Global quantification of heterochromatin-associated histone methylations in cell lines with differential sensitivity to ionizing radiation

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INTRODUCTION

Chromatin structure and function are associated with post-translational histone modifications (PTHMs) of the histone proteins which are detected on lysine as acetylation, methylation, or ubiquitination, on arginine as methylation, and on serine/threonine as phosphorylation. The detection of new PTHMs is ongoing (Turner, 2012). Along with non-coding RNAs, DNA methylation and chromatin remodeling, PTHMs are an essential part of epigenetic pathways and can directly affect the interaction of DNA and histones, and thus influence the accessibility of chromatin. For example, it is widely accepted that extensive acetylation of histone tails neutralizes their positive charge and thus reduces their interaction with negatively charged DNA (Friedl et al., 2012). The presence of PTHMs may increase or reduce binding of other proteins to histone tails and thus affect chromatin structure.

Chromatin structure does not only affect transcription, but all processes involving access to the DNA, including repair. DNA double-strand breaks (DSBs), the most deleterious type of DNA damage and other types of DNA damage induce a complex and highly coordinated DNA damage response (DDR) that is intrinsic to the suppression of genomic instability (Nagaria et al., 2013). Histone modifications play a role in sensing of the initial DNA damage and provide support for critical repair proteins (For a review see Kumar et al., 2013).

Wide application of the phosphorylation of the histone variant H2AX for visualizing the chromatin regions surrounding DSBs as well as for assessment and quantification of DSBs (Dickey et al., 2009) stimulated interest in alterations of other PTHMs in the context of DDR. One of the PTHMs involved in DDR is methylation of lysine 9 on histone H3 (H3K9me). This modification is preferentially found in heterochromatin and functions as a binding site for heterochromatin protein 1 (HP1). H3K9me-mediated HP1 binding is important for maintaining the genome stability in regions of heterochromatin (Peng & Karpen, 2009). Experimental data show that the DNA damage-induced displacement of HP1-beta from H3K9me3 is crucial for the activation of ATM via Tip60 acetyltransferase activity, the kinase responsible for H2AX phosphorylation (Ayoub et al., 2008; Sun et al., 2009) asserting the pivotal role of chromatin architecture in regulating DSB repair (Xu et al., 2012).

Different patterns of PTHMs were detected in cells with differential radiation sensitivity (Maroschik et al., 2014; Djuzenova et al., 2013). In the present study, we assessed how H3K9 methylation (H3K9me2 and H3K9me3) correlates with radiation sensitivity. Experiments were conducted using two different cell lines with different sensitivities to γ-irradiation-induced apoptosis including HeLa (sensitive) and MCF-7 cells (resistant) (Özugr et al., 2013). Another heterochromatin methylation, H4K20me3, was used as a control modification in the assay due to its stability following DNA damage. Our findings show that γ-irradiation leads to a meaningful accumulation of H3K9me2 and H3K9me3, but not H4K20me3 in MCF-7 cells. This effect is less pronounced in HeLa cells.

MATERIALS AND METHODS

Cell culture and DNA damage induction. HeLa and MCF-7 cells were obtained from the German Resource Centre for Biological Material and maintained under standard culture conditions (37°C and 5% CO₂, humidity) with DMEM culture medium (Biochrom, Berlin, Germany) supplemented with 10% fetal serum (Biochrom). Twenty-four hours after plating, 4×10⁶ cells were irradiated by a total dose of 0, 2, or 5 Gy using a Cobalt-60 γ-ray source and kept under standard growth conditions for another 24 h, when they were collected and analyzed.

Abbreviations: ATM, Ataxia telangiectasia mutated; PTHMs, post-translational modifications; DDR, DNA damage response; DSBs, DNA double-strand breaks; Gy, Gray; H3K9me2, dimethylation of lysine 9 on histone H3; H3K9me3, trimethylation of lysine 9 on histone H3; H4K20me3, trimethylation of lysine 20 on histone H4; TP53, p53 protein; OD, optical density
Cell viability assessment. Cell viability was evaluated using trypan blue dye 0.4% (Applichem, Düren, Germany). Viable and non-viable cell numbers were counted using a hemocytometer and optical microscopy. Viability was calculated through the following equation: (% cell viability = total viable cells (unstained)/total cells (viable and non-viable cells) × 100. The analysis was performed in duplicate and mean results were considered.

Measurement of TP53 protein content and apoptotic cell death. We measured TP53 accumulation as a response of cells to DNA damage induction and apoptotic cell death as a measure of radiation sensitivity. Accumulation of TP53 was measured by the pan-p53 ELISA assay (Roche Diagnostics, Mannheim, Germany). The assay was performed according to the manufacturer’s instructions using cytoplasmic lysates, and relative protein concentrations were determined from the mean absorbance values using a calibration plot. To quantify apoptotic cell death we used the photometric enzyme immunoassay Cell-Death Detection ELISA kit (Roche Diagnostics) for in vitro detection and quantitation of mono- and oligonucleosomes that are released into the cytoplasm from apoptotic cells during the early stages of cell death. The measurement was performed according to the instructions of the manufacturer using cytoplasmic lysates of irradiated and non-irradiated cells in duplicate, and relative concentrations of cytoplasmic nucleosomes were determined from the mean absorbance values.

Histone extraction from cell nuclei. In order to enable study of the histone methylation in the subsequent immunoassay, core histones were isolated by acid extraction. Briefly, cells (1×10⁶) were re-suspended in 250 µl of Tris-boric acid-EDTA buffer (90 mM Tris, 90 mM boric acid and 2.5 mM EDTA, pH 8.3) and gently shaken on ice for 10 min. Following two centrifugation steps (3400 × g for 5 min at 4°C followed by centrifugation at 11 250 × g), the supernatant was removed and the pellet was re-suspended in 50 µl of extraction solution (0.5N HCl + 10% glycerol). After incubating on ice for 30 min, it was centrifuged at 13 500 × g for 5 min at 4°C. The supernatant was transferred into a fresh tube, 150 µl acetone was added and incubated overnight at 20°C. Next day, it was centrifuged at 13 500 × g for 5 min at 4°C, the supernatant was discarded, and the pellet was dried. The pellet containing histones was re-suspended in water and stored at −80°C for subsequent analyses.

Measurement of global histone methylations. All three histone methylation markers, H3K9me2, H3K9me3 and H4K20me3, were measured on extracted histones by colorimetric Global Histone Quantification Kits (Epi- gentek, Farmingdale, NY, USA) according to the instructions of the manufacturer. We applied 100 ng of histone extracts for the assay and the absorbances were measured at 450 nm. For quantification, a graph of OD versus amount of standards was plotted and delta OD/ng was determined from the slope. Alterations in histone meth-

Figure 1. Differential radiation sensitivity and global histone methylation levels.
We exposed HeLa and MCF-7 cells to γ-irradiation (0, 2 or 5 Gy) and 24 h later measured total cellular TP53 protein (A) as a response of the cells to DNA damage induciton. At the same time we assessed cell viability (B) and mono- and oligonucleosomes released into the cytoplasm (C) as measures of apoptotic cell death. The histone markers H3K9me2, H3K9me3 and H4K20me3 were quantified by immunoassays (D). *indicates statistically significant differences.
ylation levels in treated cells relative to untreated cells were expressed as fold changes.

Statistical analyses. We compared the accumulation of p53 protein, cell death rates, and histone methylation in two different cell lines exposed to γ-irradiation. Results of 3 independent experiments were used to calculate the mean values of the above parameters. Statistical comparisons were made by the Student’s t-test. Levels of p < 0.05 were considered as significant.

RESULTS

We first measured the accumulation of TP53 as a response of cells to DNA damage induction 24 h after irradiation. Although the basal levels are different, DNA damage induction leads to a similar pattern of TP53 accumulation in HeLa and MCF-7 cells. As depicted in Fig. 1A, TP53 accumulation is induced approx. 3-fold by 2 Gy of radiation in both cell lines (p < 0.05) while higher doses (5 Gy) did not substantially increase this effect. In spite of similar TP53 accumulation, we observed differential cell viability and apoptotic cell death rates in irradiated HeLa and MCF-7 cells. Cell viability decreased in HeLa cells with increasing radiation dose while in MCF cells only a very small and statistically insignificant effect was observed (Fig. 1B). Confirming this conclusion, DNA damage induced significant release of mononucleosome in HeLa cells in a dose-dependent manner whilst no notable levels were detected in MCF-7 cells (Fig. 1C).

In view of the differential sensitivity to radiation, we measured global levels of H3K9me2, H3K9me3, and H4K20me3 upon DNA damage induction. As seen in Fig. 1D, we detected differences between both cell types. In MCF-7 cells, we detected a remarkable accumulation of H3K9me2 and H3K9me3 while no changes were found for H4K20me3 as compared to control cells. The extent of increase was nearly the same for H3K9me2 and H3K9me3. Interestingly, a low dose of radiation (2 Gy) was more effective in inducing H3K9me2 and H3K9me3 up to 5-fold (p < 0.05) in these cells. In contrast to MCF-7 cells, in HeLa cells the effect of irradiation on these methylation markers was less pronounced and only a smaller increase (approx. 1.5–1.8-fold) was visible at 5 Gy (Fig. 1D).

DISCUSSION

Detection and repair of radiation-induced DNA damage occurs in the context of chromatin. In recent years it became clear that the cellular response to radiation-induced DNA damage involves pathways of histone modifications (Friedl et al., 2012). Factors affecting the extent and duration of radiation-induced histone alterations are poorly defined and a correlation between DNA repair capacity and alterations in PTHM levels has been described (Maroschik et al., 2014). As heterochromatin is essential for chromosome organization and stability (Peng & Karpen, 2009), and several histone modifications have been linked to the formation of constitutive heterochromatin, including H3K9me3 and H4K20me3 (Rea et al., 2000; Peters et al., 2001; Schotta et al., 2004) we focus here on quantitative alterations of heterochromatic modifications in radiation-induced DNA damage. As it is known that cells differing in radiosensitivity can differ in the patterns of PTHMs (Maroschik et al., 2014; Djuzenova et al., 2013) we used two different cell lines (HeLa and MCF-7) with differential sensitivities to radiation-induced apoptosis (Özgür et al., 2013; El-Awady et al., 2003).

Our findings indicate that γ-irradiation leads to a significant accumulation of H3K9me2 and H3K9me3 in MCF-7 cells (up to 5-fold). This event was found to be site-specific as DNA damage had no effect on cellular levels of H4K20me3. In HeLa cells the extent of accumulation was less pronounced (approx. 1.5–1.8-fold) and again no changes were detected in H4K20me3 levels, confirming the specificity of the effect at H3K9. These findings confirm the ascribed function of H3K9me3 in DDR (Xu et al., 2012). We observed nearly the same pattern of accumulation for H3K9me2 as for H3K9me3, suggesting that these two heterochromatinic marks could have similar roles in DSB repair via Tip60 acetyltransferase activity (Peng & Karpen, 2009; Ayoub et al., 2008; Sun et al., 2009; Xu et al., 2012). In line with this hypothesis, a recent study found accumulation of both markers after a short interval of decrease in irradiated cells (Young et al., 2013).

The obvious difference between the two cell types in terms of H3K9 methylation may be related to their sensitivity to radiation. In consistence with the literature (Jänick et al., 2001; Essmann et al., 2004), MCF-7 cells were resistant to irradiation-induced apoptosis despite significant accumulation of TP53 protein. Resistance of MCF-7 cells to radiation-induced apoptosis is attributed to the caspase-3 deficiency (Essmann et al., 2004). It has been shown that radioresistant cell lines display higher levels of residual γ-H2AX foci 24 h after irradiation (Maroschik et al., 2014) and are endowed with a higher DNA repair potential (Luzhna et al., 2013) requiring higher levels of H3K9 methylation in DDR in MCF-7 cells than in HeLa cells. On the other hand, according to a study from Chenand coworkers (2010), heterochromatin formation has been found to affect the radiosensitivity in cancer stem cells. This leads us to speculate that the vulnerability of different genomic regions to DNA damage and the pattern of alterations of PTMs may vary.

Different approaches have been used to quantify the PTHM patterns in damaged DNA. Antibody-based immunofluorescence is applied to monitor γ-H2AX-occupied chromatin regions (Falk et al., 2007; Solovjeva et al., 2007; Löbrich et al., 2010; Rothkamm et al., 2013). ChiP is widely used for the analysis of alterations at specific damaged sites (Murr et al., 2006; Stanciu et al., 2009; Iacoboni et al., 2010). Some studies globally investigated DSB-associated alterations in PTHMs by Western blotting of nuclear lysates (Tjeertes et al., 2009; Seiler et al., 2011). ELISA immunoads are increasingly being utilized to globally measure PTHMs (Dai et al., 2011; Dai et al., 2013; Farifteh et al., 2014). To our knowledge this is the first study to measure heterochromatic modifications by ELISA in damaged cells. One of the advantages of using the ELISA-based assay over other techniques such as immunofluorescence or western blotting is the quantitative nature of measurement and its simple and robust processing. Furthermore, this approach may be particularly useful to characterize the accumulation of modifications enriched at heterochromatic regions.

In conclusion, our findings indicate that the accumulation of heterochromatin-associated methylations (H3K9me2 and H3K9me3) is related to sensitivity to the DNA damage-induced apoptosis. Further work is needed to unravel the underlying mechanisms of this finding.

Conflicts of interest

There are no conflicts of interest.
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REFERENCES


