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Advances in Brief

Expression of Phosphorylated Histone H2AX as a Surrogate of Cell Killing by Drugs That Create DNA Double-Strand Breaks[1]

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Abstract

Phosphorylation of histone H2AX on serine 139 (γH2AX) occurs at sites flanking DNA double-strand breaks and can provide a measure of both number and location of these breaks within the nucleus. Because double-strand breaks are often lethal and are produced by several chemotherapeutic agents, we examined the possibility that expression of γH2AX after treatment might be useful as a surrogate indicator of clonogenic cell kill. Chinese hamster V79 cells were exposed for 30 min to drugs known to produce DNA double-stand breaks with different efficiencies: bleomycin, tirapazamine, doxorubicin, etoposide, 4-nitro-quinoline-N-oxide, and hydrogen peroxide. Cells were then allowed 1 h to develop foci before fixation or were plated to measure colony formation ability. Anti-γH2AX antibody staining was measured using flow cytometry. Flow histograms were analyzed for the percentage of cells that showed γH2AX levels greater than untreated cells, and this percentage was compared with the clonogenic surviving fraction. H2AX expression measured 1 h after treatment predicted cell killing for all of the drugs examined over two logs of cell kill. Moreover, predictive ability was largely independent of drug type in this cell line, and γH2AX levels five times background resulted in 50–90% cell kill. This method seems to provide a useful indicator of clonogenic response to treatment with selected chemotherapeutic drugs.

Introduction

Predictive assays for tumor response have been used for more than 30 years in medical oncology and more recently in radiation oncology (1). Von Hoff posed an important question in a review of this area when he questioned why oncologists had not paid more attention to the use of *in vitro* predictive tests, especially when results based on clonogenicity tests in 2300 patients were as good as, or better than, many accepted tests such as estrogen receptor status or bacterial sensitivity tests (2, 3). Methods that require too much time or effort or show poor predictive ability are obvious reasons for loss of enthusiasm. However, potential advantages are the possibilities that this information could be used to identify treatments that have a better likelihood of success or that avoid normal tissue toxicity resulting from unnecessary exposure to therapies likely to be ineffective against that tumor.

Ionizing radiation and many chemotherapeutic agents produce a variety of types of DNA damage, most notable being the double-strand break. Complex lesions involving both strands are more difficult to repair than single-strand breaks or base damage, and their misrepair is often associated with chromosome aberrations and cell death. Recently, formation of γH2AX[3] has been identified as an early event after the production of double-strand breaks (4). Within minutes of damage, H2AX in a 2-Mb region around the break becomes phosphorylated on serine 139, producing foci that are microscopically visible when labeled with an antibody (5). The potential to detect a single focus within the nucleus makes this the most sensitive method currently available for detecting DNA double-strand breaks (6). We have recently applied flow cytometry to the detection of γH2AX (7, 8). Although less sensitive than microscopic analysis, bivariate analysis with DNA content provides useful information on ploidy and cell cycle response (7). It is also a rapid way to analyze thousands of cells to measure heterogeneity in response to a specific treatment.

Using both the alkaline and the neutral comet assays, we previously compared cell killing with DNA single-strand and double-strand breaks produced by five drugs known to cause both types of lesions (9). We found that the amount of DNA damage associated with a given level of cell killing varied for each drug. DNA damage consistent with 15,000 single-strand breaks per V79 cell produced more than 3 logs of cell kill after X-ray exposure, 2 logs of kill by etoposide, 50% cell killing by tirapazamine (3-amino-1,2,4-benzotriazine-1,3-dioxide), and no killing by hydrogen peroxide. Although calibration for each drug was therefore necessary, once known, it could be used to predict the response in more complex model systems such as multicell spheroids and murine tumors (10, 11).

The neutral comet assay allows the measurement of DNA double-strand breaks but, because these lesions are much less prevalent than single-strand breaks, supralethal doses are generally required for their detection. We were, therefore, interested in applying γH2AX antibody staining to cells treated with drugs known to produce DNA double-strand breaks, including doxorubicin, tirapazamine, bleomycin, and etoposide. Two additional drugs were examined; hydrogen peroxide produces a thousand or more single-strand breaks for each double-strand break and 4-nitro-quinoline-N-oxide produces a hundred or more single-strand than double-strand breaks (9). The sensitivity of γH2AX antibody staining allowed, for the first time, a comparison between DNA double-strand breaks and cell killing in the same treated population.

Materials and Methods

Cell Lines and Survival Assay. Chinese hamster V79–171b lung fibroblasts have been in culture in our lab for over 20 years and were maintained in exponential growth by twice weekly subculture in MEM plus 10% fetal bovine serum. Plating efficiency of this cell line was at least 95%. Cell viability was measured using a standard clonogenicity assay on cells taken from the same population that were analyzed for γH2AX. Pooled results from three independent experiments are shown.

Drug Exposure. Tirapazamine was obtained from Sanofi-Synthelabo Inc. and dissolved in PBS at a concentration of 2.5 mM. Because this drug is preferentially toxic to anoxic cells, cells were incubated at 37°C in glass

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[3] The abbreviations used are: γH2AX, serine 139 phosphorylated histone H2AX; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride hydrate; 4-NQO, 4-nitroquinoline-N-oxide.

spinner culture flasks in medium equilibrated with 95% oxygen-free N_2 and 5% CO_2. All of the other incubations were conducted with cells attached to tissue culture dishes in an air-plus-5%-CO_2 incubator. Doxorubicin and hydrogen peroxide (H_2O_2) were purchased from Sigma and were dissolved directly in the medium. Etoposide was purchased from Bristol Myers Squibb and was prepared as 3.4-mM stock solution in distilled water. Bleomycin was purchased from the same source and diluted in sterile water at 7.5 units/ml. All of the drugs were dissolved in MEM plus 5% fetal bovine serum immediately before incubation. Drug incubation was conducted for 30 min at a cell density of 10^5 cells/ml in 10 ml of complete medium. Cells incubated with bleomycin were first permeabilized using 0.0025% saponin in PBS for 2 min at room temperature, and then 230 μM ferrous ammonium sulfate was included with bleomycin during incubation. Cells were incubated with H_2O_2 in the cold room (5–6°C), and all other incubations were performed at 37°C. Some cells were also exposed to 0–10 Gy 250 kV X-rays at a dose rate of 3.1 Gy/min. After drug incubation and rinse, cells were incubated at 37°C for 1 h to allow formation of γH2AX. A single-cell suspension was prepared by a 5-min exposure to 0.1% trypsin in PBS, and a portion of the cells was used to measure clonogenic survival. Cells (5×10^5) were fixed in 1 ml of 70% ethanol and were kept at −20°C for up to 2 weeks before analysis.

Flow Cytometry for γH2AX. Staining for γH2AX was conducted as described previously (7). Briefly, fixed cells were rehydrated for 10 min and then were centrifuged and resuspended in 200 μl of mouse monoclonal anti-phospho-histone H2A.X antibody (Upstate Biotechnology; 1:500 dilution). Cells were incubated for 2 h at room temperature and were rinsed and resuspended in 200 μl of secondary antibody, Alexa 488 goat antimouse IgG (H + L)F(ab′)$_2$ fragment conjugate (Molecular Probes; 1:200 dilution) for 1 h at room temperature. Cells were rinsed and resuspended in 1 μg/ml DAPI (Sigma) before an analysis of 20,000 cells/sample with a Coulter Elite cell sorter. Analyses of flow cytometry data were conducted using WinList software. Samples were gated on DAPI for DNA content and time of flight to eliminate debris and cell doublets before the analysis of γH2AX antibody staining intensity. Fluorescence intensity, in arbitrary units, was expressed relative to the control, an untreated cell population. Two different methods of analysis were used using the same data. The average γH2AX antibody staining relative to the untreated control cells was calculated based on mean fluorescence, or the populations of γH2AX-labeled cells were gated according to control histograms to determine the percentage of cells with no γH2AX antibody labeling.

Results

Exponentially growing Chinese hamster V79 cells were examined for cell killing and γH2AX antibody staining after a 30-min exposure to six drugs (Fig. 1). On the basis of previous results in this cell line using ionizing radiation and these drugs, a 1-h posttreatment incubation period was chosen to allow for maximum phosphorylation of H2AX (8). In all cases, γH2AX levels increased in direct proportion to drug dose. Cell killing measured using the same population of treated cells showed a typical shouldered survival curve for most of these drugs. γH2AX levels that were five times the value measured for untreated control cells were consistent with a 50–90% cell kill. Ionizing radiation also caused one log of cell kill at γH2AX levels five times the background in V79 cells (data not shown).

To more accurately compare γH2AX response and cell survival, bivariate plots of γH2AX antibody staining and DNA content were analyzed because this procedure takes into account the heterogeneity in γH2AX response throughout the cell cycle. A gate was drawn to encompass the response of the untreated cells. The percentage of cells falling into this gate was defined as the percentage of cells that were negative for drug-induced double-strand breaks and therefore likely to survive treatment. An example of this procedure is shown for etoposide in Fig. 2 and for tirapazamine in Fig. 3. The untreated cells showed a pattern that we have found to be typical for γH2AX staining through the cell cycle (7). G_1 phase cells showed a significantly lower background level of γH2AX than did cells in S and G_2 phase, presumably because of foci resulting from replication-related events

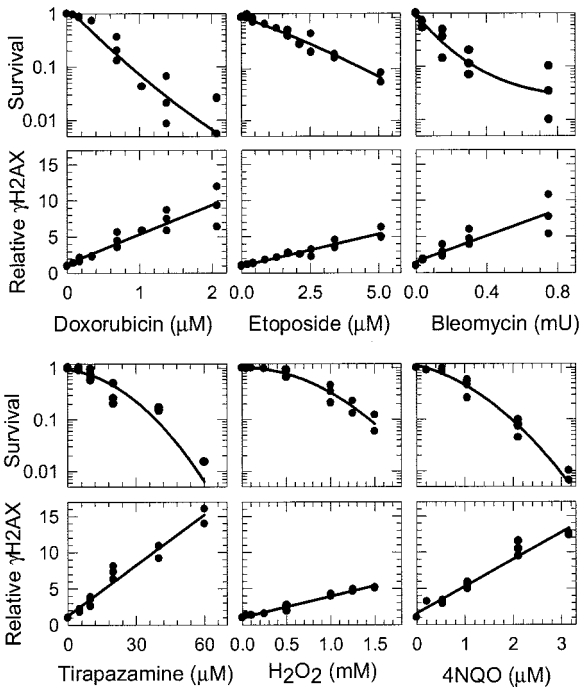

Fig. 1. Comparison between clonogenic cell survival and expression of γH2AX after exposure to six drugs. Cells were incubated with drugs for 30 min followed by a 1-h repair period before fixation for γH2AX antibody staining or plating for survival. Results from 3 independent experiments are shown. Curves for the survival data show the best-fit quadratic, and survival results below 0.005 are not shown. The line for the γH2AX data are the linear best-fit to the data.

or damage expressed as double-strand breaks in S phase. As double-strand breaks accumulated, differences through the cell cycle were reduced for three of the drugs, including etoposide, doxorubicin, and bleomycin. The pattern shown in Fig. 2 is similar to that previously reported for ionizing radiation (7). However, exposure to tirapazamine, H_2O_2, and 4-NQO gave a different pattern because γH2AX levels were significantly higher in drug-treated S-phase cells for these drugs (Fig. 3).

The comparison between measured clonogenic fraction and the fraction of cells that were negative for γH2AX is shown in Fig. 4 for the six drugs. Results for ionizing radiation are included in the panel for bleomycin. Tirapazamine exposure in air-equilibrated medium required ~80 times more drug to produce the same amount of cell killing as exposure under anoxia. However, as shown in Fig. 4, the relationship between survival and γH2AX expression was similar for air- and nitrogen-equilibrated cells exposed to tirapazamine. The fraction of cells that were negative for γH2AX agreed well with measured clonogenic fraction, with negative slopes of the six lines varying from 0.97 to 1.37.

Discussion

H2AX has been shown to be a sensitive indicator of DNA double-strand breaks produced by ionizing radiation and by drugs that cause double-strand breaks (4, 7). Using flow cytometry, we have found that γH2AX antibody labeling increases in response to damage by drugs known to produce these lesions. Although known for their ability to produce single-strand breaks, very high doses of 4-NQO and H_2O_2 can also produce DNA damage that results in an increase in γH2AX. Like results using the comet assay to measure DNA breaks (9), the γH2AX dose-response relationships were linear with dose.

The relative expression of γH2AX, when measured 1 h after a 30-min drug exposure, seems to be a useful indicator of cell killing.

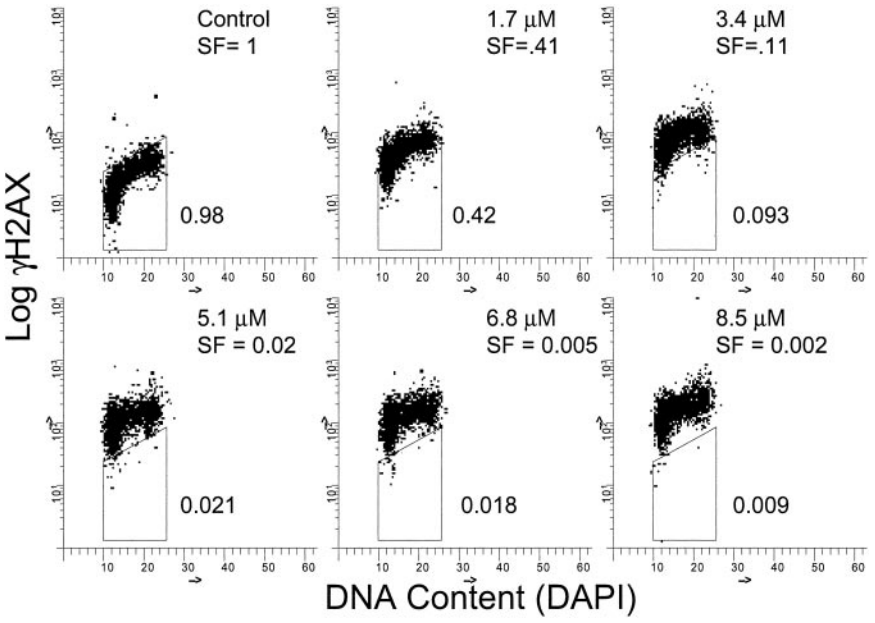

Fig. 2. Flow cytometric analysis of V79 cells stained for DNA and γH2AX after treatment with etoposide. Cells were exposed for 30 min to the indicated drug concentrations followed by a 1-h repair period. Cells were then either plated to determine the clonogenic surviving fraction (shown beneath each drug dose), or fixed for analysis of γH2AX. A gate was drawn to encompass >95% of the cells of the untreated population, and the fraction of cells within this gate is shown for each drug dose. Results are correlated in Fig. 4. SF, surviving fraction after radiation.

By gating around the population of cells that were negative for γH2AX and assuming that cells within this population contained only surviving cells, we found that we could estimate the surviving fraction for several different drugs using this same simple approach. This seems to indicate that a specific level of expression of γH2AX is associated with a specific amount of cell killing, regardless of drug. This was surprising for several reasons. Using the alkaline and neutral comet assays, we have found that the ratio of single-strand breaks: double-strand breaks varies from a factor of about 5 for bleomycin to more than 1000 for hydrogen peroxide (9). One interpretation is that double-strand breaks are responsible for cell killing by these drugs and the other lesions, such as base damage and single-strand breaks, are largely irrelevant to survival. Because a similar level of γH2AX produced by any drug appears to be associated with the same likelihood of cell death, an interpretation is that the chemical nature of the double-strand break is either unimportant or has the same likelihood of repair. Neither conclusion seems reasonable because the chemical nature of the break varies, and complex lesions are known to be more difficult to repair. Moreover, both single- and double-strand break rejoining rates differ considerably for different agents. Radiation- and H_2O_2-induced strand breaks are repaired with a half-time of less than 5 minutes, whereas repair of one-half of the breaks produced by etoposide and tirapazamine requires an hour or more (9). Additional experiments using other cell lines will be required to determine whether this relationship between surviving fraction and unstained γH2AX fraction is simply fortuitous.

Interestingly H_2O_2, tirapazamine, and 4-NQO showed increased γH2AX expression in S-phase cells. Watanabe and Horikawa (12) found that G_1 and early S-phase cells were more sensitive to 4-NQO than cells in late S or G_2 phase, and that this appeared to be attributable to differences in amounts of 4-NQO bound to the DNA. However, tirapazamine is an effective cytotoxin in nonproliferating hypoxic tumor cells, and, as with H_2O_2, there is no reason to expect greater damage to S-phase cells. In studies with the neutral comet

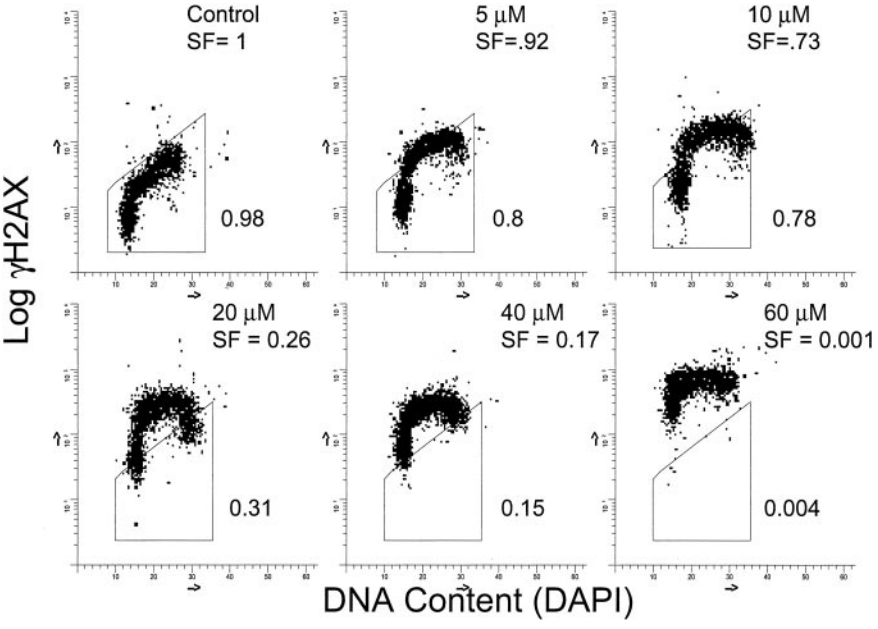

Fig. 3. Flow cytometric analysis of V79 cells stained for DNA and γH2AX 1 h after treatment with tirapazamine for 30 min under anoxic conditions. The apparent greater sensitivity of S-phase cells is notable. See legend for Fig. 2. SF, surviving fraction after radiation.

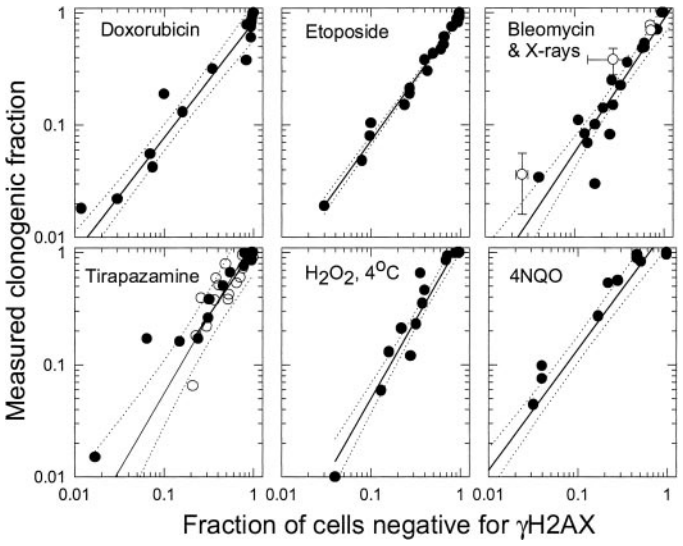

Fig. 4. Correlation between measured clonogenic fraction and the fraction of cells that were negative for γH2AX (calculated as shown in Figs. 2 and 3) for V79 cells incubated for 30 min with each drug. The response for tirapazamine (*left bottom panel*) includes cells incubated under anoxic conditions (●) and cells incubated under aerobic conditions (○). *Right top panel*, the regression line and confidence intervals are shown for bleomycin (●) along with the response of V79 cells to 0–10 Gy X-rays [○; (mean and SD bars)]. Results for three independent experiments are shown with regression lines and 95% confidence limits.

assay, we saw no cell cycle-dependent differences in double-strand break induction for H_2O_2, tirapazamine, or 4-NQO. Therefore, the increased level of γH2AX in drug-treated S-phase cells does not appear to indicate that they are more sensitive to induction of DNA damage. It could indicate that DNA single-strand breaks or base damage produced by these drugs are more likely to be interpreted as double-strand breaks in S-phase cells. The alkylating agent adozelesin also produced γH2AX foci restricted to S-phase cells (13).

We have previously used the comet assay with ^{125}IdUrd (5-iodo-2′-deoxyuridine)-labeled DNA to calibrate the neutral comet assay for V79 cells (14). The LD_{50} for V79 cells exposed to X-rays is 3.5 Gy, which is equivalent to 100 double-strand breaks/cell/LD_{50} and produces a γH2AX level 2.6 times the level of the background. A drug concentration that produce a γH2AX intensity 2.6 times the background will also kill about 50% of the cells treated with doxorubicin, etoposide, bleomycin, and H_2O_2, but values closer to 5 times the background are required to kill 50% of cells treated with tirapazamine or 4-NQO. On the basis of results using the neutral and alkaline comet assays, we previously estimated that 100–200 double-strand breaks would kill 50% of V79 cells treated with doxorubicin or etoposide, and that 300–400 double-strand breaks would be required to kill 50% of cells exposed to tirapazamine (9). Results shown here for γH2AX are reasonably consistent with these predictions. However, we also estimated that it would take only 2.5 H_2O_2- or 21 4-NQO-induced double-strand breaks to kill 50% of the cells, and our results using γH2AX suggest that 100–200 double-strand breaks are produced by the LD_{50}. Therefore, either the ratio of single-strand breaks:double-strand breaks for H_2O_2 and 4-NQO is much lower than previously reported or, more likely, large numbers of DNA single-strand lesions are sufficient to stimulate formation of γH2AX. Two single-strand breaks on opposite strands of the duplex that are not closely spaced are unlikely to produce a double-strand break in the neutral comet assay. However, it is possible that they are recognized as a double-strand break by molecules that signal the presence of double-strand damage and cause phosphorylation of histone H2AX.

In conclusion, the level of γH2AX measured 1 h after a 30-min drug exposure was able to predict the extent of cell killing in Chinese hamster V79 cells exposed to six drugs. Regardless of the drug used, levels of γH2AX antibody binding five times the background resulted in 50–90% cell kill. Preliminary results using four human tumor cell lines exposed to doxorubicin compare favorably with results for the hamster V79 cells; γH2AX levels five times the background resulted in 80–95% cell kill. This method may prove useful as a measure of cell sensitivity to drugs that cause DNA double-strand breaks.

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