Evaluation of chemical phototoxicity, focusing on phosphorylated histone H2AX

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Histone H2AX is a minor component of nuclear histone H2A. The phosphorylation of histone H2AX at Ser 139, termed γ-H2AX, was originally identified as an early event after the direct formation of DNA double-strand breaks (DSBs) by ionizing radiation. Now, the generation of γ-H2AX is also considered to occur in association with secondarily formed DSBs by cellular processing such as DNA replication and repair at the site of the initial damage, including DNA adducts, crosslinks, and UV-induced photolesions. Therefore, γ-H2AX is currently attracting attention as a new biomarker for detecting various genotoxic insults. We have determined the toxic impact of various environmental stresses such as chemicals, light and/or their coexposure using γ-H2AX, and found that the γ-H2AX assay exhibited high sensitivity and a low false-positive rate as a detection system of genotoxic potential. In this review, we introduced our recent findings concerning the evaluation of chemical phototoxicity, focusing on γ-H2AX.

Keywords: phototoxicity; γ-H2AX; DNA damage; chemicals; light

INTRODUCTION

Many chemicals are produced and released into the environment. Apart from dietary food, cosmetics and medicines, exposure to these chemicals unintentionally occurs from the emissions of industrial pollutants and automobiles. Skin, the outermost tissue that protects us from infections and trauma, is the most frequent tissue to be directly exposed to those chemicals. In addition, skin has the possibility of being exposed to sunlight concomitantly with the chemicals. Some chemicals deposited on the skin through topical applications and unintentional adhesion, or distributed through the vasculature, may act as photosensitizers [1, 2].

Acute phototoxic reactions are generally characterized by erythema and edema, followed by hyperpigmentation and desquamation [3]. Chronic repeated injuries may result in fragility, blistering, and milia formation, or even actinic keratosis and skin cancer. These injuries are attributable to the oxidation of proteins and membranes as well as DNA damage. In cosmetics, medicines and foods, in particular, which are applied and consumed by humans, the assay of phototoxicity has received much attention. Among several in vitro phototoxicity assays, the 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT) was adopted by the Organization for Economic Cooperation and Development (OECD) guideline in 2004 as the most suitable method of screening for the phototoxicity of water-soluble chemicals. On the other hand, the phototoxic mechanism and its long-term influences cannot be predicted with this assay because it evaluates cell survival and death.

We previously examined the chemically induced phosphorylation of histone H2AX (γ-H2AX) and found that several types of chemically induced DNA damage could be sensitively detected by γ-H2AX [4–7]. The phototoxicity of several chemicals could also be detected using γ-H2AX [8–10]. In this review, the usefulness of γ-H2AX for the phototoxic assay has been discussed.

Phototoxicity of chemicals

Photodynamic reaction of chemicals and toxicity

Some chemicals are known to exhibit ‘phototoxicity’ under sunlight. Phototoxicity is defined as the ability of chemicals to induce toxic effects when irradiated with ultraviolet (UV) and/or visible light. The phototoxic effects observed following the absorption of light by chemicals have been divided into two types (Fig. 1) [11]. Type I phototoxicity occurs...
when chemicals acquiring an excited state after the absorption of light directly react with an important cell constituent or transfer electrons or hydrogen atoms. Type II phototoxicity occurs when chemicals adsorbing light go from an excited singlet state to a triplet state, and react with molecular oxygen to produce singlet oxygen (\(1^O_2\)), the superoxide anion, and hydrogen peroxide.

Chemicals that exhibit phototoxicity are contained in foods, drugs, pesticides, cosmetics, industrial products, and other substances [1, 2]. Photosensitization not only occurs when some chemicals are deposited on the skin through topical applications and unintentional adhesion, but also when chemicals are distributed through the vasculature. Psolaren is a typical phototoxic compound contained in citrus plants [12], the strong phototoxic characteristics of which have been used for phototherapy [13]. Air and water pollutants released into the environment may also act as photosensitizers [9, 14–16].

Sunlight contains UV and visible light, both of which have the potential to induce phototoxicity. The UV spectrum in sunlight has been divided based on wavelength: UVA (320–400 nm) and UVB (290–320 nm). As UVB cannot penetrate the inner epidermis, UVA and visible light are important for the phototoxicity of chemicals. The action spectra for most phototoxins lie in the UVA range [3]. We have reported that coexposure to polycyclic aromatic hydrocarbons (PAHs), existing widely in the environment, and UVA generated oxidative DNA damage (8-hydroxy-2-deoxyguanosine: 8-oxodG) [14, 17] and the most serious DNA damage, DNA double-strand breaks (DSBs), via a type II reaction [8, 18]. These types of DNA damage have been associated with cancer initiation. Therefore, photosafety testing of many kinds of chemicals (such as certain drugs, cosmetic products, and chemicals released into the environment) is an important issue to address when these chemicals are produced, used, and released.

**Problems of testing for phototoxicity**

The evaluation of phototoxicity tests for chemicals *in vivo* and *in vitro* has a long history. The potential to cause phototoxicity with substances applied topically was evaluated by utilizing various animal models; however, experiments using animals have recently become difficult to perform from the view of animal protection and cost issues. In the European Union, the use of animals to test the utility and safety of cosmetics and the ingredients was prohibited from 11 March 2013 [19]. In this situation, the 3T3 NRU PT was established and validated by the European Center for the Validation of Alternative Methods (ECVAM’s) Scientific Advisory Committee in 1997 as an *in vitro* method for evaluating the phototoxic potential of chemicals shown to absorb in the UV/visible range, and has been accepted as an official test guideline of the OECD (TG432, 2004). The 3T3 NRU PT is a cytotoxicity-based assay that utilizes normal BALB/c 3T3 mouse fibroblasts to measure concentration-dependent reductions in neutral red (NR) uptake by cells following their exposure to a test chemical either in the presence or absence of UVA light. A weak cationic dye, NR readily diffuses through cell membranes and accumulates in cellular lysosomes. Once in the acidic environment of the lysosome, the NR is oxidized, becoming positively charged and trapped within the lysosome. Alterations to the cell membrane, phototoxic action caused by test chemicals, result in the loss of the NR from the lysosome. A validation test by ECVAM showed reproducibility and correlations with *in vivo* results. Considering simple technical procedures and cost efficiency, the 3T3 NRU PT is the most approved method for an *in vitro* assessment of chemicals having phototoxic potential [20]. However, the 3T3 NRU PT has some disadvantages: (i) the endpoint of the test is cell death, meaning only the detection of acute phototoxicity; (ii) the information obtained from the data is poor (the mechanism underlying toxicity is not analyzed); (iii) the test is performed using relatively high doses of chemicals and shows a high frequency of positive results [21].

Recently, validation studies by the Japanese Center for the Validation of Alternative Methods (JaCVAM) showed that a reactive oxygen species (ROS) assay had 100% sensitivity for predicting phototoxicants [22, 23]. They concluded that negative results in the ROS assay would not require further testing in animals or other tests, while positive, weakly positive, and inconclusive results would proceed to the next level of testing in an *in vitro* test system such as the 3T3 NRU PT. This method is very simple and suitable for the first screening of phototoxicity. However, this assay does not measure phototoxicity directly, but rather is a physicochemical test. In order to elucidate the mechanism underlying phototoxicity and the phototoxic influence of environmental chemicals released at low concentrations, other types of evaluation are desired.

**Photogenotoxicity testing**

As photodynamic reactions influence several cellular components, the cell membrane, proteins and DNA, we need to examine which is a target, evaluate the phototoxic mechanism involved, and also investigate the long-term fate. DNA damage may lead to cell death or tumor initiation. Assessing the action to DNA, that is the photogenotoxic potential (photogenotoxicity), is an important issue to confirm the
long-term influence of chemicals and the phototoxic mechanism involved, although photogenotoxicity testing is no longer recommended as a part of the photosafety testing strategy for medicines (the 5th International Workshop on Genotoxicity Testing in 2009) [21]. In photogenotoxicity testing, almost all of the in vitro assays used for routine genotoxicity testing have been adapted by an additional treatment with UV or visible light [24, 25]. Cell-free investigations on naked DNA [26] and in vitro assays (such as the Ames test [27], micronucleus test [26–28], and comet assay [27, 29, 30]) were performed. Hereditary consequences, point mutation and chromosome damage were also examined using the hypoxanthine–guanine phosphoribosyl transferase (HPRT) assay [31] and the chromosomal aberration test [24], respectively. Among these tests, the photo-Ames test was found to be the quickest and easiest method, but sensitivity to UV is irrelevant when compared with human exposure. The best in vitro system to analyze the phototoxic mechanism is the assay using target cells from skin. The photo-comet assay is relatively reliable and easy [25], and could be performed using target cells. In addition, the use of repair enzymes makes the assay informative, although the process becomes quite complex. More predictive and informative methods that are suitable for human skin testing are expected.

**Phosphorylation of histone H2AX (γ-H2AX)**

**Histone H2AX**

Genomic DNA in eukaryotic cells winds around histones to form nucleosomes, which are then packaged into the condensed structure of chromatin. The fundamental unit of chromatin, the nucleosome, consists of an octamer of the four core histones (two each of H2A, H2B, H3 and H4), around which ~146 base pairs of DNA are wrapped. Histones contain a large proportion of the positively charged amino acids lysine and arginine in their structure. DNA is negatively charged due to the phosphate groups. These opposite charges result in the high binding affinity between histones and DNA, and condense the structure of chromatin.

H2AX is a variant of H2A and has a carboxyl-terminal region longer than that of H2A [32]. The conserved motif centering on serine four residues from the carboxyl terminal, SQ motif (SQRY), is phosphorylated in response to ionizing radiation and other agents that can introduce DSBs (Fig. 2). Two H2A are contained in a nucleosome, in which the amounts of H2AX vary depending on the derivation of tissues. The percentages of H2AX in H2A are ~10% in human normal fibroblasts, as low as 2% in lymphocytes and HeLa cells, or as high as 20% in the SF268 human glioma tumor cell line [33, 34].

**Availability of γ-H2AX as a DNA damage marker**

γ-H2AX was originally identified as appearing early after the formation of DSBs following exposure to ionizing radiation (by Rogakou et al. in 1998 [33]). As shown in Fig. 2, hundreds to thousands of H2AX that exist in the mega bases of DNA around a DSB are phosphorylated, and foci of γ-H2AX can be detected by immunofluorescence staining [35]. As the number of foci is reported to be proportional to the number of DSBs [36], many investigators performed immunofluorescence staining for γ-H2AX, and counted the number of foci and percentages of γ-H2AX-positive cells as a marker for DSBs. H2AX is phosphorylated by ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related), and DNA-PK (DNA-dependent protein kinase), known as kinases for the SQ motif [37–39]. The role of γ-H2AX is assumed to facilitate the local recruitment and

![Fig. 2. Phosphorylation of histone H2AX. Hundreds to thousands of H2AX that exist in the mega bases of DNA around a DSB are phosphorylated, and foci of γ-H2AX can be detected by immunofluorescence staining.](image-url)
retention of DNA repair and chromatin remodeling factors to the DSBs [40, 41]; however, it has not yet been completely clarified.

Although γ-H2AX is first identified as a phenomenon that was generated following the formation of DSBs by ionizing radiation, it is now also considered to occur as a result of other types of DNA damage [34]. DSBs are caused secondarily by the collision of replication forks at the sites of DNA damage, including oxidative bases, DNA adducts, single-strand breaks (SSBs), and crosslinks, and also by the repair of this damage. In fact, H2AX could be phosphorylated following the exposure of cells to various suspected DNA-damaging agents [4–7, 42, 43], suggesting that γ-H2AX has the possibility of being a marker for several types of DNA damage induced by mutagens and carcinogens (Fig. 3). In flow cytometric analysis, DSBs (generated when the progressing replication forks collided with the base adducts and other SSBs) were clearly detected, with an increase in γ-H2AX during the S phase [44]. From the time-course formation of γ-H2AX and cell-cycle dependency, we could estimate whether DSBs were formed directly or secondary. Furthermore, γ-H2AX has the advantage of providing a more sensitive measurement of DNA damage than other traditional techniques such as pulse field gel electrophoresis and comet assays [43, 45, 46]. Focusing on γ-H2AX as a genotoxic marker is a good way to identify the genotoxic potential of chemicals. We previously used the γ-H2AX as a sensitive DNA damage marker for various chemicals, in which phototoxic DNA damage could be clearly detected [9, 10, 14]. In the next section, we introduced our recent studies on the detection of the photogenotoxicity of some chemicals using γ-H2AX.

**New evaluation method for photogenotoxicity—γ-H2AX**

**Application of γ-H2AX for the evaluation of photogenotoxicity**

To examine whether γ-H2AX is capable of becoming a marker for photogenotoxicity, the phototoxic potentials of typical chemicals were analyzed and compared with the generation of γ-H2AX using the traditional detection methods [10]. The tested chemicals are shown in Fig. 4. Human keratinocytes, HaCaT, were treated with chemicals, then exposed to UVA (320–400 nm). The UVA dose (5 J/cm²) is correlated to the outdoor level of sunlight exposure for about 1 h around noon in summer.

We first introduced the results of 8-methoxypsoralen (8-MOP), known as a strong phototoxic chemical, 6-methylcoumarin (6-MC), known as a weak phototoxic chemical, and sodium lauryl sulfate (SDS), known as a non-phototoxic chemical. It has been reported that 8-MOP forms DNA crosslinks via a type I reaction under UVA, with the production of 1O₂ via a type II reaction [47], and that 6-MC also produces 1O₂ via a type II reaction [48]. 8-MOP is the strongest phototoxic chemical, as determined by the 3T3 NRU PT, and is often used as a positive control for the phototoxic test. Fig. 5A shows the formation of γ-H2AX 4 h after treatment with 8-MOP under UVA exposure (edited figure from *J Invest Dermatol* 2011;131:1313–21 [10]). γ-H2AX foci within the nucleus were clearly detected as discrete dots. Cells having >10 foci were significantly increased by the treatment with 8-MOP and UVA, which was 8-MOP-dose-dependent and detectable at 10⁻⁹ M. The generation of γ-H2AX was
Fig. 4. Test chemicals for phototoxicity.

Fig. 5. Generation of γ-H2AX after treatment with chemicals and UVA. The generation of γ-H2AX 4 h after treatment with chemicals and UVA was analyzed by immunofluorescence staining and western blotting. (A) Images of γ-H2AX foci produced by treatment with 8-MOP and UVA. The graph shows the percentages of γ-H2AX-positive cells (cells having >10 foci). (B) 8-MOP-dose-dependent (10⁻⁹–10⁻⁴M) generation of γ-H2AX. Actin was used as a standard for the equal loading of proteins for SDS-PAGE. (C) 6-MC-dose-dependent (10⁻⁹–10⁻⁴M) generation of γ-H2AX. (D) SDS-dose-dependent (10⁻⁵–10⁻⁴M) generation of γ-H2AX. The triangles show the limit of the detection. (Edited figure from *J Invest Dermatol* 2011;131:1313–21 [10].)
also detected by western blotting from a concentration of $10^{-9}$ M, similar to the results of immunofluorescence staining (Fig. 5B). γ-H2AX was also generated after a treatment with the weak phototoxic chemical 6-MC at a concentration of $10^{-7}$ M (Fig. 5C). On the other hand, the non-phototoxic compound SDS could not generate γ-H2AX, even at the high doses (Fig. 5D).

Survival did not change throughout a wide range of 8-MOP doses as at 4 h after the treatment (Fig. 6A), at which time γ-H2AX was strongly detected. This result means that the generation of γ-H2AX 4 h after the treatment with 8-MOP and UVA, as shown in Fig. 5, was not due to apoptosis, although apoptotic DNA fragmentation significantly generated γ-H2AX. Even at 24 h after the treatment, 8-MOP at concentrations from $10^{-9}$–$10^{-7}$ M, in which the frequency of γ-H2AX-positive cells was ~50–80%, did not influence cell survival. Over $10^{-6}$ M of 8-MOP led to significant cell death. 6-MC did not cause cell death, even at the highest dose of $10^{-4}$ M [10].

Since γ-H2AX is attributed to the induction of DSBs, the induction of DSBs after treatment with 8-MOP and UVA was detected by biased sinusoidal field gel electrophoresis (BSFGE) (Fig. 6B). A single treatment with 8-MOP ($10^{-4}$ M) or UVA did not cause the migration of DNA. Migrated DNA was detected when the cells were treated with 8-MOP at concentrations ranging from $10^{-6}$–$10^{-4}$ M and UVA, the extent of which was dose-dependent. Although a significant amount of γ-H2AX was observed when the cells were treated with $10^{-9}$ M of 8-MOP and UVA irradiation (Fig. 5B), migrated DNA could not be detected by BSFGE at concentrations of 8-MOP lower than $10^{-6}$ M. Furthermore, DSBs could not be detected by BSFGE after the treatment with 6-MC and UVA [10]. These results suggested that sensitivity in terms of phototoxicity/photogenotoxicity using γ-H2AX was greater than that with DNA damage analysis with BSFGE and cell survival/death.

**Usefulness of γ-H2AX for evaluating photogenotoxicity**

Figure 7 shows the detection limits for the phototoxic effects of the chemicals assessed using (i) γ-H2AX (4 h after the treatments), (ii) DSBs detected by BSFGE (4 h after the treatments), and (iii) cell viability (24 h after the treatments) (edited figure from *J Invest Dermatol* 2011; **131**:1313–21 [10]). A total of 11 kinds of chemicals: 8-MOP, bithionol (BT), 6-MC, promethazine hydrochloride (PM), 5-methoxypsoralen (5-MOP), chlorpromazine hydrochloride (CPZ), neutral red (NR), rose bengal sodium salt (RB), tetracycline (TC), SDS, and L-histidine (L-his), as shown in Fig. 4, were compared. The most suitable concentrations for the detection of photocytotoxicity were expressed by wide bars and dark colors. For example, in the case of 8-MOP, the detectable concentrations for cell death and DSBs using gel electrophoresis were over $10^{-6}$ M, and the higher doses were more suitable. γ-H2AX could be detected even at a concentration of $10^{-6}$ M and strongly at the higher concentrations. The phototoxicity of the weak phototoxic chemical, 6-MC, was detectable only by γ-H2AX from $10^{-7}$ M, and not by survival and gel electrophoresis. On the other hand, in the cases of PM, CPZ and NR, the sensitivity of the detection of γ-H2AX was less at the excessively high concentrations. This may be due to a decrease in cellular responses (phosphorylation) by acute phototoxicity. We concluded that the limits of detection of photocytotoxicity using γ-H2AX were 100–1000 times lower than that using cell viability and DNA gel electrophoresis. In addition, neither of the non-phototoxic chemicals, SDS and L-His, generated γ-H2AX. This result means that the detection of γ-H2AX as a photogenotoxic marker is a method with a low false-positive rate, even at high concentrations, which is an essential requirement for phototoxicity testing.

![Fig. 6. Survival and DSBs generation after treatment with 8-MOP and UVA.](image-url)
As mentioned above, phototoxicity is divided into two types, I and II [11]. 8-MOP, 5-MOP, CPZ, PM, BT and NR are reported to mainly induce a type I photodynamic reaction, and 6-MC, RB and TC mainly a type II photodynamic reaction [49–54]. The patterns of generation of γ-H2AX did not differ between types, and both could be sensitively detected using γ-H2AX. The mechanism to generate γ-H2AX following each type of photodynamic reaction is assumed to be the following. Type I: excited chemicals by light-induced primary damage (such as crosslinks and adducts with DNA, and the replication fork block) and/or repair of damage results in DSBs, leading to γ-H2AX. Type II: the generation of ROS induces SSBs and oxidative base DNA damage, which changes to DSBs in the process of replication and repair, similar to type I.

Usefulness of γ-H2AX for evaluating the photogenotoxicity of environmental chemicals

Until now, we have proposed that γ-H2AX is useful for determining the genotoxicity of environmental chemicals, benzene metabolites, photooxidized polycyclic aromatic hydrocarbons (PAHs), detergents, and other substances [4–7, 55, 56]. Similarly, γ-H2AX is a sensitive phototoxic marker for environmental chemicals [9, 14]. Widespread mutagenic and carcinogenic compounds, PAHs, have been demonstrated to generate γ-H2AX under UVA irradiation [8, 9]. This was suppressed by sodium azide, an inhibitor of 1O2, suggesting that the production of 1O2 (type II reaction) is a cause of photogenotoxicity. The detection limits for the phototoxic potential of four PAHs having different numbers of benzene rings were compared using three detection methods, similar to Fig. 7. The higher the number of benzene rings, the stronger the phototoxicity/photogenotoxicity was. Toxic ability was detected at nearly two orders of magnitude greater in γ-H2AX than in cell survival and DSBs using BSFGE. Naphthalene (two benzene rings) showed no phototoxicity in any of the detection methods, indicating that a sensitive marker of γ-H2AX showed no face-positive result in non-phototoxic chemicals, as mentioned above. These results suggested that γ-H2AX is also useful for a photogenotoxic assay on environment chemicals, which would spread widely at low concentrations.

CONCLUSION

We can detect photogenotoxicity using γ-H2AX at low concentrations of several kinds of chemicals. γ-H2AX is generated based on the formation of DSBs, including secondarily formed DSBs in the process of repair and replication stress. Therefore, the analysis of γ-H2AX, dependent on the cell cycle and time, could predict the general mechanism responsible for photogenotoxicity. The methods of γ-H2AX detection (such as immunofluorescence staining) are complicated and not adequate for screening many samples. The detection method needs to be improved to put these results to practical use. Its use for an analysis of phototoxic mechanisms would be valuable following screening by the 3T3 NRU PT and ROS assay. As shown above, DNA damage has been shown to occur at doses at which cell survival is not affected by the photodynamic reaction. Survival with DNA damage is a cause of carcinogenesis. The highly sensitive detection of γ-H2AX may predict the risk of photocarcinogenesis with long-term exposure at low concentrations.

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