Q represents a quinone compound) between the clones (Table 1), which is an enzyme that can protect cells from some quinone toxicity by circumventing the semiquinone radical formation (10).

Although G4S cells were much more susceptible to HNE (Fig. 2D), aldose reductase, one of the enzymes responsible for detoxifying HNE, was four times higher in G4S cells than in D3R cells (Table 1). Also, HNE is eliminated by some isoforms of GST, particularly GST A4-4; however, GST A4-4 activity was below the limit of detection (<6 mU/mg) in both clones, although a positive control, rat lung L2 epithelial cells, had an activity of 60 mU/mg protein using the same assay conditions (2).

The PPP supplies the reductant NADPH for both the GSH and Trx systems as well as NQO and AR. The activity of the rate-limiting enzyme in the PPP, G6PD (glucose-6-P + NADP⁺ + H⁺ → 6-P-glucono-δ-lactone + NADPH), was measured; however, there was no substantial difference in G6PD activity between D3R and G4S cells (Table 1). In contrast, the activity of 6PGD (6-P-glucono-δ-lactone + NADP⁺ + H⁺ → 6-P-glucuronate + NADPH), the second NADPH-generating enzyme in the PPP, was twofold higher in D3R than in G4S cells (Table 1). Nonetheless, the difference in 6PGD activity would not affect the production of NADPH, as G6PD activity would determine the rate of the PPP under physiological conditions. Finally, we measured the activity of LDH (lactate + NAD⁺ + H⁺ ↔ pyruvate + NADH), which is not an antioxidant...
enzyme but is involved in maintaining the cytosolic NADH/NAD<sup>+</sup> ratio. Interestingly, LDH activity was almost twofold higher in D3R than in G4S cells at all measured states of confluence (Table 1).

Summarizing the antioxidant results, D3R cells had substantially higher (1.5 times) GSH, GR, GST, 6PGD, and LDH activities than G4S cells, whereas G4S cells had significantly higher catalase, GPx, and AR activities than D3R cells. There were no substantial differences (<1.5 times) between the clones in Cu,ZnSOD, MnSOD, GGT, G6PD, Trx, TrxR, and NQO activities. It may be noteworthy that although G4S cells were susceptible to DMNQ in an H<sub>2</sub>O<sub>2</sub>-dependent manner and to 4NHE, the corresponding defensive enzymes (catalase, GPx, and AR, respectively) were paradoxically higher in this clone than in the resistant D3R cells.

**Steady-state H<sub>2</sub>O<sub>2</sub> concentration in clones.** A possible consequence of the difference in catalase and GPx activities would be a difference in the intracellular hydroperoxide steady-state concentration, especially H<sub>2</sub>O<sub>2</sub>. That is, lower peroxidase activity in D3R cells could allow the H<sub>2</sub>O<sub>2</sub> steady-state concentration to be relatively higher under physiological conditions, whereas the H<sub>2</sub>O<sub>2</sub> steady-state concentration could be relatively lower for the higher peroxidase-expressing G4S cells. In cells, H<sub>2</sub>O<sub>2</sub> freely diffuses into the peroxisomes where almost all catalase is sequestered. Thus the cytosolic steady-state level of H<sub>2</sub>O<sub>2</sub> can be estimated from the rate of inactivation of endogenous catalase by ATZ (37), although it actually reflects the steady-state concentration in the peroxisomes. Catalase inactivation by ATZ was 2.5 times faster in D3R than G4S cells, with half-life values of 208 and 527 s, respectively (Fig. 5). The calculated steady-state H<sub>2</sub>O<sub>2</sub> concentrations of D3R and G4S cells were 196 and 77 pM, respectively. Thus the result was consistent with a presumable consequence of the difference in peroxidase activities between the clones, thereby substantiating the different peroxidase activities in vivo.

**Relative resistance to chemical oxidation.** High concentrations of exogenous hydroperoxides have often been used to study cell death, even though the physiological relevance is clearly questionable. Nonetheless, as the differences in the enzymes that directly interact with H<sub>2</sub>O<sub>2</sub> were strikingly different, we tested the relative resistance to what may better be considered as chemical oxidation. In marked contrast to the DMNQ and HNE results, when cells were exposed to bolus H<sub>2</sub>O<sub>2</sub> or tert-butyl hydroperoxide (t-BuOOH) for 8 h, and the viable cells were detected by crystal violet staining as described. Values are means ± SD from 6 (control) or 3 (experimental) cultures in a representative experiment. *P < 0.01 and **P < 0.001, D3R vs. G4S cells at each concentration.

### Fig. 5

**Time course of endogenous catalase inactivation by aminotriazole (ATZ).** Kinetics of catalase inactivation were measured as described. Values are means ± SD (n = 2) in a representative experiment. First-order nature of the decrease in catalase activity is shown (inset).

### Fig. 6

**Difference in the sensitivity to chemical oxidation of A549 cell subclones.** Cells were treated with the indicated concentration of H<sub>2</sub>O<sub>2</sub> (A) and tert-butyl hydroperoxide (t-BuOOH, B) for 8 h, and the viable cells were detected by crystal violet staining as described. Values are means ± SD (n = 2) in a representative experiment. *P < 0.01 and **P < 0.001, D3R vs. G4S cells at each concentration.
cells were more susceptible to DMNQ (see Fig. 2C), in which addition of catalase extracellularly protected cells by >50% (see Fig. 2F), the cells were relatively resistant to chemical oxidation by bolus hydroperoxide addition. Conversely, although D3R cells showed relative resistance to DMNQ, the cells were more susceptible to the bolus hydroperoxide treatments. Although 2 mM H₂O₂ caused more extensive cell death in D3R than G4S cells, neither caspase 3 nor caspase 9 activities could be detected in the D3R cells or in the G4S cells dying from the exposure even up to 4 mM H₂O₂ [a dose lethal to both clones (data not shown)], which demonstrates that the mode of cell death caused by this nonphysiological concentration of H₂O₂ was via a caspase-independent mechanism.

**DISCUSSION**

Cell lines of tumor cell origin are well known to readily undergo chromosomal rearrangement, multiplication, and spontaneous mutation (21). Reflecting this fact, several subclones and subpopulations that bear different morphologies as well as different sensitivities to some xenobiotics have been previously isolated from the parental population of A549 cells (14, 16). In this study, we isolated two clones (D3R and G4S cells) from the A549 cell line that differed markedly in terms of morphology (see Fig. 1), growth rate (see Fig. 2), cell death (see Figs. 2 and 6), and antioxidant profile (see Fig. 4 and Table 1).

In any single cell type, higher GR activity or GSH content renders cells resistant to cell death (13, 34). Compared with G4S cells, D3R cells had higher GSH content (1.6 times) and GR (3.1 times), GST (1.6 times), and 6PGD (1.8 times) activities and had comparable activities (<1.5 times) in Cu,ZnSOD, MnSOD, NQO, GGT, Trx, TrxR, and G6PD (see Fig. 4 and Table 1). Thus one may suspect that the difference in the activity of the GSH system (GSH, GR) may be in part responsible for the different sensitivity of the clones. However, G4S cells also had higher activities of GPx (5.5 times), catalase (3 times), and AR (4 times), which suggests that attributing the difference in sensitivity of the clones to the difference in the GSH system is incompatible with the total picture. Similarly, whereas the much-higher catalase and GPx activities in G4S cells correlate with the relative susceptibility to bolus addition of nonphysiological concentrations of hydroperoxides (H₂O₂ and t-BOOH; Fig. 6), that sensitivity is inconsistent with the GR- and GSH-profile differences. Thus the relationship between the antioxidant profiles and the relative susceptibility to the macromolecular synthesis inhibitors DMNQ and HNE or to bolus hydroperoxide is not straightforward. As such, we suggest that the prediction of relative susceptibility or the drawing of mechanistic conclusions from comparisons of antioxidant defenses between the two clones is confounded by many variables. Although we cautiously consider in the following text some of those variables, the potential remains for many other factors having nothing to do with oxidant-antioxidant balance to contribute to differences in susceptibility.

**Defective caspase activation program in D3R cells is likely a contributing mechanism for different predisposition of clones to cell death.** Apoptosis is a likely component of cell death in G4S cells induced by all four agents (actinomycin D, anisomycin, DMNQ, and HNE) because all of these activated caspase (see Fig. 3). Nevertheless, none of the agents tested induced any measurable increase in the activity of either caspase 9 or caspase 3 in D3R cells (see Fig. 3). Thus D3R cells appear to have a defect in the caspase-activation program itself whereby the cells show absolute resistance to the macromolecule synthesis inhibitors, whose toxicological mechanism is apparently more apoptotic than necrotic. On the other hand, the sensitivity of D3R to DMNQ and HNE toxicity is likely due to the induction of necrotic cell death by these agents. Although DMNQ and HNE appear to induce both apoptosis and necrosis, only the G4S cells were susceptible to both cell death mechanisms.

D3R cells are susceptible to bolus addition of very high concentrations of H₂O₂ and t-BOOH (Fig. 6), although caspases were not activated (data not shown). In this case, the defective caspase-activation program in D3R cells no longer confers a protective role because the mode of death is probably necrosis. The results here appear to parallel what has been observed in the Jurkat T-cell line in which a caspase activation-deficient clone has been shown to be defective in the expression of the proapoptotic protein Bak (44).

**Potential mechanism for paradoxes in H₂O₂ toxicity and peroxidase activity.** G4S cells were more susceptible to DMNQ than were D3R cells in a catalase-inhibitable manner (see Fig. 2, C and F) but showed relative resistance to bolus nonphysiological concentrations of hydroperoxides (Fig. 6). This apparent paradoxical contrast in susceptibility to hydroperoxide toxicity may be explained by differences in the site of H₂O₂ attack in DMNQ toxicity and in bolus hydroperoxide toxicity. Although it is probable that DMNQ could be reduced in the cell by oxidoreductases, it may actually be reduced at the outer surface of the plasma membrane by a transmembrane oxidoreductase that can reduce quinones (41). Because H₂O₂ is highly diffusible through membranes, it is therefore conceivable that DMNQ derived H₂O₂ signals for cell death by affecting a target at or near the plasma membrane. In this scenario, the level of activities of intracellular GPx and catalase would be far less relevant in preventing cytotoxicity than when hydroperoxide directly damages intracellular targets. In fact, complete inactivation of endogenous catalase by ATZ pretreatment had no effect on DMNQ sensitivity (data not shown), which suggests that the site of redox cycling of DMNQ as well as the target of H₂O₂ are unlikely to be cytosolic. In contrast, although the physiological relevance of bolus addition of high concentrations of H₂O₂ is questionable, high H₂O₂ concentrations throughout the cell are quickly reached, and differences in cytosolic H₂O₂ scavenging...
ability may then account for the differences in resis-
tance to cell death.

Caveat for studies using cell lines. Although the A549
cell line has been widely used, it is difficult to elicit
consistent cellular responses in this cell line (personal
communication from three other laboratories and our
experience). It is likely that the ratio of clones such as
those described here, which can vary with culture
conditions, would contribute to inconsistencies. As
with other cell lines from which subclones have been
shown to vary in response, so should future studies
with A549 cells. In this regard, the D3R and G4S cells,
which are now extensively characterized, would be
more useful individually in future investigations than
the parental A549 cell line. Nonetheless, all
though efforts at subcloning are expected to produce a
population with genetic homogeneity that will be pre-
served over certain generations, due to the inherent
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genetic instability of cancer cells, it is probably best to
use them for only several passages.

Although characterization of antioxidants and anti-
oxidant enzyme profiles of two A549 cell subclones
have revealed paradoxical inconsistencies between an-
tioxidant enzyme activities and sensitivities to the
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corresponding oxidants used to induce cell death, the
antioxidant enzyme profile alone cannot predict pro-
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tensity to apoptosis, especially when cells are com-
pared that are dissimilar in so many respects. Thus,
although the individual clones may be useful for future
studies, comparisons between them are fraught with
hazards.

We acknowledge Dr. Mutay Aslan for comments on the H2O2
measurement by aminotriazole. We also thank Dr. Victor Darley-
Usmar for valuable discussions.

This work was supported by National Institutes of Health
Grants HL-37556 and ES-05511.

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