Constitutive histone H2AX phosphorylation on *Ser*-139 in cells untreated by genotoxic agents is cell-cycle phase specific and attenuated by scavenging reactive oxygen species

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Abstract. DNA damage, particularly when it involves formation of double-strand breaks (DSBs), triggers phosphorylation of histone H2AX on *Ser*-139. Phosphorylated H2AX has been named γH2AX, and induction of γH2AX in cells exposed to genotoxic agents is considered a sensitive and specific reporter of DNA damage. However, in untreated normal cells as well in the cells of various tumor lines cells, a fraction of histone H2AX molecules remain phosphorylated. In the present study, we observed that the extent of this constitutive H2AX phosphorylation varies depending on the cell type (line) and on cell cycle phase and, in most cell types, S and G_2/M phase cells exhibit greater levels of H2AX phosphorylation than do cells in the G_1 phase. Furthermore, constitutive H2AX phosphorylation in human pulmonary carcinoma A549, lymphoblastoid TK6, and in normal bronchial epithelial cells was reduced following cell exposure to N-acetyl-L-cysteine, a scavenger of reactive oxygen intermediates; the reduction was most pronounced for G_2M cells. Growth of A549 cells in the presence of buthionine sulfoximine, an inhibitor of glutathione synthetase, amplified the level of constitutive H2AX phosphorylation in A549 cells. The observed constitutive H2AX phosphorylation may be a reflection of the ongoing DNA damage mediated by reactive oxygen species (ROS) generated by metabolic activity during progression through the cell cycle, leading to formation of DSBs during the S phase. Because cumulative DNA damage in proliferating cells mediated by ROS is considered the key mechanism for cell ageing, the present approach to estimate the degree of attenuation of constitutive H2AX phosphorylation by antioxidants may provide a convenient tool to assess the DNA-protective and possible anti-ageing properties of other agents.

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Introduction

Histone H2AX, one of the variants of the nucleosome core histone H2A (1-3), undergoes phosphorylation on *Ser*-139 in response to DNA damage, particularly if the damage involves formation of DNA double-strand breaks (DSBs) (4,5). The phosphorylation, which is mediated by the PI-3-like kinases ATM- (4-7), ATR- (8) and/or DNA-dependent protein kinase (DNA-PK) (9), affects H2AX molecules at megabase DNA domains on both sides of DSBs in chromatin (4,5). The *Ser*-139-phosphorylated H2AX has been defined as γH2AX (10).

The presence of γH2AX in chromatin can be detected immunocytochemically (5,10). Shortly after DNA damage (e.g. by ionizing radiation), the induction of γH2AX manifests in the form of distinct nuclear γH2AX immunofluorescent (IF) foci (5,10). Each focus is presumed to correspond to a single DSB. Numerous signaling and repair proteins including the M/R/N complex (Mre11/Rad50/Nbs1), Brca1 and the p53 binding protein 1 (53BP1) co-localize with phosphorylated H2AX at the foci (7-9,11-13). While $H2AX^{-/-}$ cells fail to form irradiation-induced foci (14), the migration of repair and signaling proteins to DSBs is not abrogated in these cells. This would suggest that the M/R/N complex itself provides the mechanism for migration of these proteins. The loss of H2AX in mice, however, leads to genomic instability. $H2AX^{-/-}$ mice are radiation sensitive, growth-retarded, and immunodeficient (15). Even H2AX haploinsufficiency compromises the genetic integrity of the mice, and enhances their susceptibility to cancer, particularly in the absence of p53 (16). H2AX is thus one of the critical proteins responsible for surveillance of genome integrity (17).

H2AX is also phosphorylated in physiological processes that involve DNA recombination. This occurs in response to

formation of DSBs in V(D)J and class-switch recombination during immune system development and also at sites of recombinational DSB formation in meiosis (18-21). The multiplicity of DSBs generated during DNA fragmentation in apoptotic cells also induces H2AX phosphorylation (22).The extent of H2AX phosphorylation during apoptosis is much greater compared to the H2AX phosphorylation induced by particular genotoxic agents (22-25).

In untreated normal cells and in cells of various tumor lines, a fraction of histone H2AX molecules remain phosphorylated. The extent of this constitutive ('programmed', 'intrinsic') H2AX phosphorylation, which can be detected immunocytochemically, varies depending on the cell type (line) and cell cycle phase (24-26). Generally, cells in S phase as well as mitotic cells exhibit much greater levels of H2AX phosphorylation than G_1 cells. However, in contrast to the cells in which H2AX phosphorylation is induced by DNA damage, there is no evidence of discrete and strongly fluorescent nuclear γH2AX foci. Instead, the constitutive H2AX phosphorylation appears in the form of diffuse, weakly fluorescent heteromorphous structures distributed over the entire nucleus (23-26).

It is generally recognized that cells are continuously exposed to oxidants generated by metabolic activity and other biochemical reactions as well as to external oxidants or oxidant-inducers. It is also recognized that oxidative stress induces DNA damage. In fact, progressive oxidative DNA damage has been considered one of the primary factors of ageing (27-29). The present study was designed to explore whether the observed constitutive H2AX phosphorylation in untreated cells may be a reflection of the ongoing DNA damage induced in untreated cells by oxidative stress during the cell cycle. If this is the case, one would expect that depletion of free radicals will attenuate the constitutive H2AX phosphorylation. We have tested, therefore, whether exposure of cells to an antioxidant, the free radicals scavenger N-acetyl-L-cysteine (NAC), may have such an effect. Furthermore, to explore whether the intrinsic oxidant scavenger, glutathione, plays a role in reducing DNA damage that can be revealed by the level of constitutive H2AX phosphorylation, we have measured the effect of inhibition of glutathione synthesis by the synthetase inhibitor, buthionine sulfoximine (BSO), on expression of γH2AX in relation to the cell cycle phase.

Materials and methods

Cells and culture conditions. Human lymphoblastoid TK6 were kindly provided by Dr Howard Liber of Colorado State University, Fort Collins Co. Jurkat cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). They were grown in 25 ml Falcon flasks (Becton-Dickinson Co., Franklin Lakes, NJ) in RPMI-1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (all from Gibco/BRL Life Technologies, Inc., Grand Island, NY) at 37˚C in an atmosphere of 5% CO_2 in air. At the onset of the experiments, there were fewer than $5x10^5$ cells per ml in culture and the cells were at an exponential and asynchronous phase of growth. DU145, HeLa, MCF-7 and A549 cells were obtained from ATCC and were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Gibco/BRL). Human normal bronchial epithelial cells (HNBE) were purchased from Cambrex BioScience, Walkersville, MD, and were cultured in complete bronchial epithelial cell growth medium (BEGM), prepared by supplementing bronchial epithelial basal medium (BEBM) with retinoic acid, human epidermal growth factor, epinephrine, transferrin, triiodothyronine, insulin, hydrocortisone, bovine pituitary extract and gentamicin by addition of SingleQuots™ . BEBM and SingleQuots™ were purchased from Cambrex BioScience. The cultures were diluted and replated every 4 days to maintain them in an asynchronous and exponential phase of growth. For experiments, the cells were trypsinized and seeded at low cell density (~$5x10^4$ cells per chamber) in 2-chambered Falcon CultureSlides (Beckton-Dickinson Labware, Franklin Lakes, NJ).

Cell treatments. NAC was obtained from Sigma Chemical Co. (St. Louis, MO). The pH of the NAC solution was adjusted to 7.0 before being applied to the cells. The cultures were incubated with various concentrations of NAC for different time intervals, as described in the figure legends. To inhibit GSH synthesis, the cultures were incubated with 100 μM BSO (Sigma) for 17 h. The cells were then fixed either in suspension or by transferring the slides into Coplin jars containing 1% methanol-free formaldehyde (Polysciences, Inc., Warrington, PA) in PBS for 15 min on ice followed by suspension in 70% ethanol, where they were stored at -20˚C for 2-24 h.

Immunocytochemical detection of γH2AX. The fixed cells were washed twice in PBS and suspended in 0.2% Triton X-100 (Sigma) in a 1% (w/v) solution of bovine serum albumin (BSA; Sigma) in PBS for 30 min to suppress nonspecific antibody (Ab) binding. The cells were then incubated in 100 μl of 1% BSA containing 1:200 diluted anti-phospho-histone H2A.X (*Ser*-139) mAb (Upstate, Lake Placid, NY). The cells were then incubated overnight at 4˚C, washed twice with PBS and resuspended in 100 μl of 1:30 diluted FITC-conjugated F(ab')2 fragment of goat anti-mouse immunoglobulin (Dako) for 30 min in room temperature in the dark. The cells were then counterstained with either 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) in PBS for 5 min, or 5 μg/ml PI in the presence of 100 μg/ml of RNase A (Sigma).

Fluorescence measurements. Cellular green (FITC) and blue (DAPI) fluorescence emission was measured simultaneously in the same cells using an iCys laser scanning cytometer (LSC) (CompuCyte, Cambridge, MA) utilizing standard filter settings; fluorescence was excited with 488-nm argon ion and violet diode lasers, respectively. The intensities of maximal pixel and integrated fluorescence were measured and recorded for each cell. At least 3,000 cells were measured per sample. Cellular green (FITC) and red (PI) fluorescence of cells in suspension was measured using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). The red (PI) and green (FITC) fluorescence from each cell were separated and quantified using standard optics and CellQuest software (Becton-Dickinson). Each experiment was run in duplicate or triplicate and repeated at least three times.

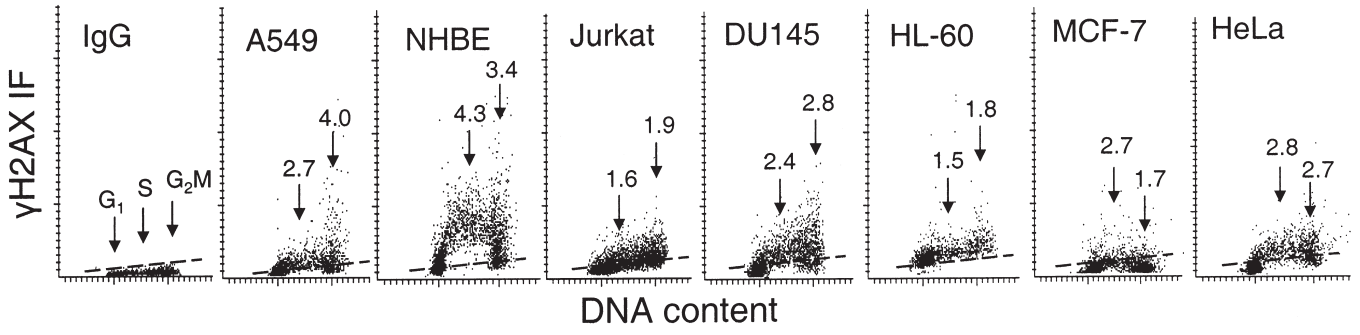

Figure 1. Different level and cell cycle phase variability of constitutive H2AX phosphorylation observed in different cell types. Bivariate distributions representing cellular DNA content vs γH2AX IF of human cells from untreated exponentially growing lung carcinoma A549, normal bronchial epithelial (NHBE), T-cell leukemic Jurkat, prostate carcinoma DU145, promyelocytic leukemic HL-60, breast carcinoma MCF-7 and cervical carcinoma HeLa cell cultures measured by laser-scanning cytometry. The negative isotype control (IgG; A549 cells) is shown in the left panel. Other cell types had similarly low nonspecific fluorescence; the maximal level of IgG IF, below which >97% of cells in the IgG panel were located, is marked in each panel by a dashed line. The figures above the arrows pointing to S and G_2M cells indicate the n-fold higher mean expression of γH2AX of S- and G_2M-phase cells, respectively, compared to G_1 cells, whose mean γH2AX for each cell type was normalized to 1.0. To allow for comparison of the differences in γH2AX IF intensity, fluorescence measurements were carried out at the same laser power output and photomultiplier sensitivity. Note differences in the extent and pattern of γH2AX expression vis-à-vis the cell cycle phase in different cell types. NHBE cells in the S phase of the cycle exhibit a particularly high expression of γH2AX.

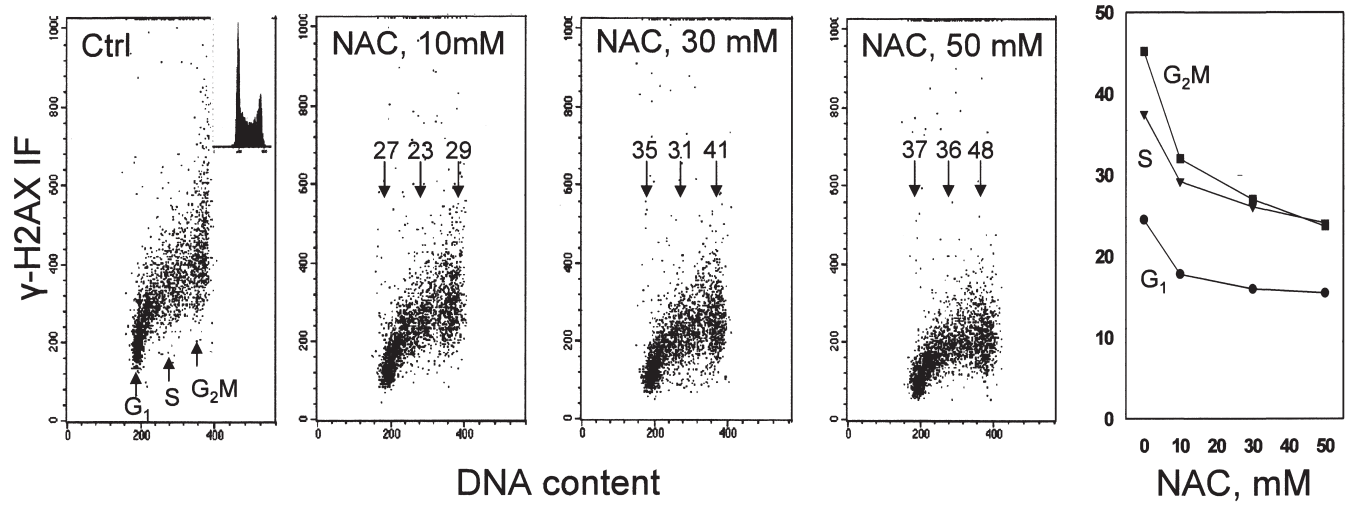

Figure 2. Decrease in expression of γH2AX in TK6 cells induced by their growth in the presence of different concentrations of NAC. The first four panels show bivariate distributions illustrating cellular DNA content vs γH2AX expression of cells growing in the absence (Ctrl) or presence of 10, 30 or 50 mM NAC, included into cultures for 1 h prior to cell harvesting. The figures above arrows pointing to G_1, S, and G_2M cells indicate percent decrease of means of γH2AX IF of cells in these phases of the cycle in the NAC-treated cultures with respect to the Ctrl. The right panel presents the plot of the mean values of γH2AX expression estimated for G_1, S and G_2M cell populations by gating analysis of the raw data shown in the left panels, as a function of NAC concentration.

Results

Using Ab that specifically binds to the epitope of histone H2AX phosphorylated on *Ser*-139, the presence of the phosphorylated form of this histone was detected immunocytochemically in a variety of cell types (Fig. 1). The cells were from cultures growing exponentially and were not subjected to treatment with any genotoxic agent. Concurrent differential staining of cellular DNA and measurement of cellular fluorescence by LSC made it possible to correlate expression of γH2AX with cell cycle phase. The data show that γH2AX expression varied markedly depending on the cell type and cell cycle phase. Some cell types, such as NHBE, A549 or DU145, showed distinctly higher levels of γH2AX expression than Jurkat, MCF-7 or HL-60 cells. Among the cell types strongly expressing γH2AX, S-phase cells and a fraction of cells in G_2M exhibited higher levels of H2AX phosphorylation compared to G1 cells.

More detailed analysis of constitutive H2AX phosphorylation vis-à-vis cell cycle phase was performed for TK6, A549 and NHBE cells. In several independent experiments, subpopulations of cells in G_1, early-, mid-, and late-S and G_2M were selected by gating analysis and the mean values of γH2AX IF for these subpopulations were estimated. The data were normalized to G_1 cells (1.00) to represent n-fold change in γH2AX expression that occurs during progression through S and G_2M. It is quite evident that the extent of phosphorylated H2AX progressively increased when cells advanced through

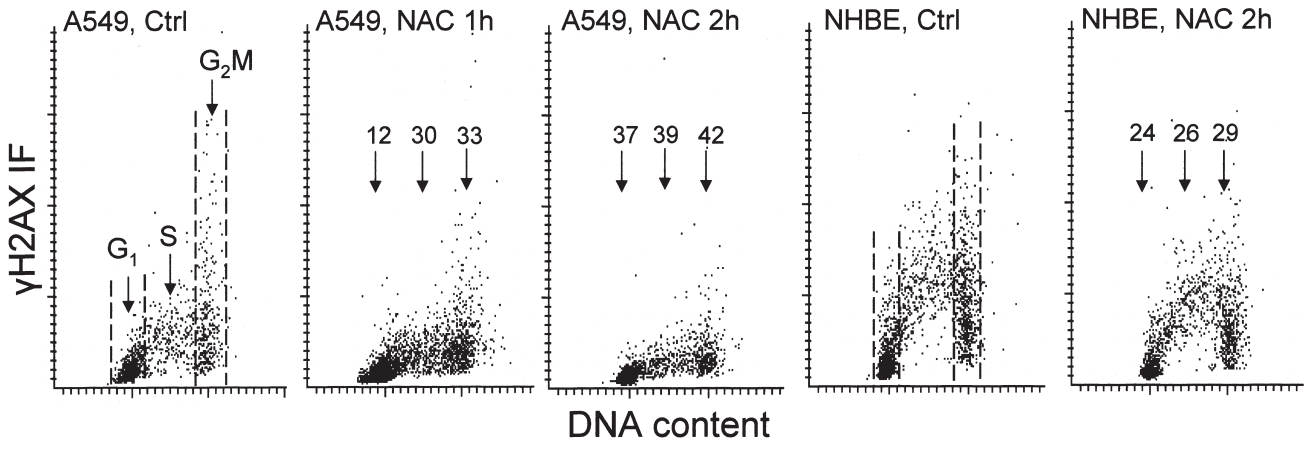

Figure 3. NAC-induced decrease in expression of γH2AX in A549 and HNBE cells. The bivariate cellular DNA content vs γH2AX IF distributions represent untreated (Ctrl) A549 or NHBE cells and the cells growing in the presence of 25 mM NAC for 1 or 2 h. As in Fig. 2, the figures over the arrows pointing towards G_1, S and G_2M cells indicate the percent decrease of means of H2AX IF of cell in these phases in the NAC-treated cultures with respect to the respective Ctrl.

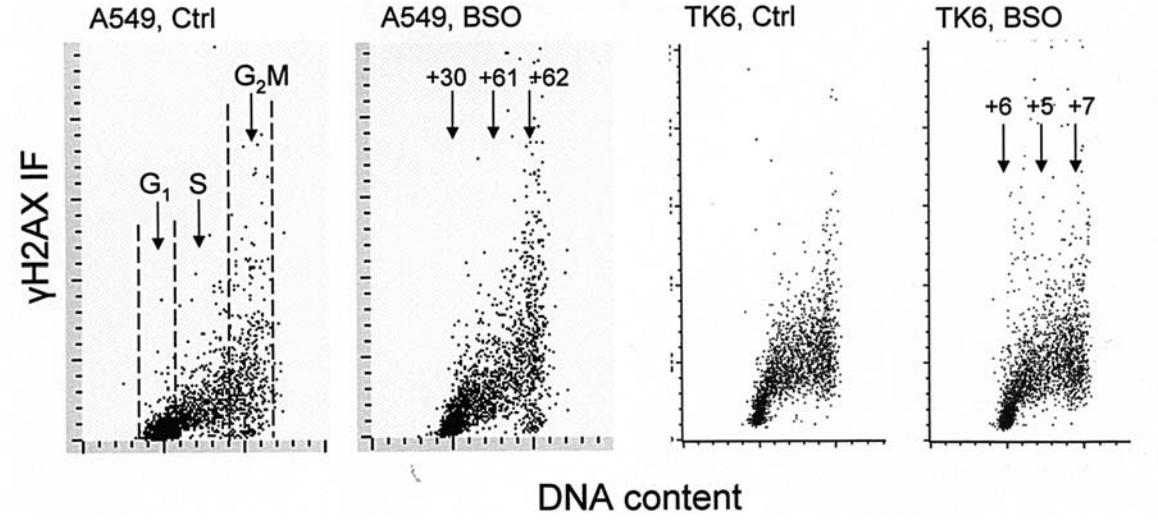

Figure 4. Effect of growth in the presence of BSO on expression of γH2AX in A549 and TK6 cells. Exponentially growing A549 and TK6 cells were exposed in cultures to 100 μM BSO for 17 or 21 h, respectively, prior to harvesting. Fluorescence of A549 cells was measured by LSC and fluorescence of TK3 cells by flow cytometry. The mean γH2AX IF of cells gated in G_1, S and G_2M phase was measured and is expressed in the BSO-treated cultures as percent increase above the respective means in Ctrl.

S and, in NHBE cells, peaked at late-S phase. In the case of TK6 and A549 cells, the maximal expression of γH2AX was in G_2M cells.

Growth of TK6 cells in the presence of NAC for 1 h reduced their expression of γH2AX in an NAC concentration-dependent manner (Fig. 2). The effect was already seen at 10 mM NAC, and was most pronounced for G_2M phase cells, whose mean γH2AX IF was reduced by 48% at 50 mM NAC. Under the same culture conditions, the decrease in γH2AX IF of G_1- and S-phase cells was 37 and 36%, respectively.

The expression of γH2AX was also reduced in A549 and NHBE cells that were grown in the presence of NAC (Fig. 3). Although the differences between G_1 vs S vs G_2M in the degree of reduction of H2AX expression were smaller than in the case of TK6 cells (Fig. 2), in every instance, the reduction was most pronounced for G_2M cells.

Growth of A549 cells in the presence of 100 μM BSO for the approximate duration of one cell cycle (17 h) led to a distinct increase in expression of γH2AX (Fig. 4). The increase was more pronounced for S- and G_2M-phase cells (61-62%) than for G_1-phase cells (30%). In the case of TK6 cells, however, the effect of BSO was minimal.

Discussion

The present data confirm and extend the earlier observations of McPhail *et al*, (26) and our own findings (22-24) that the presence of histone H2AX phosphorylated on *Ser*-139 can be detected in a variety of cell types growing in cultures and not exposed to genotoxic agents. Histone H2AX phosphorylation is being considered a specific and sensitive reporter of DNA damage, particularly the induction of DSBs (4-6). It is likely,

Table I. The cell-cycle phase differences in the level of expression of γH2AX in different cell types.

Cells	G_1	Early-S	Mid-S	Late-S	G_2M
TK6 (n=3)	1.00±0.19	1.68±0.17	1.86±0.16	2.06±0.14	2.22±0.19
A549 (n=5)	1.00±0.02	2.27±0.07	2.79±0.06	3.25±0.17	3.87±0.22
HNBE (n=5)	1.00±0.04	3.50±0.15	4.50±0.17	4.68±0.11	3.41±0.08

Expression of γH2AX was measured in TK6, A549 and NHBE cells concurrently with cellular DNA content by flow (TK6) or laser-scanning (A549 and NHBE) cytometry, as shown in the raw data in Fig. 1. The mean values of H2AX expression were then estimated for cell populations gated based on differences in DNA content (DNA index; DI) as follows: G_1 (DI=0.9-1.1), early-S (DI=1.1-1.4), mid-S (DI=1.4-1.6), late-S (1.6-1.9) and G_2M (DI=1.9-2.1). The data were normalized to the mean value of G_1 cells (1.00) of the respective cell type. The analysis was carried out on several (n=3 or 5) different cultures for each cell type and the data show mean values ± SE of these separate analyses.

therefore, that the observed H2AX phosphorylation in the untreated cells is a marker of constitutive DNA damage that occurs during progression through the cell cycle and leads to formation of DSBs. It has been estimated that ~50 DSBs per nucleus (which amounts to ~0.8 DSBs per 108 bp) are generated during a single cell cycle in human cells untreated with any exogenous genotoxic agent (30). This estimate of constitutive formation of DSBs, called 'endogenous DSBs', was based on an assumption that ~1% of single-strand lesions (SSLs) are converted to DSBs during the cell cycle (30), and also by calculating the dose of X-irradiation (IR) that doubles the extent of H2AX phosphorylation (number of γH2AX foci per nucleus), interpolating the IR to dose zero and, thereby, estimating the magnitude of the background H2AX phosphorylation in non-irradiated cells in terms of number of DSBs (30).

In the present study, we observed that the level of H2AX phosphorylation varied between cell lines and also between cells, depending on their position in the cell cycle (Fig. 1). Interestingly, normal (non-tumor) NHBE cells exhibited the highest level of constitutive H2AX phosphorylation, distinctly higher than cells of each of the six tumor lines. Compared to G_1-phase cells, which served as a yardstick (1.0) to relate the relative level of γH2AX expression in S- and G_2M-phase cells, G_2M cells of the A549 line and S-phase NHBE cells showed the highest level of H2AX phosphorylation (4.1- and 3.9-fold, respectively).

In several cell lines (A549, NHBE, DU145) a minor fraction of G_2M cells showed high levels of γH2AX expression. As shown by us (31) and others (32,33), the strongly γH2AX-positive cells were mitotic cells. H2AX phosphorylation observed in mitotic cells from cultures untreated by genotoxic agents is considered unrelated to DNA damage. Its function was postulated to be related to preservation of fidelity of the mitotic process, even in the absence of DNA damage (32). However, chromatin condensation during mitosis induces torsional stress on the DNA superhelical structure, which manifests by increased sensitivity of mitotic cell DNA to single-strand specific nucleases (34), and upon exposure to heat or acid, in increased susceptibility to denaturation (melting) (35,36). It is possible, therefore, that the observed H2AX phosphorylation in mitotic cells is triggered by this conformational DNA change that is associated with its tight packing in the condensed chromatin in mitotic chromosomes that causes torsional topological stress on DNA. Similar topological stress (melting transitions) induced in DNA by T4 gene 32 protein was recently measured by single molecule force spectroscopy (37). It is likely that local DNA pre-melting conformational changes that may occur when subjecting mammalian cells to heat shock are also responsible for the induction of H2AX phosphorylation in these cells (38).

Parallel to DNA content, histone content also doubles during the cell cycle. The observed increase in γH2AX expression in S- and G_2M-phase cells thus reflects both, the increase in H2AX content and the increased degree of its phosphorylation (per unit of H2AX protein). To estimate the mean degree of H2AX phosphorylation in a particular phase of the cell cycle, one thus has to compensate for the increase in H2AX content during the cell cycle and thereby divide the mean increases of γH2AX expression in S and G_2M cells related to G_1 cells, as marked in Fig. 1, by 1.5 and 2.0, respectively. Following such compensation, one may conclude that no significant increase in the degree of H2AX phosphorylation during the cell cycle occurred in some cell types (Jurkat, HL-60). Although, in other cell types, the increase was seen only in S-phase cells (MCF-7), in most cell types (A549, NHBE, DU145, HeLa), the elevated degree of H2AX phosphorylation (above that of G_1 cells) was seen in both, S- and G_2M-phase cells. A variety of factors associated with a cell's response to oxidative stress and DNA damage, such as the presence of wt p53 (39), or expression of other tumor suppressor- and onco-genes (40), known to vary between cell lines, may be responsible for the observed cell cycle-phase differences in constitutive H2AX phosphorylation in the studied cell types.

Cell growth in the presence of NAC distinctly reduced the extent of constitutive H2AX phosphorylation. The reduction was apparent for all three cell types that were studied, lymphoblastoid TK6 cells (Fig. 2), pulmonary carcinoma A549 and normal bronchial epithelial cells (Fig. 3). NAC is considered an effective scavenger of ROIs and is widely used as a chemopreventive agent (reviewed in ref. 41). It is most likely that the observed reduction of H2AX phosphorylation by NAC reflects a decreased rate of constitutive DNA damage by

ROIs, which are scavenged by this antioxidant. Indeed, there is strong evidence that constitutive DNA damage during the cell cycle is mediated by ROS (27-30,42).

It should be noted, however, that NAC itself may cause DNA damage (41). The DNA damaging effect, however, was generally observed after prolonged (18 h) exposure of cells to this antioxidant. It is rather unlikely, thus, that significant DNA damage was induced by NAC in the present study, in which the duration of cell exposure to NAC did not exceed 2 h.

In support of the notion that observed constitutive phosphorylation of H2AX reflects DNA damage caused by ROS are the results showing its increased phosphorylation in A549 cells growing in the presence of BSO, the agent expected to decrease the level of the intrinsic ROS scavenger, glutathione, in the cell. This effect, however, while distinct in A549 cells, was not evident in TK6 cells (Fig. 4). Perhaps the efficiency of ROS scavenging properties of the intracellular glutathione, e.g. due to differences in glutathione content per cell, or its accessibility to ROS, varies between the cell types, and is lower in TK6 than in A549 cells.

As mentioned, approximately 50 DSBs are generated during the cell cycle in human cells (30). They represent ~1% of the SSLs initially induced by ROS, which become converted to DSBs during DNA replication; the remaining 99% of SSLs are repaired by error-free mechanisms (30). The generation of DSBs is thus a two-step dynamic process: (i) abundant production of SSL, followed by (ii) conversion of some SSLs into DSBs during the S phase. Our present findings, which indicate the highest degree of constitutive H2AX phosphorylation in S and G_2M cells and a progressive increase in the level of phosphorylated H2AX in mid- and late-S phase as compared to early-S (Table 1), are consistent with this mechanism. Namely, as more and more DNA is replicated during progression through S phase there is a cumulative production of DSBs triggering progressive phosphorylation of H2AX. Some of these DSBs may remain during G_2 and be responsible for a transient delay in progression of cells at the G_2M checkpoint, where the repair of DSBs may occur.

Cumulative DNA damage in proliferating cells mediated by ROS is considered the key mechanism for cell ageing. The present data show that it is possible to measure the degree of attenuation of H2AX phosphorylation and, thus most likely, the degree of protection of DNA from the damage caused by intrinsic ROS. Therefore, this approach can be used to evaluate DNA-protective and possible anti-ageing properties of antioxidant agents other than NAC. It also can be used to assess whether oxidative stress mediates the DNA-damaging effect of the suspected genotoxic agent, as recently demonstrated in studies on the effects of tobacco smoke (43,44).

Acknowledgements

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