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Relationship between Phosphorylated Histone H2AX Formation and Cell Survival in Human Microvascular Endothelial Cells (HMEC) as a Function of Ionizing Radiation Exposure in the Presence or Absence of Thiol-Containing Drugs

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Abstract

Human microvascular endothelial cells (HMEC) were exposed to ionizing radiation at doses ranging from 0 to 16 Gy in either the presence or absence of the active thiol forms of amifostine (WR1065), phosphonol (WR255591), *N*-acetyl-L-cysteine (NAC), captopril or mesna. Each of these clinically relevant thiols, administered to HMEC at a dose of 4 mM for 30 min prior to irradiation, is known to exhibit antioxidant properties. The purpose of this investigation was to determine the relationship (s), if any, between the frequency of radiation-induced histone H2AX phosphorylation at serine 139 (γ -H2AX) in cells and subsequent survival, as assessed by colony-forming ability, in exposed cell populations as a function of the presence or absence of each of the five thiol compounds during irradiation. γ -H2AX formation in irradiated cells, as a function of relative DNA content, was quantified by bivariate flow cytometry analysis with FITC-conjugated γ -H2AX antibody and nuclear DAPI staining. γ -H2AX formation in cells was measured as the relative fold increase as a function of the treatment conditions. The frequency of γ -H2AX-positive cells increased with increasing dose of radiation followed by a dose- and time-dependent decay. The most robust response for γ -H2AX formation occurred 1 h after irradiation with their relative frequencies decreasing as a function of time 4 and 24 h later. To assess the effects of the various thiols on γ -H2AX formation, all measurements were made 1 h after irradiation. WR1065 was not only effective in protecting HMEC against γ -H2AX formation across the entire dose range of radiation exposures used, but it was also significantly more cytoprotective than either its prodrug (WR2721) or disulfide (WR33278) analogue. WR1065 had no significant effect on γ -H2AX formation when administered immediately or up to 30 min after radiation exposure. An inhibitory effect against γ -H2AX formation induced by 8 Gy of radiation was expressed by each of the thiols tested. NAC, captopril and mesna were equally effective in reducing the frequency of γ -H2AX formation, with both WR1065 and WR255591 exhibiting a slightly more robust protective effect. Each of the five thiols was effective in reducing the frequency of γ -H2AX-positive cells across all phases of the cell cycle. In contrast to the relative ability of each of these thiols to inhibit γ -H2AX formation after irradiation, NAC, captopril and mesna afforded no protection to HMEC as determined using a colony-forming survival assay. Only WR1065 and WR255591 were effective in reducing the frequencies of radiation-induced γ -H2AX-positive cells as well as protecting against cell death. These results suggest that the use of γ -H2AX as a biomarker for screening the efficacy of novel antioxidant radioprotective compounds is highly problematic since their formation and disappearance may be linked to processes beyond simply the formation and repair of radiation-induced DSBs.

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INTRODUCTION

Ionizing radiation produces a wide spectrum of molecular lesions in DNA. These can include base damages, single-strand breaks (SSBs), double-strand breaks (DSBs), and multiply damaged sites. DNA in eukaryotes is packaged along with core histone proteins into a fundamental subunit of the chromosome, the nucleosome. H2A is one of the core histone families that have been conserved throughout evolution and has as one of its subfamilies a histone variant known as H2AX (1). H2AX is reported to account for approximately 10% of the H2A histone complement and is randomly distributed and expressed throughout the genome (2). This particular histone variant is of particular interest because of the observation that, after the induction of DSBs by ionizing radiation, H2AX is phosphorylated at serine 139 within its conserved COOH-terminal region, i.e. γ -H2AX (2). The induction of γ -H2AX after exposure to radiation is reported to be mediated by ATM and occurs at the sites of DSBs in the nuclear DNA (3). The rapid phosphorylation of H2AX at these damaged sites precedes the involvement of repair enzymes involved in both homologous recombination (HR) and non-homologous end-joining (NHEJ) DNA repair (2,4,5). H2AX phosphorylated in this manner has been observed to cover approximately 2 Mb of DNA surrounding the damaged site (6); the number of γ -H2AX foci formed in this manner has been shown to be directly proportional to the number of DSBs formed, and their dephosphorylation has been correlated with repair of DSBs (7). From these studies, the rationale for using data relating to γ -H2AX focus formation and disappearance has led to the development of this end point as a sensitive and specific marker for the detection of DSB damage and repair in irradiated cells (7-9).

Ionizing radiation produces most of its DNA damage through the production of free radicals, i.e., the indirect effect. For this reason, antioxidant drugs have long been investigated as potential radioprotective drugs (10). Because free radical production by ionizing radiation is a very rapid process and is essentially complete by 10^{-2} s (11), radical scavenging drugs must be present at the time of irradiation to be effective (12). Amifostine and phosphonol are phosphorothioate drugs that need to be dephosphorylated by alkaline phosphatase to their free thiol forms to be active and effective radioprotectors (13). The free thiol forms of these agents have been studied extensively and have been found to protect against ionizing radiation-induced DSB formation in DNA and subsequent cell killing (14,15). Other free thiol-containing drugs purported to be effective free radical scavengers and therefore potential radioprotectors include captopril, mesna and *N*-acetyl-L-cysteine (NAC) (10). Because of the potential usefulness of the γ -H2AX marker in detecting radiation-induced DSB damage in biological systems, it is reasonable to propose its inclusion in mass screening systems to rapidly identify and evaluate the effectiveness of novel radioprotective compounds for future development. In this paper we describe the results of using γ -H2AX as a marker for radiation-induced DNA damage after exposure of HMEC in the presence or absence of five-well characterized antioxidant drugs administered at equal molar doses, e.g., the free thiol forms of amifostine and phosphonol, and captopril, mesna and NAC.

To facilitate this study, we have used a recently developed quantitative flow cytometry method with which to rapidly identify and measure radiation-induced γ -H2AX in cells. We have successfully compared it to a standard method of detection involving the use of an automated digital microscopic focus analysis using NIH ImageJ custom software and have published the results elsewhere (16). While each method gave similar results, flow cytometry allowed for not only a more rapid accumulation of data but also the determination of γ -H2AX formation in cells as a function of their DNA content, i.e., their relative position within each specific phase of the cell cycle.

METHODS AND MATERIALS

Cells and Culture Conditions

Human microvascular endothelial cells (HMEC) were obtained from the Biologic Products Branch, Centers for Disease Control, Atlanta, GA, and were maintained in endothelial basal medium MCDB131 (Gibco/BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS) (Sigma, St. Louis, MO), 10 ng/ml epidermal growth factor (Collaborative Biomedical Products, Bedford, MA), 1 µg/ml hydrocortisone (Sigma), penicillin and streptomycin (Gibco/BRL). HMEC were grown in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cells were subcultured weekly, and new cell stocks were used every 3 months. HMEC were grown as fed cultures to confluence for all experiments and trypsinized with 0.25% trypsin and 1 mM EDTA (EDTA/Trypsin; Gibco/BRL).

Irradiation and Drug Treatment Conditions

HMEC were irradiated at room temperature using an X-ray generator (RT250, Philips) operating at 250 kVp and 15 mA at a dose rate of 1.65 Gy/min. Doses ranged from 0 to 16 Gy. Five clinically relevant free thiol antioxidant drugs were studied: the active free thiol forms of amifostine (WR2721; {S-2-[3-aminopropylamino] ethylphosphorothioic acid}), designated WR1065 (2-[(aminopropyl)amino]ethane thiol), and phosphonol (WR3689; {S-[2-(3-methylaminopropyl)aminoethyl] phosphorothioic acid}), designated WR255591 (2-[3-{methylamino} propylamino] ethanethiol), and the ACE inhibitor captopril ((2S)-1-[(2S)-2-methyl-3-sulfanylopropanoyl] pyrrolidine-2-carboxylic acid), mesna (sodium 2-sulfanylethanesulfonate), and *N*-acetyl-L-cysteine (NAC). Each of these agents was dissolved in phosphate-buffered saline (PBS; 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 140 mM NaCl; Gibco/BRL) at a final concentration of 4 mM. Each drug was added to cells 30 min prior to irradiation and remained present during the irradiation procedure.

Cell Preparation and Measurement of γ-H2AX by Flow Cytometry

The methods for cell preparation and flow cytometry measurement of γ-H2AX in HMEC after exposure to ionizing radiation are described in detail elsewhere (16). Briefly, HMEC were suspended in complete growth medium containing 2% paraformaldehyde (PFA, Sigma) and were fixed after incubation at 37°C for 10 min, at which time they were chilled on ice, centrifuged, washed three times with 0.5% bovine serum albumin (BSA; EMD Bioscience, La Jolla, CA) in PBS, permeabilized with 90% methanol in 0.5% BSA-PBS, and then stored at -20°C. Fixed cells (1–2 × 10⁶) were washed three times with 0.5% BSA-PBS and then stained with FITC-γ-H2AX antibody (Upstate, Charlottesville, VA) for 1 h at 4°C in the dark. HMEC were then washed three times with 0.5% BSA-PBS and then counterstained with DAPI (2 µg/ml 4',6-diamidino-2-phenylindole dihydrochloride hydrate; Sigma) in TBS (14.04 g Tris-HCl, 1.34 g Tris-base, 0.4 g MgCl₂, 1 ml Triton X 100, 6.5 g NaCl in 1 liter, pH 7.4).

Cells stained with FITC-γ-H2AX and DAPI were analyzed with a three-laser LSR II (Becton Dickinson, San Jose, CA) using 488 nm excitation for FITC (530/30 BP filter) and 355 nm excitation for DAPI (450/65 BP filter). Debris was excluded from the analysis by gating a forward scatter relative to side scatter plot. Cell aggregates were excluded by gating a time of flight versus forward scatter plot. Integrated fluorescence measurements for FITC and DAPI were recorded for 10,000 single nondebris events. Logarithmic FITC fluorescence was plotted relative to linear DAPI fluorescence using FlowJo analysis software (TreeStar, Ashland, OR). Unirradiated controls were used to set the threshold gating to determine the percentage of γ-H2AX-positive cells. DNA content (DAPI) was plotted on the x axis, positioning the G₁ peak at channel 100, while the background autofluorescence of FITC was plotted on the y axis at an intensity level of 100. Contour or dot plots were used to set the gating strategy. A minimum

of three independent gating experiments were performed using contour patterns, and the data are presented as means with standard errors of the mean (SEM).

Colony-Forming Survival Assay

Immediately after exposure of HMEC to 4 and 8 Gy of radiation in the presence or absence of thiol-containing drugs, cells were washed, trypsinized, counted and plated into fresh growth medium at appropriate numbers to give rise to about 100 colonies per dish as described in detail elsewhere (17). Five dishes were used per experimental point, and experiments were repeated three times. Cells were incubated under standard growth conditions for 21 days, and the resultant colonies were stained with 20% crystal violet in methanol and colonies containing 50 or more cells were scored. Statistical analyses of surviving fractions at 4 and 8 Gy in the presence or absence of each of the thiols tested were performed using ANOVA with Tukey-Kramer multiple comparisons.

RESULTS

Bivariant Flow Cytometry Analysis of γ -H2AX Formation after Irradiation

While the identification and analysis of γ -H2AX formation in cells after irradiation can be obtained using both standard microscopy-generated image analysis and gel electrophoresis-based methods, a flow cytometry-based method offers the advantage of rapid quantification of γ -H2AX-positive cells in the population as a whole as well as a function of their relative position within the cell cycle. This latter approach has a significant advantage for the development of rapid drug screens for novel radiation protectors with the caveat that γ -H2AX formation must be a stringent marker of radiation-induced DNA DSBs. We recently developed and published the details of one such system that was found to generate comparable values that were determined in parallel using a standard microscopy-based focus counting method (16). We have applied that bivariant flow cytometry method to assess the radioprotective effectiveness of five well-characterized thiol-containing antioxidants. Presented in Fig. 1 are data describing the dose responses of radiation-induced γ -H2AX formation in HMEC at 1, 4 and 24 h after exposure to doses ranging from 0 to 16 Gy. The frequency of γ -H2AX-positive cells measured in this manner was greatest 1 h after irradiation and decreased with time to near background levels 24 h later. Because a readily observable dose response for γ -H2AX formation could be seen at 1 h, this interval was used in all subsequent experiments to evaluate the effects of the various thiol-containing drugs on radiation response.

The intensity of γ -H2AX formation has been reported to vary as a function of position of cells in the cell cycle (18,19). The use of dual staining with FITC- γ -H2AX antibodies and DAPI allows us to correct for cell cycle variations. Contour plotting was used to gate γ -H2AX-positive cells from the remainder of the population expressing cell cycle phase-specific background intensities. To standardize the system for potential use in novel radioprotector drug screening, initial studies were performed using the extensively studied free thiol form of amifostine, WR1065. The results of this gating approach on HMEC exposed to ionizing radiation in the presence or absence of 4 mM WR1065 as a function of dose for doses ranging from 0 to 16 Gy are presented in Fig. 2A and B. The frequencies of γ -H2AX-positive cells detected by this method were reduced at all radiation doses for HMEC exposed in the presence of WR1065. A fairly robust difference in the frequency of γ -H2AX-positive cells as a function of WR1065 treatment was observed at a dose of 8 Gy. For this reason all subsequent evaluations of radiation effects on γ -H2AX formation as a function of thiol exposure were made using a dose of 8 Gy. The relative effects of cell cycle position, frequency of γ -H2AX-positive cells, and WR1065 treatment are described in Fig. 2C and D. WR1065 was protective against γ -H2AX formation across all phases of the cell cycle and radiation doses used.

Effects of Amifostine and its Metabolites on Postirradiation Induction of γ -H2AX

Amifostine is an inactive prodrug that requires activation by alkaline phosphatase into its free thiol form (12,13). The free thiol form can then react with other thiols forming either a disulfide molecule structurally and functionally resembling the polyamine spermine (20) or a mixed disulfide. The disulfide, like the prodrug form of amifostine, has not been found to be cytoprotective (21). The free thiol form of amifostine, WR1065, is well recognized as the active form of the drug responsible for protection against the lethal effects of ionizing radiation exposure. All three forms were therefore evaluated to determine their relative effectiveness in protecting against γ -H2AX induction after exposure of HMEC to 8 Gy. As shown in Fig. 3, WR1065, consistent with its well-characterized cytoprotective effectiveness, was the most effective of the three forms of the drug in reducing the frequency of radiation-induced γ -H2AX-positive cells.

Approximately 70% of the damage induced by ionizing radiation is attributed to the generation of highly reactive free radical; this is known as the indirect effect. Drugs possessing antioxidant properties have routinely been identified as potential candidates for use as radioprotectors because of their ability to scavenge free radicals. Protectors that work by this mechanism are most effective when administered prior to radiation exposure with marginal if any protective effects being observed if administered after irradiation. To determine whether postirradiation administration of WR1065 could affect γ -H2AX formation, HMEC were exposed to WR1065 immediately after and 1, 3, 10 and 30 min after exposure to 8 Gy. Under these conditions WR1065 is ineffective in protecting against the lethal effects of ionizing radiation (22). Likewise, WR1065 was ineffective in reducing the frequency of γ -H2AX-positive cells when administered after irradiation (see Fig. 4).

Radioprotector Effectiveness as Assessed by γ -H2AX and Colony-Forming Ability

The active free thiol forms of amifostine and phosphonol, WR1065 and WR255591, respectively, along with captopril, mesna and NAC were each evaluated at a concentration of 4 mM with respect to their ability to affect the induction of γ -H2AX-positive HMEC exposed to 8 Gy. As shown in Fig. 5A, each of these thiols was effective in reducing the frequency of γ -H2AX-positive cells induced by radiation. Furthermore, each of these agents protected cells in each phase of the cell cycle, with WR1065 and WR255591 being the more protective of the five agents evaluated (Fig. 5B). The ability of each of these thiols to protect against γ -H2AX formation would suggest that they would all be effective in protecting against radiation-induced cell killing. To test this we exposed HMEC to 4 and 8 Gy in the presence or absence of each of these drugs under the same conditions used to assess γ -H2AX induction after radiation exposure. Cell survival was assessed using a colony-forming assay. As shown in Fig. 6, both WR1065 and WR255591, consistent with their ability to protect against γ -H2AX induction, significantly protected HMEC against radiation-induced cell killing at both doses. In contrast, captopril, mesna and NAC were incapable of protecting HMEC against the lethal effects of radiation at either dose indicating that their effects on the prevention of γ -H2AX induction were not predictive of their abilities to protect cells.

DISCUSSION

With the observation that DNA DSBs induced by ionizing radiation result in the phosphorylation of serine 139 in histone H2AX, γ -H2AX focus formation has become an important marker for use in the rapid assessment of cellular DNA damage induced by radiation exposure (2). γ -H2AX appears within minutes after radiation exposure, and it is estimated that approximately 1% of H2AX becomes γ -phosphorylated per gray of radiation exposure (2). In contrast to neutral elution methods that are used to measure the kinetics of DNA DSB induction and repair requiring doses of 25 to 250 Gy (14,15), dose responses and repair kinetics based

on γ -H2AX formation and disappearance can easily be measured in the 0.5- to 10-Gy range (16,23-26). We have successfully applied a bivariate flow cytometry method to rapidly quantify radiation-induced γ -H2AX in HMEC as a function of their relative position within the cell cycle (16). As shown in Fig. 1, the frequency of γ -H2AX-positive HMEC increased as a function of dose over the range of 2 to 16 Gy when measured 1 h after irradiation. A qualitatively similar radiation dose response could be observed when measurements were made 4 and 24 h later, but the overall frequency of γ -H2AX-positive cells fell significantly as a function of time after radiation exposure, presumably due to the cellular repair of DSB lesions.

To better assess the usefulness of the bivariate flow cytometry method as a first-pass screen for evaluating the relative protective effectiveness of novel radioprotective drugs, HMEC were exposed to a range of radiation doses in the presence of 4 mM of WR1065, the well-characterized active free thiol form of amifostine (13). Consistent with DSB analysis using neutral elution techniques and cell survival assays (14,15), a 4 mM dose of WR1065 was effective in protecting against radiation-induced γ -H2AX-positive cells across the entire dose range tested (see Fig. 2). HMEC in all phases of the cell cycle were protected by WR1065.

A number of studies have been performed by numerous investigators to identify the most radioprotective form of amifostine. In particular, Smoluk *et al.*, using cell survival based on a classical colony-forming assay as an end point, clearly demonstrated that radiation protection was found to correlate with cellular levels of the free thiol form of the drug (SH) but not with the disulfide (SS) or the parent prodrug form of amifostine (27). This relationship was also evident with regard to protection against radiation-induced γ -H2AX induction, as shown in Fig. 3. Furthermore, administration of WR1065 after irradiation is well known to be ineffective in protecting against cell killing induced by radiation, but it can protect against mutation induction at a number of loci (22,28). Administration of WR1065 to HMEC immediately after or up to 30 min after exposure to 8 Gy resulted in no significant change in the percentage of γ -H2AX-positive cells (see Fig. 4). This finding is consistent with the paradigm that DSBs are considered to be potentially lethal lesions in mammalian cells. Since WR1065 does not affect cell survival when administered after radiation exposure, its inability to significantly alter γ -H2AX induction, a marker of potentially lethal DSB damage after irradiation, was not unexpected.

Having characterized a flow cytometry-based γ -H2AX detection system as a potential screening mechanism for novel radioprotector development using WR1065 as a radioprotector standard, we applied the system to evaluate four additional thiol-containing drugs purported to possess cytoprotective properties. WR255591, the active free thiol form of phosphonol, captopril, mesna and NAC all have been investigated extensively as cytoprotective agents. WR255591 has been reported to be comparable to WR1065 in protecting against radiation-induced DSBs and cell killing (15). Captopril is an angiotensin-converting enzyme (ACE) inhibitor that is used to treat hypertension. It has been used successfully to prevent radiation-induced nephropathy and fibrosis as well as pneumopathy and lung fibrosis in a rat model (29,30). Mesna has been developed for use as a cytoprotector against the toxicity of acrolein, a urotoxic metabolite of oxazaphosphorine-based alkylating agents (31,32). NAC is a well-characterized antioxidant that is known to stimulate glutathione biosynthesis (33) and to affect numerous cellular redox systems (33-35). Each of these agents was effective in protecting against radiation-induced γ -H2AX in HMEC, as shown by a significant reduction in the frequency of γ -H2AX-positive cells, regardless of their relative position within the cell cycle (see Fig. 5A, B). If radiation induction of γ -H2AX is reflective of DSB formation and radiation-induced DSBs are potentially lethal lesions in cells, the ability of these five thiol-containing agents to protect against γ -H2AX induction by radiation in HMEC should be predictive of their relative abilities to protect against radiation-induced cell killing. Only WR1065 and WR255591, however, were observed to be cytoprotective. Captopril, mesna and NAC afforded

no protection against radiation cytotoxicity as determined by a colony-formation survival assay (Fig. 6).

Unrepaired DSBs are considered to be lethal lesions in irradiated cells. Neutral elution methods have been used in past studies to quantify not only the induction of DSB by ionizing radiation in cells but also their rates of repair after exposure to both WR1065 and WR255591 (14,15). While both WR1065 and WR255591 were very effective in protecting against radiation-induced DSBs and the protection against DSB induction was closely correlated with protection against cell death, neither agent affected either the rate of DSB repair or the amount of residual unrepaired damage. Using the technique of neutral elution to measure radiation-induced DSBs, the predictor for the efficacy of a radioprotector as defined by enhanced cell survival was related to the relative ability to protect against initial DSB damage and not the rate of DNA repair or residual damage. The inability of captopril, mesna and NAC to affect cell survival after radiation exposure has also been reported in other studies (10,35). The relative ability of thiols to protect in this manner has been investigated extensively, resulting in a theory that postulates that it is the net charge of a thiol compound that is a determining factor (36,37). The basis of this theory is that because DNA is negatively charged, thiol compounds that have a net positive charge will be more effective in migrating to the microenvironment of the DNA. Since approximately 70% of the damage to the DNA by ionizing radiation is due to free radical generation, the ability to concentrate free radical scavenging agents within the microenvironment of the DNA at the time of radiation exposure would enhance the effectiveness of radioprotectors. Both WR1065 and WR255591 have a net +2 charge, while captopril, mesna and NAC have a net charge of 0, -1 and -1, respectively (10). While the theory of the net charge of the thiol has been useful in predicting the efficacy of a radioprotector relating to enhanced cell survival after irradiation in the presence of thiol drugs, the ability to affect the phosphorylation state of H2AX by these thiols after radiation exposure has not been a very useful predictor in this study.

The use of γ -H2AX as a surrogate marker of radiation-induced DNA DSBs has been found to be problematic by several investigators in their attempts to correlate this end point with differences between cell strains and relative levels of clonogenic survival after irradiation (24-26). It has been proposed that differences between cell lines in p53 status and defects in ATM can strongly influence not only γ -H2AX levels but also the kinetics of their disappearance with time. Because in this study we used only HMEC, cells that are wild-type p53, these cell-specific factors are not relevant to the discrepancy we have observed between the relative level of protection against γ -H2AX formation and cell survival after irradiation in the presence of the five thiol compounds evaluated (38). Furthermore, WR1065 has been demonstrated to be an effective cytoprotective agent regardless of the p53 status of cells (39).

Each of the agents evaluated in this study, by virtue of having a free SH group in its structure, shares in an ability to affect the phosphorylation state of proteins that can be affected by SH-dependent protein phosphatases (40). WR1065 has been demonstrated to inhibit topoisomerase II α activity and phosphorylation levels (41). Captopril has been reported to be effective in inhibiting tyrosine phosphorylation (42) in human mesangial cells and Ras-regulated myosin light chain phosphorylation in the smooth muscle cells of SHR rats (43). Mesna has been demonstrated to affect phosphorylation of HSP27 in human lymphocytes (44), while NAC has been studied extensively as an antioxidant with the ability to affect receptor tyrosine kinases, MAP kinases and I κ B kinases (45,46). Thus the ability of these five thiol compounds to affect γ -H2AX formation induced by ionizing radiation is not surprising. However, what is unknown at present is how these agents might differentially affect the formation and persistence of γ -H2AX foci that are unrelated to or decoupled from the formation and/or persistence of frank DSBs in irradiated cells. The very nature of thiols in general to affect the phosphorylation state of proteins, either directly or indirectly, may make this class of compounds problematic for

initial screening by any method that employs the γ -H2AX marker as a measure of DNA DSBs. In our present study, the use of this marker would have resulted in three false positives out of the five agents tested. The earlier demonstration that the two effective radioprotectors of the five evaluated in this study, WR1065 and WR255591, had no effect on either the kinetics or magnitude of DSB repair using neutral elution methods indicates that assessing the kinetics of the disappearance or persistence of γ -H2AX after longer periods after irradiation would not be very effective in identifying effective protectors in this class of drugs (14,15). If such a system were used, neither WR1065 nor WR255591 would have been identified as being radioprotective.

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FIG. 1. Radiation dose response of γ -H2AX-positive cells using bivariate flow cytometry analysis. Confluent HMEC were irradiated with 0–16 Gy X rays and the percentages of γ -H2AX-positive cells were assessed 1, 4 and 24 h after irradiation. The percentages of γ -H2AX-positive HMEC $\pm$ 1 SEM are plotted as a function of dose and time after irradiation.

FIG. 2.

Radiation dose response of γ -H2AX-positive cells 1 h after treatment as a function of radiation dose and the presence or absence of 4 mM WR1065 (WR) during irradiation. All cells were exposed to FITC- γ -H2AX antibody and stained with DAPI. Panel A: Two-dimensional flow cytometry histograms showing the percentages of γ -H2AX-positive cells after bivariate analysis. Panel B: Relative changes in the percentages of γ -H2AX-positive cells $\pm$ SEM as a function of radiation dose (IR) and WR1065 treatment. Panel C: Percentage of γ -H2AX-positive HMEC as a function of radiation dose in the absence of WR1065. Panel D: Percentage of γ -H2AX-positive HMEC as a function of radiation dose in the presence of 4 mM WR1065.

FIG. 3.

Relative effectiveness of amifostine's prodrug (WR2721), free thiol (WR1065), and disulfide (WR33278) forms, each administered 30 min prior to irradiation at a concentration of 4 mM, on the induction of γ -H2AX-positive cells by 8 Gy. Error bars represent $\pm$ SEM. Statistical analysis of γ -H2AX-positive in the presence or absence of each of the drug forms tested was performed using ANOVA with Tukey-Kramer multiple comparisons (not significant = ns; ** = <0.01 ; *** = <0.001).

FIG. 4.

Effect of WR1065 on the induction of γ -H2AX-positive cells by 8 Gy as a function of the timing of administration before (B) or various times starting immediately (I) after (A) irradiation. Cells were exposed to 4 mM WR1065 for 30 min in all cases. Error bars represent $\pm$ SEM. Significance was determined after pairwise comparisons of the percentage of γ -H2AX-positive cells at 8 Gy with each of the WR1065 treatments (not significant = ns; *** = <0.001).

FIG. 5.

Effect of the free thiol forms of amifostine (WR1065) and phosphonol (WR255591) and the drugs *N*-acetyl-L-cysteine (NAC), captopril and mesna on the induction of γ -H2AX-positive cells (panel A) and as a function of their relative position within the cell cycle (panel B). All thiols were administered 30 min prior to radiation exposure at a dose of 4 mM. Error bars represent $\pm$ SEM. Significance was determined after pairwise comparisons of the percentage of γ -H2AX-positive cells at 8 Gy with each of the thiol treatment conditions (* = <0.05; ** = <0.01; *** = <0.001).

FIG. 6.

Bar graphs on a semilogarithmic plot depicting the surviving fraction of HMEC exposed to (panel A) 4 Gy and (panel B) 8 Gy of 250 kVp X rays after a 30-min treatment with 4 mM of WR1065, WR255591, NAC, captopril and mesna. Each bar represents the mean $\pm$ SEM of three separate experiments. Significance was determined after pairwise comparisons of surviving fractions at 4 or 8 Gy, respectively, with each of their corresponding thiol treatment conditions (not significant = ns; *** = <0.001).