The γH2AX Assay for Genotoxic and Nongenotoxic Agents: Comparison of H2AX Phosphorylation with Cell Death Response

Teodora Nikolova,* Mirek Dvorak,* Fabian Jung,* Isabell Adam,* Elisabeth Krämer,* Aslihan Gerhold-Ay,† and Bernd Kaina*†

*Institute of Toxicology, University Medical Center, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany; and †Institute of Medical Biometry, Epidemiology and Informatics, University Medical Center, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany

1To whom correspondence should be addressed. Fax: +49-6131-17-8499. E-mail: kaina@uni-mainz.de

Received December 7, 2013; accepted March 30, 2014

DNA double-strand breaks (DSBs) and blocked replication forks resulting from bulky adducts and inhibitors of replication activate the DNA damage response (DDR), a signaling pathway marked by phosphorylation of histone 2AX (H2AX). The phosphorylated form, γH2AX, accumulates at the site of the damage and can be visualized as foci by immunocytochemistry. The objective of this study was to assess if γH2AX is a reliable biomarker for genotoxic exposures. To this end, we selected 14 well-known genotoxic compounds and compared them with 10 nongenotoxic chemicals, using CHO-9 cells because they are well characterized as to DNA repair and DDR. We quantified γH2AX foci manually and automatically. In addition, total γH2AX activation was determined by flow cytometry. For all chemicals the cytotoxic dose response was assayed by a metabolic cytotoxicity assay. We show that (1) all genotoxic agents induced γH2AX dose-dependently whereas nongenotoxic agents do not; (2) γH2AX was observed for genotoxicants in the cytotoxic dose range, revealing a correlation between cytotoxicity and γH2AX for genotoxic agents; for nongenotoxic agents cytotoxicity was not related to γH2AX; (3) manual scoring of γH2AX and automated scoring provided comparable results, the automated scoring was faster and investigator independent; (4) data obtained by foci counting and flow cytometry showed a high correlation, suggesting that γH2AX scoring by flow cytometry has the potential for high-throughput analysis. However, the microscopic evaluation can provide additional information as to foci size, distribution, colocalization and background staining; (5) γH2AX foci were colocalized with 53BP1 and Rad51, supporting the notion that they represent true DSBs. Collectively, the automated analysis of γH2AX foci allows for rapid determination of genetic damage in mammalian cells. The data revealed that the induction of γH2AX by genotoxicants is related to loss of viability and support γH2AX as a reliable bio-indicator for pretoxic DNA damage.

Key words: genotoxicants; testing; γH2AX assay; flow cytometry; MTT assay; DNA damage.

Exposure of cells to chemical mutagens, replication inhibitors, ultraviolet light, or ionizing radiation leads to replication stress and, directly or indirectly, to DNA double-strand breaks (DSBs). These breaks represent a severe form of DNA damage because they result in chromosomal changes (Durante et al., 2013) and are powerful inducers of cell death by apoptosis (Roos and Kaina, 2013). Even a low number of unrepair ed DSBs is cytotoxic (Jackson, 2002; Sonoda et al., 2006). Because of the default nature of DSBs, a complex signaling and DNA damage response network is operating in cells, in order to maintain genomic integrity and to protect against genotoxic effects (Ciccia and Elledge, 2010). Central players in this network are the MRN complex, consisting of MRE11, NBS1 and RAD50, which monitors free DSBs as well as breaks at collapsed replication forks, and the PI3-like kinases ATM, ATR, and DNA-PK. Once activated by critical DNA damage, these kinases target multiple substrates, one of them being histone 2AX (H2AX) in the immediate vicinity of the DNA break (Ismail and Hendzel, 2008; Jackson, 2002). Phosphorylation occurs at serine 139 (Rogakou et al., 1998), which is thought to have biological relevance as it alters the chromatin conformation in the damaged area and allows better access of repair enzymes in order to reach the site of the lesion (Chapman et al., 2012). Phosphorylated H2AX (designated as γH2AX) is rapidly accumulated over megabase domains at the sites of DSB (Shrivastav et al., 2008) and, therefore, can be microscopically visualized as discrete intranuclear “foci.” These foci are detected by indirect immunofluorescence using specific primary antibodies and secondary antibodies coupled with fluorescent dyes. It has been shown that other proteins involved in DSB repair also form intranuclear foci, e.g., 53BP1 (Anderson et al., 2001; Rappold et al., 2001; Schultz et al., 2000), MRE11/RAD50/NBS1 (Furuta et al., 2003), MDC1 (Stucki and Jackson, 2004) and RAD51 (Haaf et al., 1995), all of which usually colocalize with γH2AX (Dimitrova and de Lange, 2006; Furuta et al., 2003; Lukas et al., 2004; Paull et al., 2000; Stucki et al., 2005). This colocalization was taken to indicate that γH2AX has a pleiotropic function, which ranges from recruitment signaling, fixation of broken ends and facilitation of DSB repair (Fernandez-Capetillo et al., 2004).

There are different methods for quantifying DSBs, e.g., the neutral comet assay (Fairbairn et al., 1995), pulse field electrophoresis (Whitaker et al., 1991), and TUNEL assay (Hewitson et al., 2006). Although well established, these methods
are quite insensitive for the detection of DSBs, e.g., the neutral comet assay delivers a significant signal at a dose >2 Gy (own unpublished data), i.e., above a damage level of about 80 DSBs per cell (Rothkamm and Lobrich, 2003). In contrast, immunofluorescence analysis of γH2AX foci allows DSB detection even in the milligray range (Rothkamm and Lobrich, 2003). Furthermore, it has been reported that γH2AX foci actually depend on the presence of DSBs; thus even a single DSB can be detected (Rothkamm and Lobrich, 2003; Thompson, 2012). The high sensitivity and mechanistic understanding of the origin of γH2AX foci make them highly attractive as a biomarker for the presence of DNA damage.

The phosphorylation of H2AX after genotoxic stress can be measured by counting the nuclear foci by fluorescence microscopy or determining the number of γH2AX positive cells by flow cytometry (Banath and Olive, 2003; Smart et al., 2011). Flow cytometry evaluates the signal intensity per cell whereas immunofluorescence allows the evaluation of both the number of foci and the average nuclear staining intensity. It appears that in most cases, the number of intranuclear foci corresponds in a 1:1 ratio with the actual number of DSBs (Sedelnikova et al., 2002).

The objective of the current study was to compare the dose-response of γH2AX with viability/cytotoxicity for a set of well-known genotoxic and nongenotoxic compounds. We selected 14 well-characterized genotoxicants and compared them with 10 nongenotoxic substances (Brambilla and Martelli, 2009; Kirkland et al., 2008; van Delft et al., 2004). Replicating Chinese hamster ovary cells (the line CHO-9, which is well characterized as to DNA repair and DDR) were exposed to the tested chemicals and DNA damage was analyzed using the microscopic γH2AX foci assay and the flow cytometric assay. Additionally, we compared conventional microscopic (“manual”) scoring of γH2AX foci with automatic scoring, making use of a slide scanning platform supplied with counting software, in order to evaluate the precision of foci detection by automatic scoring. Inhibition of growth and cytotoxic effects of the tested chemicals were measured by the MTT assay. γH2AX levels and viability data were compared as to their interrelation for genotoxic and nongenotoxic compounds. The data revealed that γH2AX induction corresponds to reduction of cell viability for all genotoxicants tested.

**MATERIALS AND METHODS**

**Cell lines, culture conditions, and treatment.** The well-characterized Chinese hamster cell line CHO-9 (Tjio and Puck, 1958) was used in this study. Cells were cultured in DMEM Ham’s F-12 containing 5% fetal calf serum (FCS) in a humidified atmosphere with 7% CO2 at 37°C. Cells were checked before experimental use for mycoplasma contamination. Cells were treated with increasing concentrations of genotoxicants and nongenotoxicants. The treatment occurred for 1 h and, for temozolomide, for 2 h. Thereafter, the medium was changed and cells were incubated at 37°C until further processing by the γH2AX assay.

**Tested compounds.** The following genotoxic compounds were tested in the MTT and γH2AX foci assay: methyl methanesulfonate (MMS), temozolomide, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), lomustine (ACNU), benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), mitomycin C (MMC), mafosfamide, cisplatin, oxaliplatin, topotecan, etoposide, 4-nitroquinoline 1-oxide (4NQO), tert-butyl hydroperoxide (t-BOOH), and hydrogen peroxide (H2O2). For nongenotoxic agents, the following chemicals were used: sodium chloride (NaCl), urea, diclofenac sodium salt, curcumin, phthalic acid, menthol, benzyl alcohol, dymenhydrinate, enalapril maleate salt, and metformin hydrochloride. The compounds were previously described as being genotoxic and nongenotoxic (Brambilla and Martelli, 2009; Kirkland et al., 2008; van Delft et al., 2004). The sources of the chemicals and dissolvants are shown in Supplementary table 1.

**MTT cell growth/viability assay.** The cytotoxicity of compounds was tested by the MTT assay, which measures cellular metabolic activity by means of the dye formazan (tetrasodium salt). Cells were seeded in 96-well dishes (n = 3000 for all drugs except MNNG and temozolomide for which 1500 cells were seeded). After 48 h (72 h for MNNG and temozolomide), the medium was removed and 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were mixed with 100 μl RPMI supplied with 5% FCS and added to each well. One hundred twenty microliters of the mixture were added to wells without seeded cells, which were used as negative control. Cells were incubated for 3 h at 37°C. The medium was removed and cells were fixed in 100 μl of a 40mM solution of HCl diluted in isopropanol. The 96-well dish was incubated for 15 min with 500 rpm. Absorption was measured at 570 nm (reference 650 nm) with a Tecan Sunrise Reader. All experiments were performed in triplicate (three wells per treatment level) and fully repeated at least two times. Results were presented as MTT staining intensity compared with the untreated control and expressed as mean value (%) using the term “viability.”

**Quantification of γH2AX by flow cytometry.** For γH2AX analysis by flow cytometry (FACS), a modified version of a previously described assay (Banath and Olive, 2003) was used. Forty thousand cells were seeded for a short period of time in 6-cm Petri dishes for each treatment variant. The cells were treated with the drug 2–3 days after seeding for 60 min, then the medium was changed. After incubating for 6 h, cells were harvested by trypsinization, fixed in 70% ethanol and stored overnight at –20°C. On the day of FACS measurement, cells were centrifuged for 5 min at 1,500 rpm at 4°C. The pellet was resuspended in 1 ml blocking reagent (20 mMTris, 137 mM NaCl, pH 7.4 (= Tris buffered saline, TBS) containing 0.1%
used to acquire the images for each sample. The stored galleries according to the classifier settings. The signals are acquired as a classifier. The system scans and focuses using the DAPI system automatically scans a preselected area and captures the signals and, after recognizing the presence of a cell, captures red (TRITC, Cy3) or green (FITC, Alexa Fluor 488) fluorescent signals and, after recognizing the presence of a cell, captures red (TRITC, Cy3) or green (FITC, Alexa Fluor 488) fluorescent signals. The nuclei were then counterstained with TBS + DAPI (1 μg/ml DAPI in TBS) and measured immediately using the FACSCanto II Flow Cytometer (BD Biosciences).

Quantification of γH2AX foci by microscopy. Cells (n = 20,000 per well) were seeded onto precleaned sterile cover slips in six-well culture plates. Two days later, they were treated for 60 min with the tested chemical. After treatment, the medium was changed and cells were further incubated for 6 or 24 h. Finally, the cells grown on cover slips were washed in PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, cells were incubated with 1–2 ml ice-cold methanol at −20° C. After three washing steps with PBS, the blocking reagent (100 μl PBS + 0.25% Triton X-100 + 10% NGS) was added for 1 h. The primary antibody (mouse anti-phospho-H2AX Ser139, Millipore, cat. no. 05-636) was diluted to 1:1000 in the same buffer. The samples were stored in the dark at room temperature for 1 h. After washing, 10 μl antifade medium (Vectashield) with DAPI was dropped onto clean slides and the cover slips were transferred onto the slides and fixed with nail polish. For each treatment level, 50–100 cells were analyzed in each experiment. All experiments were repeated at least twice.

Microscopic images were screened and captured using Zeiss Axio Imager M1 (Carl Zeiss) supplied with the Metafer4 Software (MetaSystems, Altussheim, Germany). The slide scanning platform “Metafer” and the accompanying software “Metafer4” have been used, which were approved for automatic scoring of micronuclei, dicentrics, and FISH stained samples (Rossnerova et al., 2011; Schunck et al., 2004; Schunck and Mohammad, 2011; Varga et al., 2004). At brief intervals, the system automatically scans a preselected area and captures the images according to the settings defined by the user and saved as a classifier. The system scans and focuses using the DAPI signal and, after recognizing the presence of a cell, captures red (TRITC, Cy3) or green (FITC, Alexa Fluor 488) fluorescent signals and, after recognizing the presence of a cell, captures red (TRITC, Cy3) or green (FITC, Alexa Fluor 488) fluorescent signals according to the classifier settings. The signals are acquired as a z-stack with a total of five planes. The same classifier is used to acquire the images for each sample. The stored galleries of images can be reloaded on a review screen, where every captured cell can be relocated and the automatic analyses can be verified. In addition, we compared the automatic analyses of the saved images by the Metafer4 Software with visual analysis using ImageJ (Fiji) with suitable batch-macro.

Colocalization of γH2AX foci with other DSB marker. To confirm that γH2AX foci actually mark DSB, we performed double-stainings with specific primary antibodies against other DSB markers: rabbit anti-phospho-53BP1 (Ser1775) from Cell Signalling (final concentration 1:100) and rabbit anti-RAD51 from Abcam (1:10 000). For γH2AX, the mouse anti-phospho-H2AX Ser139 (Millipore) was used. Secondary antibodies were antimouse Alexa Fluor 488 (Invitrogen) and antirabbit Cy3 (Jackson ImmunoResearch). Nuclei were counterstained with 1μM To-Pro-3 for 15 min. Foci scoring and colocalization analyses were done using the LSM 710 (Carl Zeiss, Jena, Germany) equipped with LSM software ZEN 2009.

Statistical analyses. All experiments were performed in triplicates. Mean values and standard deviations were calculated by Microsoft Office Excel. Cell viability and γH2AX were presented as a function of concentration of the chemicals. Spearman’s correlation coefficients, r, were calculated using the software program GraphPad Prism 6. The r value is a measure of linear association between two variables. The closer the r value is to one, the stronger the linear association. The counts of γH2AX positive cells measured in FACS were compared with the microscopically determined number of γH2AX foci, and r values were calculated as well. For detecting possible groupings of agents, Finite Mixture Models were used. These models were fitted with the CRAN-package “FlexMix” (version 2.3-11) within the statistical software program R (version 2.15.2). This package also provided values for the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). When comparing models, the lower the AIC and BIC values the more appropriate the model.

RESULTS

For most chemical DNA damaging agents DSB formation depends on the progression of cells through S-phase. In order to be sure to collect cells that have passed through S-phase, we decided to use two fixation times following the addition of the chemical to the medium, 6 and 24 h. An example of γH2AX foci induced following treatment with MNNG and cisplatin is shown in Figure 1A. For high content scoring of γH2AX foci actually mark DSB, we performed double-stainings with specific primary antibodies against other DSB markers: rabbit anti-phospho-53BP1 (Ser1775) from Cell Signalling (final concentration 1:100) and rabbit anti-RAD51 from Abcam (1:10 000). For γH2AX, the mouse anti-phospho-H2AX Ser139 (Millipore) was used. Secondary antibodies were antimouse Alexa Fluor 488 (Invitrogen) and antirabbit Cy3 (Jackson ImmunoResearch). Nuclei were counterstained with 1μM To-Pro-3 for 15 min. Foci scoring and colocalization analyses were done using the LSM 710 (Carl Zeiss, Jena, Germany) equipped with LSM software ZEN 2009.

Manual Versus Automatic Analysis of γH2AX Foci

For high content scoring of γH2AX, an automatic screening and evaluation system would be highly desirable. Therefore, we tested for several compounds whether visual scoring by counting each focus of a size above a virtual threshold (“manual
FIG. 1. Formation of γH2AX after treatment with different genotoxicants. (A) Representative images of nuclei from CHO-9 cells not treated (control) and treated with MNNG or cisplatin. Cells were fixed and stained 6 and 24 h after treatment. (B to I) Dose response for γH2AX foci. (B) The number of foci was determined automatically using ImageJ Fiji (B, D, and F), and foci were manually counted (C, E, and G). In panels (H) and (I), the γH2AX nuclear staining intensity was measured in the microscope using Metafer4 software.
scoring) delivers results that are comparable to scoring in which foci above a particular size and fluorescence intensity are automatically scored (“automatic” scoring). Figure 1 shows results obtained following treatment with cisplatin (Fig. 1B versus C), MNNG (Fig. 1D versus E) and NaCl (Fig. 1F versus G). The data demonstrate that automatic scoring delivers data comparable to manual scoring. Because automatic scoring is precise and reliable, we decided to utilize the automatic scoring system in subsequent experiments. We should note that each automatic analysis was followed by a complete visual analysis of all captured images in order to evaluate the staining quality and intensity and to recheck the data delivered by software for nonspecific background staining, overlapping foci, too small foci or pan staining.

We also wished to know whether the overall intensity of the green Alexa Fluor 488 fluorescence signal of nuclei inspected by microscopy is a trustworthy measure of induced γH2AX. As shown in Figure 1H (for MNNG) and Figure 1I (for NaCl), measurement of the overall fluorescence intensity is not superior over γH2AX foci counting. Therefore, we have not pursued this strategy further.

Impaired Viability and γH2AX as a Function of Dose for Genotoxic and Nongenotoxic Agents

The chemicals used in this series of experiments were recommended as well-known reference compounds for genotoxicants (Brambilla and Martelli, 2009; Kirkland et al., 2008; van Delft et al., 2004), which have a well-described mechanism of action (see Supplementary Table 1). For all of them, we determined the cell viability (using the MTT assay 48 h after treatment; for MNNG 72 h after treatment) and the number of γH2AX foci 6 and 24 h after the onset of treatment. The data presented in Figure 2 show that all tested genotoxicants reduce viability and induce γH2AX foci formation in the same dose range. The number of foci increased with each dose up to a saturation level. For many, but not all, agents the number of foci was higher at 24 h compared with 6 h after treatment.

For the tested nongenotoxic chemicals, the dose response for reduction of viability and H2AX foci formation is shown in Figure 3. All these chemicals reduced viability in a given dose range, but did not induce, even at the highest toxic dose level, γH2AX foci. The data clearly show that nongenotoxic compounds even in the toxic dose range do not induce γH2AX foci. This indicates that γH2AX foci formation is not bound on cytotoxicity per se, but exclusively on the DNA damage inducing capability of the compound.

Correlation Analysis of Cytotoxicity and γH2AX Foci

Different types of DNA damage are able to trigger DDR and downstream cell death pathways (Roos and Kaina, 2013). Because mutants defective in the removal of adducts from DNA or defective in repairing DSB are generally hypersensitive to genotoxicants, it is generally accepted that genomic DNA damage is the main source of cell death. There is, however, a great uncertainty as to the killing potential of specific types of DNA damage. Because the type of DNA damage induced by the genotoxicants used here is quite different in structure and a systematic study comparing DDR and cytotoxicity induced by different genotoxicants is lacking, we wondered whether there is a correlation between cytotoxicity and γH2AX induction for the agents tested. Thus, we compared cytotoxicity with the number of γH2AX foci for all genotoxicants included in this study. According to the data, there is a high correlation for almost all agents (r value > 0.9 except for MNNG) between cytotoxicity and the number of induced γH2AX foci per cell (Figure 4).

Cytotoxicity as a function of γH2AX foci per cell for all tested genotoxicants is shown in Figure 5. Here, we fitted Finite Mixture Models to the data with 1, 2, and 3 linear mixture components to determine if there was more than one underlying pattern of relationship to induced γH2AX. According to this analysis, the agents can be grouped into two clusters, which include group 1: cisplatin, MNNG, MMC, topotecan, ACNU, temozolomide, and group 2: MMS, BPDE, mafosfamide, oxaliplatin, etoposide, H2O2, t-BOOH, and 4NQO. The correlation analysis revealed that for cisplatin, MNNG, MMC, topotecan, ACNU, and temozolomide cytotoxicity is high compared with the induced γH2AX formation, whereas for oxaliplatin, etoposide, H2O2, t-BOOH, and 4NQO cytotoxicity is low compared with the induced γH2AX formation. Irrespective of these differences, for all genotoxicants γH2AX was quantitatively correlated with reduction in viability.

γH2AX Foci Versus Signal Intensity as Measured by Flow Cytometry

We also considered the possibility to quantify H2AX phosphorylation by flow cytometry, which might be an even faster and more robust method. To this end, we compared the number of induced γH2AX foci (determined microscopically) and the fraction of γH2AX positive cells by flow cytometry for several genotoxicants. The data shown in Figure 6 revealed a correlation between both parameters (with a Spearman correlation coefficient between r = 0.66 for MMS, 24 h and r = 1.00 for MMC and temozolomide, 6 h). Obviously, γH2AX foci counts and γH2AX positive cells measured by FACS give similar, but not identical results.

γH2AX Foci Induced by Genotoxicants Represent True DSB

γH2AX foci result from the activation of DNA damage dependent PI3-like kinases, i.e. ATM, ATR, and DNA-PK. These kinases, once activated by DSBs, single-stranded DNA or blocked replication forks (at which DSBs can be generated), phosphorylate H2AX and, at the same time, other proteins such as 53BP1 and RAD51. These proteins are recruited to the site of the damage and form distinct intranuclear foci, which are used as a supportive marker for DSBs. Therefore, we determined the colocalization of γH2AX with phospho-53BP1 and RAD51 for some genotoxicants (here shown for MMS and cisplatin). Representatively stained nuclei are shown in Figure 7. They demon-
FIG. 2. Viability and γH2AX formation in CHO-9 cells following treatment with different genotoxicants. Viability (resp. survival) was determined by the MTT assay 48 h after adding the agents to the medium except for MNNG and temozolomide, for which 72 h was used as fixation time. γH2AX was determined 6 and 24 h after adding the agents to the medium. The amount of γH2AX foci was analyzed using ImageJ (Fiji). Each measure point represents the mean of three independent experiments ± standard deviation (SD).
FIG. 3. Viability and γH2AX foci formation in CHO-9 cells following treatment with different nongenotoxic agents. Survival was determined by the MTT assay 48 h after adding the compounds to the medium. γH2AX was determined 6 and 24 h following the onset of treatment. The amount of γH2AX foci was analyzed using ImageJ (Fiji). Data are the mean of three independent experiments.
FIG. 4. Correlation analysis between cytotoxicity determined by the MTT assay and the number of γH2AX foci following treatment with increasing concentrations of the genotoxins. Data are from Figure 2. Each measure point represents the mean of three independent experiments. The correlation coefficient $r$ was calculated using the using the Spearman test (GraphPad Prism 6).
FIG. 5. Plot of cytotoxicity versus induced γH2AX foci levels induced by the genotoxicants included in this study. We fitted Finite Mixture Models to the data with 1, 2, and 3 linear mixture components to determine if there was more than one underlying pattern of relationship to γH2AX. To decide which of the three models fits best, the three AIC and BIC values were compared, leading to the following results: model with one mixture component: AIC: 790.01; BIC: 797.31; model with two mixture components: AIC: 756.28; BIC: 773.28; model with three mixture components: AIC: 753.19; BIC: 779.94. The parameters of the model used are for cluster 1: intercept 3.45, slope 1.27 [1.05 1.49]; for cluster 2: intercept 17.50, slope 2.44 [2.01–2.88]. The estimated values are presented along with a 95% confidence interval. The two lines represent the best-fitting model (two mixture components). Compound cluster 1 consists of cisplatin, MNNG, MMC, topotecan, ACNU, temozolomide, and compound cluster 2 consists of MMS, BPDE, mafosfamide, oxaliplatin, etoposide, H$_2$O$_2$, t-BOOH, and 4NQO.

strate that most, if not all, γH2AX foci colocalize with p53BP1 and RAD51, supporting the notion that the observed γH2AX foci represent true DSBs, which were formed in response to a variety of genotoxicant-induced DNA lesions.

**DISCUSSION**

The standard battery of assays used in mammalian genotoxicity testing includes tests for gene mutations, chromosomal aberrations, sister chromatid exchanges (SCE) and gain or loss of whole chromosomes (Burlinson, 2012). The assays do not always deliver nonambiguous results and, under certain conditions, are inadequate to achieve a decision as to whether a chemical is genotoxic. The assays are also laborious, time consuming and expensive and, thus, they are not suitable for screening large amounts of compounds in a short period of time. Obviously, there is a need for additional assays that represent a reliable and sensitive bio-indicator of critical DNA damage. The comet assay was an answer to this need, providing the opportunity to test cells in vitro and in vivo for the presence of DNA damage (Olive and Banath, 2006). It is applied in several modifications, detecting different types of DNA damage. The neutral comet assay detects single-strand breaks and alkali-labile sites. If lesion specific enzymes (e.g., FPG) or DNA fragmentation by X-ray treatment is included, the assays can be applied to detect ROS-induced lesions such as 8-oxo-guanine (Collins, 2014) and DNA interstrand crosslinks, respectively (Spanswick et al., 2010). The alkaline assay is quite sensitive and often applied for experimental purposes, including the assessment of DNA repair activities (Azqueta et al., 2013). Although the assay has been used for many years, an OECD guideline for its application is not yet available (Moller, 2006; Burlinson, 2012).

In conventional genotoxicity assays, the genotoxic endpoints are far away from the critical primary DNA damage. Thus, chromosomal aberrations and SCEs require a complex metabolic pathway to be generated (Kaina, 2004) and for most of the critical genotoxic lesions, the pathways leading to the genotoxic endpoints are still unknown. This is different from γH2AX, for which the biochemical basis has been intensively explored and described in numerous reports (Bekker-Jensen and Maitland, 2010; Ciccia and Elledge, 2010; Ismail and Hendzel, 2008; Lukas et al., 2011). There is consensus that γH2AX represents an early response of cells to genotoxic stress that results directly from DSBs and/or blocked replication forks that generate single-stranded DNA and indirectly, upon replication fork collapse, that generates DSBs. Because H2AX is phosphorylated in an amplified way around the damaged site, the assay allows the detection of even a single DSB (Rothkamm and Lobrich, 2003), a resolution that was impossible to achieve with the conventional assays, including the neutral comet assay. Thus, γH2AX is to our best knowledge the most sensitive and most specific assay for detecting DSBs. As DSBs are most critical lesions leading to chromosomal aberrations (Durante et al., 2013; Natarajan et al., 2008) and cell death (Lips and Kaina, 2001) and because DSBs can persist, e.g., in lymphocytes, their high-resolution detection is of utmost importance for screening purposes. Thus, it is reasonable to utilize γH2AX as a bio-indicator for the presence of DNA damage, either directly induced or formed in response to replication of a damaged DNA template (genotoxic replication stress) or other types of replication stress (e.g., upon virus infections and oncogene activation), leading to genomic changes and cell transformation.

In this study, we aimed to show (1) the reliability and specificity of the γH2AX assay for the detection of genotoxic substances and (2) its relation to cytotoxicity/proliferation inhibition measured by a metabolic cytotoxicity assay (MTT). To this end, we compared well-known chemical genotoxicants with nongenotoxic agents. We show that all genotoxicants exhibited positive results, confirming that the foci assay is able to detect genotoxic agents. Although the assay is highly sensitive, no foci were detected above background level once cells were treated with nongenotoxic chemicals, indicating that the assay is highly specific for DNA damaging agents. Our data are in accordance with data reported by others (Hernandez et al., 2013; Mathijs et al., 2009; Runge et al., 2012; Zhou et al., 2006). Recently, several papers appeared that describe automated meth-
FIG. 6. Correlation analysis between the fraction of γH2AX positive cells measured by flow cytometry (y-axis) and the number of γH2AX foci per cell (x-axis) determined by immunocytochemistry. CHO-9 cells were treated with the genotoxicants for 60 min. Thereafter, the medium was changed and cells were harvested and processed for immunohistochemistry and flow cytometry 6 and 24 h later. Each measure point represents the mean of three independent experiments. The correlation coefficient \( r \) was calculated using the Spearman test.
FIG. 7.  (A) Representative LSM images of nuclei with γH2AX (green) and RAD51 (red) foci induced by treatment of CHO-9 cells with 2mM MMS (60 min) and stained 24 h thereafter. (B) Representative LSM images of nuclei with γH2AX and 53BP1 foci induced by treatment of CHO-9 cells with 10μM cisplatin (60 min) and stained 24 h later. To-Pro-3 was used for nuclear DNA staining. Control, without mutagen treatment.
ods for DNA damage assessment after ionizing radiation in various cell types based on counting γH2AX foci (Hernandez et al., 2013; Runge et al., 2012). The full automation (we have used Metafer 4) and fast performance of γH2AX foci assay surely facilitates integration of this sensitive assay in an optimized strategy of genotoxicity testing.

The flow cytometry assay for measuring γH2AX provides another option to detect and quantify DNA damage. In a recent study, flow cytometry was used for comparing six prototypical genotoxicants with three nongenotoxic cytotoxins (Smart et al., 2011). It was shown that, at similar cytotoxicity levels, all substances classified as genotoxicants induced concentration-dependent increases in the γH2AX level compared with nongenotoxicants. In the same study, it was shown that the γH2AX flow cytometry assay was comparable regarding sensitivity, specificity and concordance to the Ames test and the in vitro mammalian genotoxicity tests like mouse lymphoma aberration assay. The flow cytometry analysis of γH2AX was used in another study to confirm the genotoxic potential of selected compounds in HepG2 cells. The compounds were classified as true genotoxicants, nongenotoxicants and false genotoxicants showing positive results in in vitro genotoxicity assays, but negative results in in vivo genotoxicity assays. The authors found a concordance between results obtained in the flow cytometry assay and the classical genotoxicity tests (Magkoufopoulou et al., 2011). It appears that both microscopic and FACS γH2AX scoring can be applied for genotoxic screening. Each of the methods manifests specific advantages: microscopic scoring allows direct visualization of the phosphorylated HAX protein at sites of DSB and provides data describing multiple characteristics of the foci like absolute area of the spot, fluorescence intensity and distribution of cells with a different number of foci as well as costaining with other DSBs markers.

In addition, the saved image galleries provide the opportunity for re-evaluating the data, e.g., in order to exclude apoptotic cells, which can be distinguished by the saturated fluorescence signal due to the massive degradation of DNA forming chromatin (apoptotic) bodies visualized in the DAPI channel. The flow method cannot discriminate between γH2AX labeled nuclei due to massive DNA degradation during apoptosis, but it can be combined with an additional staining technique for apoptotic cells, e.g., annexin V labeling. The greatest advantage of the FACS analysis lies in its fast performance and, in combination with DAPI staining, the possibility of assessment of distribution of γH2AX positive cells in different phases of the cell cycle.

In the present study, we determined for all chemicals the cytotoxicity/proliferation inhibition (using the MTT assay) in conjunction with γH2AX. The data revealed that γH2AX induction occurred in the same dose range in which cytotoxic/proliferation inhibiting effects were induced. As the MTT assay measures the metabolic activity of cells, we cannot exclude that the outcome was dependent on impaired mitochondrial function. For genotoxic agents, multiple effects are expected to be provoked including mitochondrial and nuclear DNA damage, the latter triggers DDR, which regulates both cell cycle progression and cell death pathways. The finding that γH2AX induction and cell death are highly correlated (Fig. 4) is actually expected, based on our current knowledge of the mechanism and consequences of DDR (Roos and Kaina, 2013). Thus, DSBs trigger PI-3-like kinases that phosphorylate H2AX and, at the same time, p53, CHK1, CHK2, and other substrates that trigger downstream cell cycle inhibition and proapoptotic responses, giving rise to cytotoxic effects (Ciccia and Elledge, 2010). The correlation between γH2AX and cell death suggests that for dose finding, cytotoxicity assays should be performed to make sure that the proper dose range was used. The MTT assay is robust and can be performed on microtiter plates, which allows for high-throughput screening within a large dose range. Recently, an assay has been described for measuring γH2AX and cytotoxicity on the same microtiter plate, and γH2AX formation has been found in non-cytotoxic dose ranges if both endpoints were determined 24 h after the begin of treatment with the agents (Khoury et al., 2013). We should note, however, that cytotoxicity following DNA damage (including DSBs) is usually a late response, which occurs hours and even days after the induction of the critical primary DNA damage (Quiros et al., 2010). Therefore, the proper timing of determination of γH2AX and subsequent killing effects is of utmost importance.

Regarding the optimal time points for investigation of γH2AX after treatment, we often observed stronger effects 24 h after the addition of the compound to the culture medium, compared with 6 h. The early response is likely a consequence of replication blocking DNA damage that activates ATR. At later times this damage might be converted into DSBs that activate ATM and DNA-PK. Because all these PI-3-like kinases phosphorylate H2AX at Ser139, their activation gives rise to γH2AX, although with different efficiency and at different time points after exposure. An example is given by S21 and S21-2 methylating agents, represented by MNNG, temozolomide and MMS, respectively, which were compared in this study. MNNG and temozolomide produce rather high amounts of O6-methylguanine compared with MMS (~8% versus 0.3%). Although highly mutagenic, O6-methylguanine requires processing in which both mismatch repair causing a futile repair cycle and DNA replication are essentially involved (Quiros et al., 2010). The resulting DSB eventually provide the signal for activation of ATM, ATR, and DNA-PK, which activate the DDR and give rise to γH2AX and programmed cell death (Roos and Kaina, 2006). Because O6-methylguanine needs two cell cycles to be converted into DSBs that trigger cytotoxic and genotoxic effects (Quiros et al., 2010), we anticipated γH2AX to occur at later times after treatment, which was indeed the case. Cytotoxicity in the MTT assay for S21 alkylating agents was also observed at later times (72 h posttreatment) compared with other agents for which cytotoxicity was induced 48 h posttreatment incubation. The example shows that complex recognition and repair mechanisms are involved in activating the DDR that trig-
gers downstream cell death pathways, including apoptosis and necrosis. Therefore, screening should be performed at multiple time points; i.e., at least an early and late time point should be chosen after treatment for assessing the dose response of γH2AX induction.

What is the advantage of the γH2AX foci assay? The advantage relies on its specificity (very specific if containing occurs with 53BP1) and sensitivity for detecting DSBs (which is the most severe form of DNA damage), the small number of cells required, the applicability to all types of eukaryotic cells in vitro and, in principle, also in vivo, and the combination with other manipulations (e.g., costainings with repair markers such as Rad51, Ku80, NBS1, and others) that allow a more precise insight into the genotoxic mechanism triggering the DDR and downstream genotoxic effects. The biggest advantage rests on the mechanistic insights. The S139 phosphorylated form of H2AX is the result of an interplay of three PI3-related kinases (ATM, ATR and DNA-PK), which become activated by critical types of DNA damage and DNA replication stress that lead to genomic instability. Thus, γH2AX is an indicator not of any type of DNA damage, but of lesions that are critical as to proliferation inhibition, cell death including replicative senescence, apoptosis and necrosis and chromosomal instability. An example of this is provided by O6-alkylguanine, which is converted to DSBs that trigger all these effects (Knizhnik et al., 2013). Thus, with γH2AX we have a bio-indicator on hand for critical DNA lesions and severe genomic replication stress. Hence, the γH2AX assay is not an assay for detecting any type of DNA damage, but for biologically relevant and severe DNA damage (including DNA interstrand crosslinks that give rise to DSBs; Nikolova et al., 2012). “Neutral” lesions, such as N7-methylguanine, which does not block replication, will likely not be detected. What about the sensitivity? Although for some compounds such as MMS data are available obtained with the neutral and alkaline comet assay and γH2AX (own unpublished data), more comparative studies are required. Thus, data obtained in the same laboratory with the same cell type and experimental conditions (treatment and recovery time, medium composition and pH are important) are needed and a large group of agents/treatments should be tested. In conclusion, the γH2AX assay will probably not replace established genotoxicity tests, but may serve as a reasonable extension to them.

Overall, we show that genotoxic agents are efficient in inducing γH2AX, which occurs in the cytotoxic dose range, whereas nongenotoxic treatments applied at cytotoxic doses were completely ineffective in inducing γH2AX. This supports the notion that γH2AX is a reliable bio-marker useful for screening for agents that damage DNA. The γH2AX assay is highly sensitive and reproducible. It can be applied as an automated foci scoring assay or can be performed as a flow cytometric assay, which facilitates screening. On the basis of the presented data, we recommend screening first for cytotoxicity in order to determine the proper dose range and subsequently for γH2AX. If in the cytotoxic range γH2AX foci are not formed, the agent/treatment can most likely be considered as nongenotoxic. Overall, the data confirm γH2AX as a suitable DNA damage indicator useful for screening for genotoxicants.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org.

ACKNOWLEDGMENTS

We acknowledge the support of the imaging and FACS unit by the NMFZ Mainz, project 32 (2012) and in part by the German Research Society (NI 1319/1-2). We thank Ms Anna Frumkina for technical assistance and Dr Mazur for their help with biostatistics.

REFERENCES


