Extent of constitutive histone H2AX phosphorylation on Ser-139 varies in cells with different TP53 status

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Abstract. In response to DNA damage by genotoxic agents, histone H2AX is phosphorylated on Ser-139. However, during the cell cycle, predominantly in S and G₂M phase, histone H2AX is also phosphorylated in untreated normal and tumour cells. This constitutive H2AX phosphorylation is markedly reduced by exposure of cells to the reactive oxygen species scavenger N-acetyl-L-cysteine. Therefore, it appears likely that constitutive H2AX phosphorylation reflects the ongoing oxidative DNA damage induced by the reactive oxygen species during progression through the cell cycle. Because the tumour suppressor p53 (tumour protein p53) is known to induce transcription of genes associated with cell response to oxidative stress, we have compared the intensity of constitutive H2AX phosphorylation, and the effect of N-acetyl-L-cysteine on it, in cells with different tumour protein p53 status. These were human lymphoblastoid cell lines derived from WIL2 cells: TK6, a p53 wt line, NH32, a tumour protein p53 knock-out derived from TK6, and WTK1, a WIL2-derived line that expresses a homozygous mutant of tumour protein p53. Also tested were the tumour protein p53-null promyelocytic HL-60 cells. The degree of constitutive H2AX phosphorylation was distinctly lower in NH32, WTK1 and HL-60 compared to TK6 cells in all phases of the cell cycle. Also, the degree of attenuation of constitutive H2AX phosphorylation by N-acetyl-L-cysteine was less pronounced in NH32, WTK1, and HL-60, compared to TK6 cells. However, the level of reactive oxygen species detected by the cells' ability to oxidize carboxyl-dichlorodihydrofluorescein diacetate was not significantly different in the cell lines studied, which would suggest that regardless of tumour protein p53 status, the level of oxidative DNA damage was similar. The observed higher level of constitutive H2AX phosphorylation in cells harbouring wt tumour protein p53 may thus indicate that tumour protein p53 plays a role in facilitating histone H2AX phosphorylation, an important step in the mobilization of the DNA repair machinery at the site of DNA double-strand breaks.

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INTRODUCTION

Histone H2AX, one of the variants of histone H2A (West & Bonner 1980; Thatcher & Gorovsky 1994), is considered to be one of the critical proteins responsible for the surveillance of genome integrity (Celeste *et al.* 2002; Bassing *et al.* 2003; Celeste *et al.* 2003). In response to DNA damage that involves formation of DNA double-strand breaks (DSBs), histone H2AX becomes phosphorylated on Ser-139 (Rogakou *et al.* 1998; Sedelnikova *et al.* 2002). The phosphorylation, mediated by the PI-3-like kinases ataxia telangiectasia mutated (ATM)- (Anderson *et al.* 2001; Burma *et al.* 2001), ataxia telangiectasia mutated and Rad3-related protein (ATR)- (Furuta *et al.* 2003), and/or DNA-dependent protein kinase (Park *et al.* 2003), takes place on H2AX molecules on both sides of DSBs along a megabase length of DNA (Rogakou *et al.* 1999). The Ser-139-phosphorylated H2AX has been named γ H2AX. It should be noted that H2AX is also phosphorylated during the formation of DSBs in physiological processes that involve DNA recombination such as V(D)J class-switch recombination during immune system development and at sites of recombinational DSB formation in meiosis (Jackson 2001; Modesti & Kanaar 2001; Fernandez-Capetillo *et al.* 2002). DSBs generated during DNA fragmentation in apoptotic cells also induce H2AX phosphorylation (Huang *et al.* 2003; Huang *et al.* 2004; Huang *et al.* 2005).

The presence of γ H2AX can be detected immunocytochemically in the form of distinct nuclear γ H2AX immunofluorescent (IF) foci, each focus presumed to correspond to a single DSB (Rogakou *et al.* 1999; Sedelnikova *et al.* 2002). A variety of signalling and repair proteins including the M/R/N complex (Mre11/Rad50/Nbs1), Brcal and the p53-binding protein 1 (53BP1) colocalize with γ H2AX at the foci (Paull *et al.* 2000; Anderson *et al.* 2001; Furuta *et al.* 2003; Downs & Cote 2005; Foster & Downs 2005).

Cells are continuously exposed to oxidants generated by metabolic activity and other biochemical reactions as well as to external oxidants or oxidant inducers and it is generally recognized that oxidative stress induces DNA damage. In fact, progressive oxidative DNA damage has been considered one of the primary factors of ageing (Nohl 1994; Parrinello *et al.* 2003; Barzilai & Yamamoto 2004; Moller & Loft 2004; Schriener *et al.* 2005). Endogenous reactive oxygen species (ROS) modify approximately 20 000 bases of DNA per day in a single cell (Beckman & Ames 1997). It has been estimated that approximately 50 DSBs per nucleus (which amounts to approximately 0.8 DSBs per 10^8 bp) are generated during a single cell cycle in human cells untreated with any exogenous genotoxic agent (Vilenchik & Knudson 2003).

Given the previous discussion, it would be expected that DNA damage by endogenous ROS will be reflected by the presence of γ H2AX reporting formation of DSBs continuously throughout the cell cycle. Indeed, a fraction of histone H2AX molecules remains phosphorylated in untreated normal cells as well in the cells of different tumour lines (MacPhail *et al.* 2003; Huang *et al.* 2005). The extent of this constitutive H2AX phosphorylation (CHP), also called 'programmed' or 'intrinsic' γ H2AX, tends to be higher in S, G₂ and mitotic cells compared to G₁ cells and varies depending on the cell type (line) (MacPhail *et al.* 2003; Huang *et al.* 2005; Huang *et al.* 2006b). CHP manifests in the form of diffuse, weakly fluorescent heteromorphous structures distributed over the entire nucleus (Huang *et al.* 2005) rather than discrete and strongly fluorescent γ H2AX foci. The heterogeneity may reflect a large dispersion in the 'age' of individual γ H2AX foci, including the ones diminished in size resulting from γ H2AX dephosphorylation as DNA repair progresses. In cells of different cell types, a large portion of this CHP can be prevented by cell exposure to an antioxidant, the free radical scavenger N-acetyl-L-cysteine (NAC) (Albino *et al.* 2004; Albino *et al.* 2006; Huang *et al.* 2006b). Furthermore, cell growth in the presence of the glutathione synthetase inhibitor buthionine sulfoximine raised the level of

CHP (Huang *et al.* 2006b). These results taken collectively implicate ROS as a mediator of DNA damage that, in turn, is being reported by CHP throughout the cell cycle.

During studying CHP in a variety of cell types (lines), we noticed that its level in NHBE and pulmonary carcinoma A549 cells that both express wt TP53, was distinctly higher than in several other cell lines that had mutated TP53 or were TP53 null (Huang *et al.* 2006b). There is evidence that TP53 plays a dominant role in modulating oxidative stress (Polyak *et al.* 1997; Achanta & Huang 2004; Sablina *et al.* 2005). Thus, for example, it was observed that in the absence of severe stress, normal levels of expression of TP53 up-regulated several genes coding for proteins with antioxidant activity and was associated with a reduction of ROS (Sablina *et al.* 2005). Conversely, down-regulation of TP53 led to excessive oxidation of DNA, increased mutation rate and genome instability, all of which could be prevented by NAC (Sablina *et al.* 2005). There is also evidence that TP53 phosphorylated at *Ser*-15 directly binds to sites of DNA breaks most likely facilitating DNA repair (Al Rashid *et al.* 2005). In light of the evidence that TP53 may modulate the generation of ROS and affect DNA repair, the present study was designed to compare the level of CHP and its sensitivity to NAC in cells with different p53 status. Specifically, we have tested human B lymphoblastoid cell lines derived from WIL2 cells: TK6, a TP53 wt line, NH32, a TP53 knock-out derived from TK6, and WTK1, a WIL2-derived line that expresses a homozygous mutant of TP53 (Schwartz *et al.* 2003). Also tested were the TP53-null human promyelocytic HL-60 cells. The data show distinct differences in the extent of CHP among the lines studied, with wt TK6 having the highest level of NAC-sensitive CHP.

MATERIALS AND METHODS

Cells and culture conditions

Human B-cell lymphoblastoid TK6, WTK1 and NH32 were kindly provided by Dr Howard Liber of the Colorado State University (Fort Collins CO). HL-60 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). The cells were grown in 25 ml Falcon flasks (Becton-Dickinson Co., Franklin Lakes, NJ) in RPMI 1640 supplemented with 10% foetal 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% CO₂ in air. At the onset of the experiments, there were fewer than 5×10^5 cells per ml in culture such that the cells were at an exponential and asynchronous phase of growth.

Cell treatments

N-acetyl-L-cysteine 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 as described in the figure legends. Intracellular level of ROS was measured with the fluorescent probe carboxyl-dichlorodihydrofluorescein diacetate (H2DCF-DA) obtained from Molecular Probes (Eugene, OR). Briefly, the cells were washed twice with phosphate buffered saline (PBS), then resuspended in prewarmed PBS containing 10 µM H2DCF-DA and incubated at 37 °C for 30 min or 1 h. The cells were then washed in PBS, resuspended in PBS, and their fluorescence immediately measured by flow cytometry. For analysis of histone H2AX phosphorylation, the cells were fixed in suspension in a solution of 1% methanol-free formaldehyde (Polysciences, Inc., Warrington, PA) dissolved in PBS for 15 min on ice followed by suspension in 80% ethanol, where they were stored at -20 °C for 2–24 h.

Immunocytochemical detection of γ H2AX phosphorylation

The fixed cells were washed twice in PBS and suspended in 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 μ L of 1% BSA containing 1 : 100 diluted antiphospho-histone H2AX (Ser-139) mAb (Upstate, Lake Placid, NY). The cells were then incubated for 2 h at room temperature, washed twice with PBS and resuspended in BSA in 100 μ L of 1 : 30 diluted fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ fragment of goat antimouse immunoglobulin (Dako, Carpinteria, CA, USA) for 30 min at room temperature in the dark. The cells were then counterstained with 5 μ g/ml propidium iodide (PI) in the presence of 100 μ g/ml of RNase A (Sigma). Other details of the experimental procedure have been presented previously (Halicka *et al.* 2005; Kurose *et al.* 2005; Tanaka *et al.* 2006).

Fluorescence measurements

Cellular green (FITC) and red (PI) fluorescence of cells in suspension were measured using a FASCscan flow cytometer (Becton-Dickinson, San Jose, CA). The red (PI) and green (FITC) fluorescence from each cell were separated and quantified using the standard optics and CELLQuest software (Becton-Dickinson). Gating analysis was carried out to obtain mean values of γ H2AX IF for G₁ (DI = 0.9–1.1), S (DI = 1.2–1.8) and G₂M (DI = 1.9–2.1) cells in each experiment. The mean values ($\pm$ SD) of the measurement from three independent experiments are presented in Figs 2 and 3. Oxidation of H2DCF-DA (Sheng-Tanner *et al.* 1998) was assayed by measuring green fluorescence of the cells exposed to these reagents with the FACScan flow cytometer. Each experiment was run in duplicate or triplicate. All experiments were repeated at least three times.

RESULTS

Figure 1 illustrates expression of γ H2AX with respect to cellular DNA content (cell-cycle phase) in TK6, WTK1, NH32 and HL-60 cells, untreated (control) or exposed to different concentrations of NAC. The intensity γ H2AX IF of cells of all four cell lines was measured under identical conditions (with the same, laser and photomultiplier voltage settings and the same flow rate). Therefore, the observed differences reflect variability in expression of γ H2AX. The dashed line was plotted in all panels of Fig. 1 to outline the position of the mean value of γ H2AX IF of the S-phase cells from untreated TK6 samples, to allow for better visualization of the differences in the level of γ H2AX expression between control and NAC-treated cells, and between the cell lines. It is evident that among the untreated cells, when compared in their respective cell-cycle phases, the intensity of γ H2AX IF of TK6 cells was higher than that of the cells of the other three cell lines. It is also apparent that in all four cell lines the S- and G₂M-phase cells had higher expression of γ H2AX compared to cells in G₁ phase. These raw data also demonstrate that exposure of cells to NAC led to a decrease in intensity of γ H2AX IF. In the case of TK6 and NH32 cells, the decrease was distinctly NAC-concentration dependent. For WTK1 and HL60 cells, the decrease was much less pronounced and, thus, any NAC-concentration dependency was less evident.

Figure 2 shows the data that reveal the differences in expression of γ H2AX between G₁ versus S versus G₂M cells, and between different cell lines. To allow the comparison to be made, the data were normalized to express means of γ H2AX IF of the untreated cells, in respective phases of the cell cycle, in relation to the mean γ H2AX IF of G₁ cells of the TK6 cell line defined as 100. As is evident, the mean values of γ H2AX IF of S- and G₂M-phase cells were distinctly higher compared to G₁ cells in each of the cell lines studied. Furthermore, in each phase of the cell cycle, WTK1, HL-60 and NH32 cells had lower expression of γ H2AX compared to TK6 cells in their respective cell cycle phases.

Figure 1. Bivariate cellular DNA content vs. γ H2AX IF distributions (scatterplots) of TK6, WTK1, NH32 and HL-60 cells, untreated (ctrl) and treated with NAC at a concentration of 10–50 mM for 2 h. The intensity of γ H2AX IF of cells in all four lines was measured under identical settings of photomultiplier sensitivity. The dashed-line provides the position marker that represents the mean value of γ H2AX IF of S-phase TK6 cells from the untreated culture. The line is positioned at the same level in other panels, for comparison with γ H2AX IF of NAC-treated cells and between the other cell lines studied.

Figure 2. Differences in γ H2AX IF between untreated (Ctrl) TK6, WTK1, NH32 and HL-60 cells, at different phases of the cell cycle. For comparison, the data were normalized to express mean values of γ H2AX IF of cells in G₁, S and G₂M subpopulations of each of the cell lines as a fraction of the mean γ H2AX IF of G₁-phase cells from TK6 cultures ($x = 100$). Because of the large number of cells measured per sample (10 000) the SEM was very small (marked by ticks that are barely visible).

Figure 3. Changes in mean γ H2AX IF of G₁ and S cells as a function of cell treatment with different concentration of NAC. The cells were untreated or were treated with 10, 30 or 50 mM NAC for 2 h, then fixed, and by gating analysis their mean γ H2AX IF and the DNA content were estimated for subpopulations of cells in G₁ and S phases of the cell cycle. The percent of maximal decrease of the mean γ H2AX IF of the NAC-treated G₁ or S-phase cells, with respect to the untreated cells in S or G₁ phase, respectively, is shown for each cell line. As in Fig. 2, the ticks representing SEM for each measurement can barely be seen.

The statistical significance of the observed differences in expression of γ H2AX between the cell lines (untreated cells) was estimated by comparing the mean values of γ H2AX IF of subpopulations of G₁, S and G₂M cells, respectively, in each experiment (10 000 cells measured per sample). The difference between TK6 and each of the other cell lines, for subpopulations of cells in G₁ and S phase, respectively, was statistically significant at $P < 0.001$ in each of the three separate experiments.

The decrease in expression of γ H2AX for TK6, WTK1, NH32 and HL-60 cells treated with different concentrations of NAC is illustrated in Fig. 3. Changes in γ H2AX IF were calculated separately for subpopulations of cells in G₁ and S phases of the cell cycle. The data show that for TK6, WTK1 and NH32 cells, a decrease in the expression of γ H2AX was NAC-concentration dependent, and was most pronounced for TK6 cells (36–38%). Furthermore, in all four cell lines the cells in S phase of the cell cycle showed a somewhat greater decrease compared to G₁ cells. The least affected were HL-60 cells, for which a drop in γ H2AX expression (12–13%) was seen at 10 mM, rather than at a higher NAC concentration.

The level of endogenous ROS in TK6, WTK, NH32 and HL-60 cells was assayed by measuring oxidation of H2DCF-DA in these cells (Fig. 4). As it is evident, the differences between the cell lines in this study were minor, with a large overlap between the lines. These experiments were repeated several times. No significant differences in oxidative properties between the cell lines were observed in all these measurements.

DISCUSSION

The present findings confirm our earlier observation (Huang *et al.* 2006b) that CHP, as manifested by the presence of a fraction of phosphorylated histone H2AX, varies depending on the cell type and cycle phase, and is sensitive to NAC, the ROS scavenger.

Figure 4. Intracellular ROS in different cell lines. TK6, WTK1, NH32 and HL-60 cells were treated in cultures with 10 μ M H2DCF-DA for 1 h and their green fluorescence was subsequently measured by flow cytometry. The plot shows results of a single experiment. The bar graph (inset) shows mean values of H2DCF-DA fluorescence of the cell lines measured in five independent experiments ($\pm$ SD; $n = 5$).

It is likely that CHP reflects oxidative DNA damage caused by endogenous ROS, a product of metabolic activity during progression through the cell cycle. As mentioned, about 50 DSBs are being generated in the course a single cell cycle in normal, untreated human cells (Vilenchik & Knudson 2003). DSBs are formed predominantly during the S phase when replication forks collide with the ROS-induced DNA single-strand lesions such as DNA single-strand breaks, apurinic/apyrimidinic sites or oxidation-modified bases (Vilenchik & Knudson 2003). Given such a mechanism of DSB formation, it would be expected that the expression of γ H2AX, known to be elevated for several hours following induction of DSBs (Sedelnikova *et al.* 2003; Halicka *et al.* 2005), would be maximal during S and G₂M. Indeed, the NAC-sensitive CHP was at higher levels in S and G₂M cells than in G₁ cells in the present study.

Among different cell types/lines studied by our group previously (Huang *et al.* 2006b), CHP was most pronounced in NHBE and pulmonary carcinoma A549 cells, both of which have wt TP53, and was distinctly lower in cell lines lacking TP53 or that expressed in its mutated form. To reveal whether indeed the CHP level is associated with expression of wt TP53, we compared the cells of three cell lines derived from the same B-lymphoblastoid line, differing with respect to TP53 status. Also included were TK53-null HL-60 cells. Consistent with our earlier observation (Huang *et al.* 2006b), the cells expressing TP53 (TK6) had the highest level of NAC-sensitive CHP of all four lines.

What mechanism could explain the observed differences in CHP that may be related to p53 status? TP53 is one of the key molecules involved in response to DNA damage, facilitating DNA repair (Iwabuchi *et al.* 1998; Squires *et al.* 2004; Kang *et al.* 2005). DNA damage-induced phosphorylation of TP53 on Ser-15 is directly mediated by activation of ATM and ATR (Banin *et al.* 1998; Canman *et al.* 1998). Its phosphorylation on Ser-20 is indirectly mediated by ATM through activation of Chk2 (Chehab *et al.* 2000; Hirao *et al.* 2000). It was also reported that DNA damage induces TP53 acetylation by inhibition of both the histone deacetylase complex (HDAC1) and Sir2-mediated deacetylation (Luo *et al.* 2004). Acetylated TP53 binds to DNA with higher affinity and is more effective as a transcriptional inducer (Luo *et al.* 2004). Depending on the nature of the DNA-damaging agent and the extent of the damage, these modifications

of TP53 trigger transcription of target genes that either activate pathways arresting cells in the cell cycle to allow for damage repair before it may be transferred to progeny cells, or alternatively, the apoptotic pathway that leads to cell demise (Hofseth *et al.* 2004). However, none of these interactions involving TP53 following DNA damage discussed previously, although they are pertinent to central role of TP53 in facilitating repair, provides any clue to explain the observed differences in the extent of CHP in cell lines with different TP53.

Interestingly, among its broad range of activities, TP53 induces transcription of numerous genes that not only trigger the cell's response to DNA damage, but also modulate oxidative stress (Polyak *et al.* 1997; Achanta & Huang 2004; Sablina *et al.* 2005). Early studies demonstrated that activation of TP53 following DNA damage-induced transcription of the genes coding for proteins with oxidative function and rising endogenous ROS (Polyak *et al.* 1997). In these studies, however, the cells were stressed by extensive DNA damage, TP53 was up-regulated above its normal level, and the induced oxidative stress provided the mechanism to trigger apoptosis. However, TP53 appears to have an entirely different effect when expressed at lower levels, in cells not treated with genotoxic agents. In systematic studies, Chumakov and his collaborators observed that normal levels of expression of TP53 were actually required for transcription of the genes coding for proteins with antioxidant activity (Sablina *et al.* 2005). Expression of these genes led to a distinct reduction of endogenous ROS. Conversely, down-regulation of TP53 resulted in excessive oxidation of DNA that increased mutation rate and genome instability, all of which could be prevented by treatment with NAC (Sablina *et al.* 2005). In support of these observations are findings of Erker *et al.* (2005) who reported that cancer chemoprevention by some antioxidants is mediated by TP53. However, we were unable to observe significant differences in ROS production between the cell lines used in the present study (Fig. 4). Apparently, the antioxidant effect of TP53 may vary depending on other attributes of particular cell lines.

Because no significant differences in ROS were seen in our cell lines differing in TP53 status (Fig. 4), one would expect similar levels of oxidative DNA damage, and thus similar levels of CHP, indicative of the frequency of DSBs. Yet in cells bearing wt TP53, CHP was significantly elevated. Given the previous discussion, one may speculate that one of the many functions of TP53 may be to facilitate phosphorylation of H2AX on Ser-139. Thus, with the same level of DNA damage, histone H2AX may be more effectively phosphorylated in cells bearing wt TP53. Such a function for TP53 may, in turn, facilitate activation and translocation of other DNA repair components, known to be recruited by phosphorylated H2AX (Anderson *et al.* 2001; Furuta *et al.* 2003; Downs & Cote 2005), to the sites of DSBs, thereby increasing the efficiency of DNA repair. Such a function for TP53 would be compatible with its role in sensing oxidative DNA damage and facilitating base excision repair (Offer *et al.* 2001; Zhou *et al.* 2001; Achanta & Huang 2004) as well as its direct binding to sites of DSBs and interaction with γ H2AX (Al Rashid *et al.* 2005).

It should be stressed that although constitutive histone H2AX phosphorylation in G₁, S and G₂ may primarily reflect oxidative DNA damage, H2AX phosphorylation in mitotic cells may also be triggered by additional mechanisms, as discussed elsewhere (Ichijima *et al.* 2005; McManus & Hendzel 2005; Huang *et al.* 2006a). Thus, the γ H2AX IF of the G₂M cell subpopulation, as measured in the present study, represents the mean of G₂ and M cells, and induction of γ H2AX in the latter cells may involve different mechanisms from those operating in G₂, G₁ or S phase.

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