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Review

γH2AX as a novel endpoint to detect DNA damage: Applications for the assessment of the *in vitro* genotoxicity of cigarette smoke

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ABSTRACT

Histone H2AX is rapidly phosphorylated to become γH2AX after exposure to DNA-damaging agents that cause double-strand DNA breaks (DSBs). γH2AX can be detected and quantified by numerous methods, giving a direct correlation with the number of DSBs. This relationship has made γH2AX an increasingly utilised endpoint in multiple scientific fields since its discovery in 1998. Applications include its use in pre-clinical drug assessment, as a biomarker of DNA damage and in *in vitro* mechanistic studies.

Here, we review current *in vitro* regulatory and non-regulatory genotoxicity assays proposing the γH2AX assay as a potential complement to the current test battery.

Additionally, we evaluate the use of the γH2AX assay to measure DSBs *in vitro* in tobacco product testing.

Contents

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1. Introduction

DNA damage can be caused by products from internal metabolism such as reactive oxygen species, but also by a range of exogenous agents, from energetic radiations such as UV light to chemicals. There are multiple forms of DNA damage; DNA single-strand breaks (SSBs), DNA–DNA crosslinks or DNA–protein crosslinks or covalent binding to DNA bases, nucleotide substitution, DNA frameshifts, double-strand breaks (DSBs), etc. DSBs are one of the most deleterious lesions since they affect both strands of the DNA helix. This lesion can lead to cell death by triggering apoptosis but if the lesion fails to repair or it is repaired incorrectly, DNA information can be compromised leading to mutation and ultimately cancer and/or heritable damage (Jeggo and Lobrich, 2007).

Histones are highly conserved proteins which play a role not only in DNA packing but also in DNA repair and gene regulation. There are 5 families of histones: 1, 2A, 2B, 3 and 4. Histone 2AX (H2AX) from the histone 2A family becomes rapidly phosphorylated (γH2AX) at serine-139 in response to DSBs (Rogakou et al., 1998). There are mechanisms that get activated after DNA damage has occurred to avoid genomic instability; they are known as DNA damage response (DDR). One of the earliest DDRs is the activation of γH2AX as a result of a DSB. This response occurs within minutes of the damage, thus making it a useful marker of DNA damage. The description of events involved in this activation in mammalian cells leading to γH2AX and beyond is a complex process that has been described in detail in previous reviews (Riches et al., 2008; Paull et al., 2000; Fernandez-Capetillo et al., 2004; Cann and Dellaire, 2011; Bekker-Jensen and Mailand, 2010; Srivastava et al., 2009; Svetlova et al., 2010).

Briefly, the earliest responding proteins are those of the phosphatidylinositol 3-kinase-like family of kinases (PIKK) including ataxia telangiectasia-mutated (ATM), ATM- and Rad3-related (ATR) and the catalytic subunit of DNA-dependent protein kinase (DNA-PKc). The proteins are activated by DNA damage and are rapidly recruited to the site of damaged chromatin. Once there, they phosphorylate the histone 2AX at serine residue 139 located at the C-terminal tail resulting in the formation of γH2AX. However, to date it is still not fully understood how DNA damage is detected by the cellular machinery. Cann et al. suggested two models. The first postulates that changes in the chromatin structure following a DSB release topological constraints on the DNA helix that ultimately activate ATM. The second model, however, postulates that the MRE11-RAD50-NBS1 (MRN) complex in its task of keeping both ends of the broken DNA together is the critical DSB sensor but also the initial repair force, recruiting ATM to the site where it becomes activated (Cann and Dellaire, 2011).

Some investigations with cell lines deficient in DNA-PK and ATM showed a limited increase in H2AX phosphorylation after DSB damage (Paull et al., 2000). The roles played by the PI3K enzymes are thought to be different depending on toxic stimulus or cell type (Yan et al., 2011; Riches et al., 2008).

Either way, after the initial γH2AX activation, a positive feedback loop is created between γH2AX and the PIKKs for further DDR. The signal amplification acts as a repair signal calling for the repair systems to move to the location of the damage (Nakamura et al., 2010). Within minutes of the damage occurring, γH2AX can be detected in high quantities in the areas surrounding the DSB (Rogakou et al., 1999). These areas are known as nuclear foci and could extend several megabases of chromatin around the site of damage (Riches et al., 2008). Multiple studies (Cann and Dellaire, 2011; Xu and Price, 2011) suggest that γH2AX foci formation is mainly limited to euchromatin considered transcriptionally active and moderately compacted. Heterochromatin representing the transcriptionally inactive and highly compacted chromatin could be inaccessible to phosphorylation or more resistant to DNA damage. One could also hypothesise that DNA damage in the heterochromatin does not lead to genomic instability as there is no active transcription. Therefore, repair resources are not invested.

The formation of nuclear foci in response to DNA DSBs differs from the formation of the "apoptotic γH2AX ring" (Solier and Pommier, 2009). They demonstrated that γH2AX ring staining is an early apoptosis indicator that precedes a global nuclear staining or pan-nuclear staining and apoptotic body formation. The main driver of this particular phosphorylation is DNA-PK in contrast to ATM and ATR associated with γH2AX nuclear focus formation. This morphology variation could potentially be used to discriminate DNA DSBs from other forms of DNA damage.

γH2AX could also act as a cell cycle checkpoint (Downey and Durocher, 2006). H2AX could become phosphorylated at any point during the cell cycle, including during mitosis while other DDR proteins are limited to interphase cells (Nakamura et al., 2010). It has been suggested that DSB repair mechanisms may be suspended during mitosis. However, γH2AX foci continue to form during mitosis. The foci act as indicators to activate the repair mechanisms as soon as the cell has finished the division process. If the DNA DSB occurs in G1, the cell cycle would stop to prevent the cell moving into S-phase with damaged DNA. Likewise, DNA replication could be slowed if the DNA DSB has occurred in S-phase, so that the repair mechanisms could act before the DNA polymerase reaches the damaged section. Finally, when the damage occurs in G2-phase, the cell is prevented from moving into mitosis, avoiding the fracture of chromosomes during anaphase and cytokinesis (Jackson, 2002).

1.1. Kinetics of phosphorylation

Following the induction of DSBs, phosphorylation of the serine 139 residue starts within minutes, reaching a plateau at around 30 min after damage occurs (Paull et al., 2000). The phosphorylation then decreases over a period of hours (Rogakou et al., 1998).

The mechanism of γH2AX elimination has not been fully unravelled. There are multiple phosphatases involved in γH2AX dephosphorylation. Dephosphorylation could occur directly on the chromatin or could happen after the histone has been displaced from the nucleosomes (Chowdhury et al., 2005; Redon et al., 2011a). Both mechanisms could potentially occur simultaneously, independent of the location of the γH2AX in the foci. Other mechanisms mentioned by Bao involve histone chaperone proteins in the process of γH2AX elimination (Bao, 2011). Experiments carried out by Keogh and colleagues suggest that the loss of γH2AX could be triggered not only by DSB repair but also by the activation of steps that precede DSB repair (Keogh et al., 2006). However, some of their results seem to indicate that γH2AX loss is not mediated by single-stranded DNA resection, one of the cellular responses to DSBs.

1.2. Rationale for measuring γH2AX

There are several reasons why γH2AX is used to detect DSBs. The formation of γH2AX is proportional to the amount of DSBs, giving a direct 1:1 correlation to existing damage (Sedelnikova et al., 2002). This correlation indicates that for every DSB one nuclear focus would be created. Moreover, H2AX is distributed throughout the mammalian chromatin and when it becomes phosphorylated it covers a large area of the chromatin producing the easily detectable nuclear foci (Rogakou et al., 1999). The foci can be measured by different techniques in what is known as the γH2AX assay to give an account of the DSBs. In addition, this marker is conserved across eukaryotic evolution, giving the γH2AX assay potential use not only in human studies but also in other organisms including plants (Redon et al., 2011b).