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Cytometry of ATM Activation and Histone H2AX Phosphorylation to Estimate Extent of DNA Damage Induced by Exogenous Agents

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Abstract

This review covers the topic of cytometric assessment of activation of Ataxia telangiectasia mutated (ATM) protein kinase and histone H2AX phosphorylation on *Ser139* in response to DNA damage, particularly the damage that involves formation of DNA double-strand breaks. Briefly described are molecular mechanisms associated with activation of ATM and the downstream events that lead to recruitment of DNA repair machinery, engagement of cell cycle checkpoints, and activation of apoptotic pathway. Examples of multiparameter analysis of ATM activation and H2AX phosphorylation vis-a-vis cell cycle phase position and induction of apoptosis that employ flow- and laser scanning-cytometry are provided. They include cells treated with a variety of exogenous genotoxic agents, such as ionizing and UV radiation, DNA topoisomerase I (topotecan) and II (mitoxantrone, etoposide) inhibitors, nitric oxide-releasing aspirin, DNA replication inhibitors (aphidicolin, hydroxyurea, thymidine), and complex environmental carcinogens such as present in tobacco smoke. Also presented is an approach to identify DNA replicating (BrdU incorporating) cells based on selective photolysis of DNA that triggers H2AX phosphorylation. Listed are strategies to distinguish ATM activation and H2AX phosphorylation induced by primary DNA damage by genotoxic agents from those effects triggered by DNA fragmentation that takes place during apoptosis. While we review most published data, recent new findings also are included. Examples of multivariate analysis of ATM activation and H2AX phosphorylation presented in this review illustrate the advantages of cytometric flow- and image-analysis of these events in terms of offering a sensitive and valuable tool in studies of factors that induce DNA damage and/or affect DNA repair and allow one to explore the linkage between DNA damage, cell cycle checkpoints and initiation of apoptosis.

Keywords

ionizing radiation; DNA topoisomerase inhibitors; DNA double-strand breaks; carcinogens; tobacco smoke; replication stress; genotoxins; DNA photolysis

Activation of ATM and Phosphorylation of Histone H2AX Triggered by DNA Damage

Ataxia telangiectasia mutated (ATM) is a protein kinase that becomes activated in response to DNA damage, particularly when the damage involves formation of DNA double-strand breaks (DSBs) (1–9; Fig. 1). Interestingly, the initial activation of ATM does not take place at the exact site of the DSB but at some distance from it, and appears to be triggered by a change in the higher order of chromatin structure caused by unwinding and relaxation of the topological stress of the DNA double helix upon induction of the DSB (4). Activation of ATM occurs through its autophosphorylation on Ser1981 and it requires prior ATM acetylation that is mediated by the Tip60 histone acetyltransferase (13). Ser1981 ATM phosphorylation causes dissociation of the inactive ATM dimer or multimer into single monomeric units that have kinase catalytic activity (3,4). Whereas ATM phosphorylation on Ser1981 is required for dissociation of the dimer, the catalytic domain of ATM is located outside of the Ser1981 site and becomes accessible to substrates only if ATM is in the form of a monomer (3). The MRE11-Rad50-NBS1 (MRN) complex is essential in the process of ATM activation, as it recognizes DNA damage, recruits ATM to the damage site, and also functions in targeting ATM to initiate phosphorylation of the respective substrates (5–9). At the site of the DSB, activated ATM phosphorylates several substrates, including NBS1, SMC1, and BRCA1. Phosphorylation of NBS1 targets ATM towards Chk1, phosphorylation of SMC1 engages the S-phase checkpoint, while BRCA1 phosphorylation is essential to activate this protein along the DNA repair pathway. Upon exposure of cells to ionizing radiation (IR) activation of ATM was shown to correlate strongly with the number of DSBs but not with the number of single-strand DNA breaks (SSBs) or other ssDNA lesions (14). It should be noted that DSBs constitute only a few percent of the IR-induced DNA damage, SSBs and base damage account for over 98% of the overall damage (14). However, the cells, in which DSBs are induced by certain radiomimetic agents, such as eneidine C-1027, can robustly activate DNA damage responses in the absence of ATM (15).

ATM is the key component of the signal transduction pathways that are mobilized by DNA damage and is essential for safeguarding genome integrity (1–7,16,17). Among the downstream target substrates phosphorylated by ATM, are p53 (TP53), checkpoint proteins Chk2, Chk1, BRCA1, and H2AX (4,17). The product of BRCA1 E3-ubiquitin ligase is implicated in several biochemical processes primarily related to DNA repair. The structural maintenance chromosome protein 1 (SMC1), which is a component of the cohesion complex and involved in the S-phase checkpoint, is also phosphorylated in an ATM-dependent manner (10,12,18,19). It is quite evident that phosphorylation of these ATM downstream effectors is critical for successful DNA repair, suppression of cell progression through the cell cycle and/or prompt induction of apoptosis. These activities prevent transmission of DNA damage to cell progeny that could lead to genomic instability, and thus they play an important role in suppression of tumorigenesis.

Development of the phospho-specific Ab towards ATM phosphorylated on Ser1981 (ATM-S1981^P) provided a convenient tool to detect ATM activation immunocytochemically in tissue sections (20) and to measure expression of ATM-S1981^P in individual cells by cytometry (21–25). It should be noted that when activation of ATM is induced by formation of DSB the activated ATM is localized in individual discrete foci. However, when other

DNA lesions or chromatin alterations trigger ATM activation, ATM-S1981^P appears diffusely scattered throughout the nucleoplasm and does not form distinct foci (3,12).

As mentioned, one substrate phosphorylated by ATM is histone H2AX, the variant of histone H2A (5). Similar to ATM, H2AX plays a critical role in maintaining genome integrity. The loss of H2AX in mice leads to genomic instability (26). H2AX ^{-/-} mice are radiation sensitive, growth retarded, and immune deficient (26). H2AX haploinsufficiency compromises genetic integrity, and in the absence of p53, enhances susceptibility to cancer (27). H2AX phosphorylation on Ser139 is induced in response to DNA damage, and phosphorylated H2AX has been termed γ H2AX (28–30). Expression of γ H2AX when it is mediated by ATM has often been considered to be a reporter of such DNA damage that generates formation of DSBs (3,5–7,28–30). As in the case of ATM-S1981^P, the development of the phospho-specific γ H2AX Ab allows one not only to assess H2AX phosphorylation in individual cells (29,30), but also provides one the ability to quantify DNA damage by measuring H2AX phosphorylation in individual cells by cytometry (31–37).

H2AX can also be phosphorylated by other phosphoinositide 3-kinase related protein kinases (PIKKs) such as ATM-and Rad3-related (ATR), and/or DNA dependent protein kinase (DNA-PKcs) (2,38). For example H2AX phosphorylation in cells subjected to replication stress by hydroxyurea or aphidicolin appears to be mediated not by ATM (23) but by ATR (39). In hypertonically-stressed cells (40,41), or in response to DNA fragmentation during apoptosis (42) DNA-PKcs is the protein kinase that phosphorylates H2AX. Extensive activation of ATM, however, also accompanies DNA fragmentation in apoptotic cells, which would suggest that ATM may mediate H2AX phosphorylation during apoptosis as well (22,43). It should also be noted that in response to DNA damage caused by IR all three PIKKs, ATM, ATR, and DNA-PKcs, may function redundantly to phosphorylate H2AX (44,45). Most publications, however, point out that the induction of DSBs triggers phosphorylation of H2AX that is mediated by ATM (1,3–8,10,12,14).

Phosphorylation of H2AX is the most easily observed upon induction of DSBs by exogenous genotoxic agents such as radiation or radiomimetic drugs. The presence of DSBs manifests then by appearance of distinct γ H2AX immunofluorescent (IF) foci, easily identifiable by fluorescence microscopy. Thus, each focus reports the presence of a single DSB (28,29). It should be noted, however, that intermediates in repair of DNA damage as well as DSBs that are formed during DNA replication also trigger H2AX phosphorylation (36,46). H2AX is also phosphorylated in response to DNA damage induced by endogenous oxidants, likely the by-product of oxidative metabolic activity (24,25,47,48). In addition, H2AX phosphorylation takes place during immune system development in response to the formation of DSBs occurring in V(D)J and class-switch recombination (49) as well as during meiotic recombination (50). While in most studies γ H2AX IF foci were counted visually, cell imaging and computer algorithms have also been used to provide their quantitative analysis (51–53).

Detection of DNA Damage Induced by IR and UV

Among the variety of lesions induced by X-radiation, DSBs are the most critical in terms of their role in inducing reproductive cell death as well as delayed genomic instability, which may lead to tumorigenesis (54). Quantitative analysis of DSBs in individual cells is therefore of primary interest in various disciplines of biomedicine, in particular radiation biology. Unfortunately, single cell gel electrophoresis (neutral comet assay), which until now was the primary analytical tool to quantify DSBs, lacks the desired sensitivity, is cumbersome and generally does not allow one to relate DSB to cell position in the cell

cycle. It should be noted, however, that with the use of laser scanning cytometry some authors were able to obtain information regarding DNA content of the cells subjected to the comet assay, though the number of measured cells/comets was rather limited (55,56). The demonstration that radiation-induced expression of γ H2AX (measured by immuno-blotting) was linearly correlated with the dose of radiation (28) provided impetus to develop assays, in which H2AX phosphorylation can be used as a sensitive reporter of DNA damage induced by radiation, primarily the formation of DSBs. Olive and her collaborators (31,33,57–60) pioneered the assessment of γ H2AX expression by cytometry to detect and measure DNA damage induced by X-irradiation. While they observed variable level of constitutive H2AX phosphorylation in the untreated cells, more prominent during S- than G₁-phase (57), the increase in intensity of γ H2AX IF after cell irradiation was strongly correlated with the dose of radiation (57–60). Using flow cytometry these authors detected the induction of γ H2AX, measured 60 min after irradiation with as low as a 20 cGy dose of X-rays (58). The half-times of disappearance of the radiation-induced γ H2AX IF ranged from 1.6 to 7.2 h, was associated with a rate of decrease in the number of foci, and was correlated with clonogenic survival for 10 cell lines (59). Also strongly correlated with cell death, assessed by clonogenicity, was expression of γ H2AX IF measured 60 min after treatment of Chinese hamster V79 cells with several radiomimetic drugs examined in a range of over 2 decades of cell kill (31). This strong relationship between the induction of γ H2AX expression and loss of clonogenicity led the authors to postulate that the assessment of H2AX phosphorylation by flow cytometry can be used as a surrogate of the estimate of cell kill (31). In still another study of six human cervical cancer cell lines, these authors observed the cell line-dependent differences in the rate of disappearance of γ H2AX IF induced by X-irradiation was associated with the status of p53 (wt vs. p53 deficient) and also related in part to intrinsic radiosensitivity of these lines (59).

In contrast to the data of Banath and Olive (31), Mahrhofer et al. (61) studying 10 different cell lines were unable to find a correlation between the X-radiation-induced expression of γ H2AX and loss of clonogenicity. However, there is evidence that response to X-irradiation of normal cells in terms of induction of γ H2AX depends on the cell type, being different for example in epithelial cells of the intestine when compared with thymus or testis (62). The induction of γ H2AX by endogenous oxidants at similar levels was also shown to vary significantly depending on p53 status of the cell (63). The apparent discrepancy between Mahrhofer et al. (61) data and the earlier reports (31) may be due to the fact that cells of different types/lines, also differing in p53 status, were used in the respective studies. It should be pointed out that, using photo-multipliers to measure the intensity of fluorescence emission, flow cytometry, as shown by Banath and Olive (31), offers a greater dynamic range of sensitivity and thus better accuracy, compared with the type of image analysis employed by Mahrhofer et al. (61).

It should also be noted that the rate of disappearance of radiation-induced γ H2AX correlates with the rate of DNA repair only at low levels of DNA damage, namely when fewer than 150 DSBs per genome are generated (64). However, at extremely low X-ray doses, no DNA repair can be detected, as evident by the persistence of γ H2AX foci for several days (65). At such low number of DSBs, it is difficult, if not impossible to distinguish constitutive DNA damage and formation of DSBs as a result of oxidative metabolism (24,25) from DNA damage by radiation, or even from background radiation (66,67).

The data reviewed earlier raise the important question of whether the measurement of intensity of X-radiation-induced γ H2AX (integrated value, per cell or nucleus) by flow or laser scanning cytometry reflects the number of DSBs and thus can provide an estimate of DNA damage and repair in quantitative terms. The answer to this query appears to be affirmative provided that one limits the analysis to a particular cell type/line and does not

directly compare different cell types/lines with each other. It is also imperative to operate within the limited range of X-radiation doses at which the induction of γ H2AX expression is linearly correlated with the dose. At a certain dose range of X-radiation (2–16 Gy) a good correlation was seen between the γ H2AX IF measured by flow cytometry and the frequency of microscopic foci detected by digital image analysis (37). It should be pointed out, however, that at a higher range of X-irradiation doses (e.g., >2 Gy) one would anticipate rather large number of γ H2AX IF foci per nucleus with the possibility of foci overlap that may impede their accurate count by image analysis. It is therefore somewhat unexpected that the authors (37) observed such good correlation. It was recently reported that cytometric assessment of γ H2AX in blood cells of X-irradiated patients offers a sensitive measure of DNA damage in vivo (68). The latter authors stress that cytometric assessment of γ H2AX expression is 100-fold more sensitive in detecting DNA damage induced by X-irradiation or calicheamicin compared with the alkaline comet assay (68).

Similar to that observed with X-irradiation, the key target of UV radiation is DNA. The main classes of the primary, potentially mutagenic lesions induced by UV are cyclobutane–pyrimidine dimers and 6-4 (T-C) photoproducts (69). The principal mechanism for repair of UV induced damage is nucleotide excision repair (NER), a complex process involving about 30 different proteins; defects in genes coding some of these proteins lead to the sun light-sensitive, cancer-prone disorders, such as Xeroderma pigmentosum, Cockayne's syndrome, or trichothiodystrophy (70). Compared with X-irradiation, significantly fewer studies were done on cytometric analysis of H2AX phosphorylation induced by UV irradiation. We observed that following exposure of HeLa or HL-60 cells to a wide range (6.1 J/m²–3.45 kJ/m²) of doses of UV-B light, the highest level of γ H2AX was induced in S-phase cells particularly during the early portion of S (34). In cells that did not replicate DNA (G₁, G₂) the degree of H2AX phosphorylation was distinctly lower than that in S-phase cells, and was UV dose-dependent (33, see Fig. 2). Apoptotic cells (predominantly S-phase) become apparent >2 h after exposure to UV and they had an order of magnitude higher intensity of γ H2AX IF than that initially induced by UV (34). While the suppression of DNA replication with aphidicolin prevented the induction of H2AX phosphorylation by UV in most S phase cells, it had no effect on a small cohort of cells that appeared to be entering S-phase that expressed very high levels of γ H2AX. Our data were consistent with the notion that H2AX phosphorylation observed throughout S phase could reflect formation of DSBs resulting from the collision of replication forks with the UV-induced primary DNA lesions and that replication initiation was more sensitive to UV than the elongation step. We speculated that induction of γ H2AX in G₁ and G₂ cells could reflect a response to the primary DSBs generated during UV exposure and/or DNA repair. Marti et al., (36), however, postulated that H2AX phosphorylation seen in UV-irradiated G₁ cells depends on NER rather than being a reflection of primary DSBs. However, our present data (Fig. 2) show no induction of γ H2AX for most G₁ cells treated with UV; the treated cells with DNA content slightly above DI = 1.0 and with the progressively increasing level of γ H2AX (enS) are most likely the cells that were entering S phase during 30 or 60 min in cultures following irradiation. No increase in ATM-S1981^P IF was seen 30 min after irradiation, while γ H2AX was already induced at that time. This would suggest that ATM did not initially induce H2AX phosphorylation after UV exposure, although its activation became apparent after 60 min. The lack of ATM activation prior to H2AX phosphorylation (Fig. 2) is consistent with the reported data that UV-induced replication stress triggers activation of ATR and DNA PKcs rather than ATM (38,72). It appears, however, that ATM also is activated, although subsequently to ATR and DNA PKcs

Detection of DNA Damage Induced by Antitumor Drugs

DNA topoisomerase I and II (topo1 and topo2) inhibitors are among the most effective antitumor drugs currently available. Their mode of action is thought to involve stabilization of “cleavable” complexes between topo1 or topo2 and DNA that are transiently formed during DNA replication or untangling (73,74). Collisions of the progressing DNA replication forks or the progressing RNA polymerase molecule with the stabilized complexes convert them into DSBs, which are recognized as lethal lesions and trigger apoptosis. Predominantly S-phase cells undergo apoptosis after exposure to topo1 inhibitors (75,76).

Figure 3 illustrates distinctly different pattern of response of HL-60 cells to the topo1 inhibitor topotecan (Tpt) and topo2 inhibitor mitoxantrone (Mxt) in terms of cell cycle phase specific induction of H2AX phosphorylation. While Tpt induced γ H2AX almost exclusively in S-phase cells, Mxt triggered H2AX phosphorylation in all phases of the cell cycle, and maximally during G₁. In fact, 1 h after administration of Tpt, essentially all cells with an S-phase DNA content exhibited γ H2AX IF well above the maximal threshold of γ H2AX IF of the untreated S-phase cells; at the same time, most G₁ and G₂M phase cells had unchanged γ H2AX IF compared with control. The cells with a G₁ or G₂M DNA content but with variable expression of γ H2AX marked as *enS* or *exS* most likely are the cells that during the 1-h treatment with Tpt were entering or exiting S phase, respectively. Thus, these cells were exposed for a variable length of time to Tpt, while replicating DNA, which led to a variable extent of DNA damage, proportional to the length of time of DNA replication in the presence of Tpt. Interestingly, despite the fact that G₁ cells were the most affected by Mxt in terms of induction of γ H2AX, the S-phase cells preferentially underwent apoptosis, while G₁ cells did not. This observation implied that DSBs induced in the cells replicating DNA were much more effective in triggering apoptosis than in G₁ or G₂M phase cells (79,80). However, etoposide (VP-16), which is also a topo2 inhibitor, like Mxt, induced maximal DNA damage (H2AX phosphorylation and ATM activation) during G₁ but, unlike Mxt, triggered apoptosis indiscriminately, in all phases of the cycle (81). It should be noted that mitoxantrone belongs to the anthraquinone family of topo2 inhibitors and binds directly to DNA, while etoposide is a member of the podophyllotoxin family, does not bind to DNA but binds stoichiometrically to topo2 (81). These data suggested, therefore, that in addition to the generally accepted mechanism involving collision of replication forks with the “cleavable complexes,” other mechanisms, which appear to be different for etoposide versus mitoxantrone, may contribute to formation of DSBs and to the triggering of apoptosis (79,81).

Tri-variate gating analysis of cellular DNA content versus ATM-S1981^P IF versus γ H2AX IF is illustrated in Figure 4. The untreated A549 cells (left panels) are compared with the cells that were exposed to 150 nM Tpt for 1 h (right panels). The gating analysis was performed to color (in red) the cells that expressed γ H2AX above the upper level of γ H2AX IF for 97% of G₁- and S-phase cells in the untreated culture. The data clearly indicate that a fraction of G₂M cells in the untreated culture expressed both γ H2AX and ATM-S1981^P. However, the G₂M cells that expressed maximum levels of ATM-S1981^P had lower expression of γ H2AX (Panel E) and vice versa. Based on cell relocation using the LSC, these cells were identified as metaphase cells (E; m). The untreated G₂M cells that had maximal expression of γ H2AX and somewhat lower of ATM-S1981^P were mostly prophase and prometa-phase (E; p). The data also clearly indicate that a large majority of the S-phase cells from Tpt treated culture with induced expression of γ H2AX also had induced expression of ATM-S1981^P. The bivariate analysis of γ H2AX IF versus ATM-S1981^P of the Tpt-treated cells (F) showed that most γ H2AX IF positive cells also expressed ATM-S1981^P (quartile b). The correlation between the expression of γ H2AX and ATM-S1981^P

for these cells (within the dashed line boundary in Panel F) is very strong ($R = 0.97$). A small number of cells were located in quartile *a*, i.e., showing activation of ATM but no H2AX phosphorylation. Since H2AX phosphorylation is mediated by ATM and thus ATM activation precedes H2AX phosphorylation these cells may represent subpopulation of cells responding to DNA damage, which had already activated ATM but were yet to phosphorylate H2AX.

Figure 5 illustrates an analysis of ATM activation and H2AX phosphorylation in TK6 cells induced by nitric oxide-releasing acetylsalicylic acid (NO-ASA; NO-aspirin). This drug, initially developed as an anti-inflammatory agent specifically to avoid the adverse effects of aspirin (82), was recently shown to be cytotoxic to cells of different tumor lines (83). The aim of our studies was to reveal whether the cytotoxicity induced by NO-ASA was mediated by damage to DNA. A brief exposure of human TK6 cells to $5 \mu\text{M}$ NO-ASA led to DNA damage revealed by both ATM activation and histone H2AX phosphorylation (Fig. 5), confirmed by alkaline and neutral comet assays (84). The induction of H2AX phosphorylation was preferential to S-phase cells and was markedly reduced by NAC, a scavenger of reactive oxygen species. The data implied that the NO-ASA induced DNA damage through oxidative stress; the oxidation-generated lesions provided a signal for induction of ATM activation and H2AX phosphorylation during DNA replication, likely when the progressing replication forks collided with the primary lesions (ss breaks, base adducts) converting them to DSBs. Because neither induction of H2AX phosphorylation nor apoptosis were observed at equimolar concentrations of ASA, the NO moiety appeared to mediate these effects (84).

DNA Damage by Replication Stress

DNA replication stress (stalled DNA replication forks) often leads to DNA damage. Such damage, involving formation of DSBs, was seen in the cells treated with the antitumor drug hydroxyurea (Hxu), an inhibitor of ribonucleotide reductase that inhibits DNA replication through its effects on cellular deoxynucleotide pools (85). Aphidicolin (Aph), an inhibitor of α -like DNA polymerases, also was reported to cause DNA damage (86). It is unclear, however, whether Aph-induced DNA damage may involve formation of DSBs (87). Both Hxu and Aph are widely used to synchronize the cells in the cell cycle (88,89). We have undertaken studies, therefore, to reveal whether H2AX is phosphorylated in cells exposed to HU or APH and whether its phosphorylation may be mediated by ATM (22). Figure 6 illustrates the changes in expression of ATM-S1981^P and γ H2AX of HL-60 treated with $4 \mu\text{M}$ Aph or 0.5 mM Hxu for 2 h. After 2 h of treatment, the apoptotic cells became apparent and they expressed very high level of ATM-S1981^P and γ H2AX. To be able to locate on the bivariate distributions both apoptotic and nonapoptotic cells, it was necessary, therefore, to use a logarithmic scale for the coordinate representing IF in Figure 6.

The data (Fig. 6) show that the induction of H2AX phosphorylation by either Aph or Hxu was limited to S-phase cells and was most pronounced in early-S. In contrast, there was no evidence of ATM activation. As is evident, apoptotic cells, identified by extremely high expression of H2AX and high ATM-S1981^P IF (their apoptotic mode of death was confirmed by the presence of activated caspase-3; not shown), had an S-phase DNA content. The data imply that DNA damage-related H2AX phosphorylation induced by replication stress, as expected, affected S-phase cells, which shortly underwent apoptosis. The data also imply that, during replication stress, H2AX phosphorylation was not mediated by ATM but most likely by ATR and/or DNA-PKcs PIKKs (23,85). The corollary to this finding is the caveat that synchronization of cells in the cycle by inhibitors of DNA replication results in DNA damage and cells synchronized in this manner, including the widely used “double-

thymidine block,” cannot be considered to be “healthy” and representative of untreated cells (11).

DNA Damage Induced by Potential Carcinogens and Mutagenic Agents

Exposure of cells to potential environmental carcinogens that target DNA generates relatively little DNA damage during a single cell cycle. However, this damage, particularly induction of DSBs, has serious consequences for the cell progeny and may even promote tumorigenesis. Hence, the comet assays may not provide adequate sensitivity to detect and measure the extent of DNA damage upon cell exposure to such agents. By virtue of its high sensitivity, the cytometric assessment of H2AX phosphorylation and ATM activation is therefore of particular value for monitoring the effects of carcinogens and mutagens. We have carried out comprehensive studies to assess the potential of tobacco smoke in inducing DNA damage (35,90).

Exposure of cells to cigarette smoke is known to cause DNA damage inducing base adducts and a variety of other ssDNA lesions as well as DSBs (reviewed in 90). Figure 7 illustrates the changes in expression of ATM-S1981^P and H2AX in A549 cells upon their exposure to cigarette smoke for 5 or 20 min, under conditions as described elsewhere (35,90). The data distinctly show markedly increased levels of ATM activation and H2AX phosphorylation in the cigarette smoke-treated cells, well above the level of the mock-treated cells. The latter were exposed for 20 min to the ambient-air under the same conditions as the smoke-treated cells. The time of exposure to smoke obviously relates to the dose of the DNA damaging agents absorbed by the cell, and not surprisingly 20 min exposure led to much higher levels of ATM activation and H2AX phosphorylation than the 5 min treatment. Interestingly, while the smoke-induced increase in expression of ATM-S1981^P does not show any cell cycle phase-specificity after either 5 min or 20 min exposure, the induction of γ H2AX is distinctly cell cycle-phase specific. Namely, as it is evident (Fig. 7), the S phase cells were more affected in terms of H2AX phosphorylation after 5 min exposure to smoke than are G₁ or G₂M cells. At a higher (20 min) dose, however, the cell cycle phase specificity was no longer apparent. It should be noted that γ H2AX induced by cigarette smoke had typical pattern of IF foci (90) consistent with the notion that it reports the induction of DSBs. The data shown in Figure 7, thus, suggests that at a lower dose (5 min exposure) the DNA damaging agents in the smoke induced DSBs preferentially in DNA replicating cells. Most likely, the DSBs were generated when the progressing replication forks collided with the base adducts and other ssDNA lesions; the collisions resulted in formation of DSBs, as was described in the case of oxidative DNA damage by endogenous radicals (91). It is possible that at the higher dose of the carcinogen, when ATM activation is at a very high level, the SMC1 became phosphorylated by ATM triggering the S-phase checkpoint (10,18,19), which in turn halts movement of replication forks and thereby formation of DSBs by this mechanism.

The cytometric assay of DNA damage based on detection of ATM activation and/or H2AX phosphorylation has been used to assess the dose and kinetics of cell response to cigarette smoke or smoke condensate (35), and to test the role of ROS as the agents mediating DNA damage upon exposure to smoke (90). The activation of ATM in response to tobacco smoke implies that cell exposure to it triggers the cell cycle checkpoints and other downstream effectors mediated by ATM (5–9,91,92). This cytometric assay (35), which is sensitive and relatively simple, can be used to compare different brands of cigarettes and tobacco types, or assess the effectiveness of cigarettes filters in removing the smoke constituents responsible for DNA damage, including the formation of DSBs. Since DSBs are obvious DNA lesions that are potentially carcinogenic and mutagenic, the cytometric assay (35) can be of practical value for FDA and other agencies controlling carcinogenicity of tobacco products.

The natural toxin juglone, a component of roots, bark, and leaves of walnut trees, has been reported to be a mutagen (93) and carcinogen (94). Using multiparameter cytometry, its ability to induce H2AX phosphorylation as a reporter of DNA damage was tested on human fibroblasts in relation to the cell cycle phase and expression of p53 (95). In another study the potential mutagenic effect of oxidative stress on human spermatozoa has also been probed by flow cytometric assessment of H2AX phosphorylation (96). This finding could be rather surprising in light of evidence that most histones are replaced by protamines during late stages of mammalian spermiogenesis (97). Histone H2AX, however, is to a large extent preserved, and in fact undergoes phosphorylation during the final rearrangement of chromatin (98).

DNA Photolysis

Exposure of cells that contain halogenated precursors such as BrdU incorporated into DNA to IR induces rapid DNA photolysis (99). The photolysis generates a multiplicity of DNA strand breaks, which can be directly labeled with fluorochrome-conjugated nucleotides using exogenous terminal deoxynucleotidyl transferase in the TUNEL reaction (100,101). This approach provides the basis for a commercially available reagent kit that offers the possibility of detecting DNA proliferation (BrdU incorporation) without the necessity of DNA denaturation by heat or acid or partial DNA hydrolysis by DNase (ABSOLUTE-STM Proliferation Kit, Phoenix Flow Systems, San Diego, CA).

The DNA photolysis approach has been recently extended to have the DSBs generated by UV irradiation detected via the induction of γ H2AX (102). In this assay the cells are pulse-incubated with BrdU, then rinsed free of the precursor, and subjected to UV irradiation in the presence of Hoechst 33342, a fluorochrome which through fluorescence resonance energy transfer mechanism, additionally increases the proclivity of DNA containing incorporated BrdU to undergo photolysis upon UV irradiation (103). Thus, DSBs are generated at the sites of BrdU incorporation when such cells are irradiated with UV. The presence of DSBs triggers H2AX phosphorylation which is then detected immunocytochemically; DNA may be concurrently labeled with another color fluorochrome (102). Thus, the DNA photolysis-induced expression of γ H2AX serves as a marker of BrdU incorporation during DNA replication (Fig. 8).

When BrdU pulse-labeled cells are allowed to progress through the cell cycle the cohort of labeled cells traverses G₂, then M, and after cytokinesis, re-enters G₁. The bivariate analysis of cellular DNA content versus presence of BrdU as a function of time after pulse-labeling provides information about the kinetics of cell cycle progression, allowing one to measure the duration of the cell cycle and length of individual phases of the cell cycle (104). This approach found wide utility in analysis of cell kinetics both in vitro and in vivo (105). Similar to the ABSOLUTE-STM method (99–101), the DNA photolysis approach, utilizing expression of γ H2AX as a marker of BrdU incorporation, does not require harsh conditions for DNA denaturation or its partial digestion by DNase that often preclude immunocytochemical detection of other cell attributes or accurate analysis of the cell cycle. This method, therefore, is compatible with other immunocytochemical procedures and can be applied when the detection of BrdU incorporation has to be correlated with cell immunophenotype or with expression of intracellular immuno-markers, vis-à-vis cell cycle phase.

DNA Fragmentation During Apoptosis

Internucleosomal DNA fragmentation that leads to a multiplicity of DSBs is the hallmark of apoptosis (106,107). The classical TUNEL method developed to identify apoptotic cells is

based on labeling these DSBs with fluorochrome-conjugated nucleotides (78,100). The DSBs generated during apoptotic DNA fragmentation trigger H2AX phosphorylation (21–23,42,108). Phosphorylation of H2AX is a rather early event of apoptosis, occurring concurrently with the activation of caspase-3 (81), with appearance of nucleosomal/oligonucleosomal DNA fragments detected by gel electrophoresis (“DNA laddering”) and with externalization of phosphatidyl-serine at the outer leaflet of plasma membrane (108). At early stage of apoptosis the level of H2AX phosphorylation (intensity of γ H2AX IF) exceeds by an order of magnitude the expression of γ H2AX induced by primary DSBs generated by UV-radiation (34), DNA topoisomerase inhibitors (32,80) or other agents, such as inhibitors of DNA replication that trigger apoptosis through induction of replication stress (Fig. 9).

This extremely high intensity of γ H2AX IF allows one to distinguish apoptotic cells (Ap) from nonapoptotic cells, whether untreated or drug (e.g., aphidicolin) treated (Fig. 9). In fact, because of such large differences in IF intensity, a logarithmic scale has to be used to display γ H2AX IF when apoptotic cells are present concurrent with nonapoptotic cells, untreated or treated with DNA damaging drugs. However, the expression of γ H2AX significantly drops during progression of apoptosis and late apoptotic cells may have modest or low levels of γ H2AX IF and then cannot be distinguished by this marker (32). It is unclear whether the decrease in expression of γ H2AX during apoptosis is a result of its dephosphorylation or proteolytic cleavage.

The fact that γ H2AX IF can be a marker of both early apoptosis (very high levels of γ H2AX IF) and of the extent of the drug- or radiation-induced primary DNA damage (moderate or low level of γ H2AX IF) offers some advantages. Namely, by multiparametric analysis of γ H2AX IF and DNA content as a function of time after induction of DNA damage, one can obtain information on the relationship between the extent of DNA damage versus induction of apoptosis, with respect to the cell cycle phase. Using this strategy, we observed that when the damage was induced by DNA topo2 inhibitor mitoxantrone, the S-phase cells exclusively underwent apoptosis (79). In contrast, when the damage was caused by another topo2 inhibitor, etoposide (VP16) with a different chemical structure than mitoxantrone, no S-phase preference in terms of induction of apoptosis was apparent (81).

It is not entirely clear which PIKK is responsible for H2AX phosphorylation during apoptosis. While Mukherjee et al., (42) reported that DNA-PK is the one that phosphorylates H2AX in apoptotic cells, we observed that upon induction of apoptosis by various agents, ATM activation occurred concurrent with H2AX phosphorylation, suggesting that ATM plays a role (22; e.g., see Fig. 9). It is possible that, depending on cell type, different PIKKs may be involved in phosphorylating H2AX, or perhaps they act redundantly during apoptosis.

To assess whether H2AX phosphorylation is triggered by the primary DNA damage caused by a genotoxic agent or is a reflection of DNA fragmentation during apoptosis caused by DNA damage it is critical to distinguish the apoptosis-associated H2AX phosphorylation from its phosphorylation triggered by the primary DNA damage. Three strategies can be used to address this issue. As mentioned earlier, early apoptotic cells have so high γ H2AX IF that this marker by itself is adequate to identify them. This may not be the case with late apoptotic cells, which show diminished expression of γ H2AX IF. However, since caspase activation and DNA fragmentation is precluded by caspase inhibitors such as z-VAD-FMK (32) cell treatment with the DNA damaging agent in the presence of z-VAD-FMK eliminates the apoptosis-associated H2AX phosphorylation but does not affect the induction of γ H2AX triggered by primary DNA damage. Thus, administration of z-VAD-FMK concurrently with the genotoxic agent allows one to eliminate the contribution of apoptosis-

associated DNA fragmentation and directly measure the effects of the genotoxic agent on H2AX phosphorylation. The third strategy uses multiparameter analysis to combine the detection of caspase-3 activation with H2AX phosphorylation to gate out the cells demonstrating activated caspase-3. This strategy is illustrated by the data shown in Figure 10. In this experiment the HL-60 cells were exposed to the topo1 inhibitor Tpt that induces DSBs in S-phase cells, which subsequently undergo apoptosis (76,77). Bivariate analysis of γ H2AX IF versus caspase-3 activation (caspase-3* IF) allows one to distinguish four cells sub-populations as marked by the *a-d* regions in panel 4th of this figure, namely: (*a*) the γ H2AX IF negative/caspase-3* negative; (*b*) γ H2AX IF positive/caspase-3* negative; (*c*) γ H2AX IF positive/caspase-3* positive, and (*d*) γ H2AX IF negative/caspase-3* positive. Apoptotic cells (Ap) can be discriminated by extremely high value of their maximal pixel of γ H2AX IF as measured by laser scanning cytometry (LSC; 111,112). It is quite clear that essentially all cells with such high expression of γ H2AX are caspase-3* positive. The cells, in which H2AX phosphorylation was triggered by the primary DSBs induced by Tpt (prDSBs), demonstrate increased γ H2AX IF but show no evidence of caspase-3 activation prior to undergoing apoptosis (located in quartile *b*; Tpt 3h). The late apoptotic cells were still located in region *c* although their expression of γ H2AX would be lower compared with early apoptotic cells (32). Thus, several different approaches can be used to eliminate the contribution of apoptosis-associated DNA fragmentation to assess H2AX phosphorylation or ATM activation triggered exclusively as primary events by genotoxic agents.

Advantages and Limitations of Cytometric Analysis

Cytometric assessment of H2AX phosphorylation and ATM activation provides a very sensitive and convenient tool to estimate DNA damage either induced by radiation, anticancer drugs or environmental carcinogens/mutagens. Its sensitivity in terms of DSB detection is nearly two orders of magnitude greater when compared with the traditional method of DNA damage analysis based on the single cell DNA electrophoresis (comet) assay (31,68). Most importantly, by offering the possibility of multiparametric analysis, cytometry allows one to correlate, within the same individual cells, the extent of DNA damage with other cell attributes such as cell cycle position, initiation of apoptosis or expression of any other protein, such as tumor suppressors, oncogenes, transcription factors, signal transduction proteins, etc., that can be detected immunocytochemically. Analysis of a correlation between these measured attributes may provide important information on the mechanism of their interactions in response to DNA damage vis-à-vis recruitment of DNA repair machinery, engagement of G₁, S, and G₂-phase checkpoints, or initiation of apoptosis. One may expect, therefore, that multiparameter cytometry will be the methodology of choice in analysis of reporters of DNA damage such as ATM activation and H2AX phosphorylation.

One advantage in cytometric assessment of induction of γ H2AX or activation of ATM by a particular agent is that one actually measures the increase (Δ) in IF above the background value of the nontreated cells reflecting primarily their constitutive phosphorylation (25,47,63). The measured radiation-, drug-, or carcinogen-induced increase in IF, thus, is not affected by the initial background level of fluorescence, which may also have a nonspecific component of cell autofluorescence or nonspecific fluorochrome binding. The corollary of this is that there is no critical need to use isotypic IgG as the background control; its use however is needed to assess the level of constitutive H2AX phosphorylation or ATM activation.

Histone doubles in content during the cell cycle at the same rate as DNA content doubles. To make the measurements of treatment-induced γ H2AX IF independent of histone doubling during the cycle one may express the treatment-induced increase (Δ) in mean of

γ H2AX IF for particular phase of the cell cycle as the percent increase above the untreated cells at the same phase. After such compensation the data reflects the degree of H2AX phosphorylation in terms of frequency of phosphorylated H2AX molecules per total histone content (32). In the case of constitutive expression of γ H2AX, the data can be compensated by multiplying the mean S-phase and G₂/M phase γ H2AX IF by 0.75 and 0.5, respectively, to express γ H2AX IF per unit of DNA (histone), e.g., to compare the degree of H2AX phosphorylation in different phases of the cycle (32).

It should be stressed that there are several critical issues pertaining to the interpretation of the cytometric data. One important point is the relationship between induction of DSBs and H2AX phosphorylation detected by cytometry. It is quite evident that H2AX may be phosphorylated in the absence of the DSB induction, e.g., as it is in the case of replication stress (22, Fig. 6). Thus, the induction of γ H2AX per se cannot be considered as a reporter of DSBs. As mentioned earlier there is strong evidence that in response to DSBs, H2AX phosphorylation is mediated by ATM (3,5–7,28–30). The concurrent activation of ATM and induction of γ H2AX is therefore more assuring that it reflects the response to formation of DSBs. Perhaps even more assuring is the detection of characteristic γ H2AX or ATM-S1981^P IF foci, each focus presumed to represent a response to a single DSB (30). One should always be careful with interpreting this data, however, since ATM activation and H2AX phosphorylation seen concurrently may also be a reflection of extensive chromatin condensation unrelated to DSBs formation, such as observed during mitosis in some cell types (71) or during premature chromosomes condensation (80).

The count of individual IF foci per nucleus, especially if double-labeled with γ H2AX and ATM-S1981^P Abs (43), may provide the ultimate quantitative analysis of the number of DSBs induced in a single cell. This can be accomplished by image analysis utilizing the fluorescence in situ hybridization detection strategy provided by the LSC (109,110) or similar instruments. The image analysis indeed provides an opportunity to detect minimal extent of DNA damage, e.g., such as occurs in vivo in lymphocytes of patients subjected to computed tomography examinations (111). As mentioned before, however, that the count of individual foci per nucleus is technically limited to relatively small (about <40) numbers. With greater number of IF foci per nucleus, their proximity to each other and the possibility of overlap creates problems that may prevent their accurate contouring.

Provided that the induction of γ H2AX is in response to formation of DSBs, it is important to know whether the integrated value of intensity of γ H2AX IF per nucleus, as measured by cytometry, is a good representation of the number of DSBs inflicted by cell treatment. Since DSBs are potentially lethal lesions the cytometric assessment of γ H2AX may then be predictive of cell survival and used to assess the effectiveness of the treatment as a surrogate for more cumbersome methods (e.g., clonogenicity). Several findings indicate that, within certain limits, the intensity of the γ H2AX IF measured is indeed proportional to the frequency of DSBs. Thus, Rogakou et al. (28) observed that the γ H2AX IF integrated on Western blots strongly correlated with the dose of X-radiation and with the induced DSBs. The data of Olive and her collaborators (31,33,59,60) also provide evidence of a good correlation between the dose of radiation, cell survival and intensity of γ H2AX IF measured by flow cytometry. Our observations indicated that within certain ranges of concentration (dose) of DNA damaging agents expected to induce DSBs, the intensity of γ H2AX IF measured by flow cytometry or LSC correlates with their dose (32,34,43,81). However, there are significant differences between cell types/lines in terms of intensity of γ H2AX induced in response to DNA damage, e.g., related to p53 status (63). It is not surprising, therefore, that correlation between the extent of DNA damage (or cell survival) and the induction of γ H2AX, when applied to compare different cell types/lines, is not always apparent (61).

Expression of γ H2AX in response to the induction of DSB is a kinetic event, occurring rapidly (few minutes), peaking at about 1 h and when DNA repair continues, subsiding as a result of its dephosphorylation (112). Thus, the intensity of γ H2AX IF measured by cytometry is expected to vary depending on the time after induction of DSBs. It is possible to prevent dephosphorylation of γ H2AX by treatment of cells with phosphatase inhibitors such as calyculin A (32). However, such treatment also induces PCC (113), an event that triggers sequentially phosphorylation of histone H3, activation of ATM and phosphorylation of H2AX (80). Caution should be exercised, therefore, in data interpretation, to exclude the possibility that PCC contributes to the observed ATM activation or H2AX phosphorylation.

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Figure 1.

The model of the signaling pathway mediated by ATM in response to DSBs induced by IR, as proposed by Kitagawa et al. (10). Upon induction of DSB the MRE11, RAD50, and NBS1 proteins (MRN complex), as well as BRCA1, are recruited to its site. The MRN complex, when recruited to a DSB, combined with the “structural” change in chromatin resulting from DNA breakage (unwinding and altered torsional stress of the DNA double helix) activate ATM by triggering its auto-phosphorylation, which causes dissociation of the ATM dimer into two enzymatically active monomers, located at some distance from the DSB. After activation, ATM may phosphorylate several nucleoplasmic substrates such as p53, Chk1, Chk2, E2F1. The activated, monomeric ATM is recruited to the DSB when NBS1 and BRCA1 are already at the break site, at which point it phosphorylates several substrates, including NBS1 and SMC1. Phosphorylation of NBS1 targets ATM to Chk1 (11), phosphorylation of SMC1 activates the S-phase checkpoint (10,12), while BRCA1 phosphorylation engages this protein in DSB repair involving either nonhomologous end joining (NHEJ) or homology-directed repair (HR). Phosphorylation of NBS1 is also essential for MRN stimulation of ATM activity towards Chk2 (6). The MRN complex is critical for recruitment of ATM to the site of DSB and in mediating phosphorylation of p53 and H2AX by ATM (6). Phosphorylation of p53 may lead to upregulation of p21 or Bax thereby arresting the cell in G₁ or inducing apoptosis, respectively.

Figure 2.

Induction of γ H2AX and ATM-S1981^P expression in HL-60 cells following exposure to UV light. Exponentially growing HL-60 cells untreated (Ctrl) or exposed to 57.5 J/m² of UVB light were left in culture for 30 or 60 min after the exposure before harvesting, as described (34). The expression of γ H2AX or ATM-S1981^P was measured concurrently with cellular DNA content by flow cytometry, and the data are shown as bivariate γ H2AX IF (or ATM-S1981^P IF) versus DNA content distributions. The dashed skewed lines represent the maximal level of γ H2AX IF or ATM-S1981^P IF for over 95% G₁- and S-phase cells from the nonirradiated (Ctrl) cultures. The inset in the left panel shows the cellular DNA content frequency histogram of the untreated cells; the cells from the UV irradiated cultures displayed nearly identical histograms. Mitotic cells (M) from untreated cultures show higher expression of γ H2AX and ATM-S1981^P than G₂ cells (21,71) and are marked, respectively, in Ctrl samples.

Figure 3. Effect of topotecan (Tpt) and mitoxantrone (Mxt) on histone H2AX phosphorylation and induction of apoptosis in HL-60 cells. The cells were untreated (0 h) or treated with 150 nM Tpt (top panels) or 0.2 μ M Mxt (bottom panels) for 1, 3, or 4 h. γ H2AX IF along with DNA content was measured in the untreated cells and 1 h after administration of Tpt or Mxt, by flow cytometry. Apoptotic cells were identified as the cells expressing activated caspase-3 (77) or by the TUNEL assay (78) in the cultures treated with Tpt or Mxt for 3 or 4 h, respectively. The skewed dashed lines represents the upper level of γ H2AX expression for 97% of the cells from the untreated (0 h) culture, representing constitutive H2AX phosphorylation (47,63). Based on differences in DNA content, the cells in G₁, S, and G₂M phases of the cell cycle were identified as shown in the left and right panels. The DNA content frequency histograms of the untreated cells are presented as an inset in the left panels. The plots show an increase (Δ) in mean γ H2AX IF, estimated by gating analysis for G₁, S, and G₂M cell populations, as a function of treatment with different concentration of Tpt or Mxt for 1 h (32,79).

Figure 4.

Induction of ATM activation and H2AX phosphorylation in A549 cells treated with Tpt. Untreated (Ctrl, Panels **A**, **C**, **E**) and Tpt-treated (150 nM, 1 h, Panels **B**, **D**, **F**) A549 cells were subjected to immuno-staining to differentially label γ H2AX and ATM-S1981^P using Alexa Fluor 488 and Alexa Fluor 670 Abs, respectively. Cellular DNA was counterstained with DAPI; the emitted blue, green, and far red fluorescence was measured by three-laser scanning cytometry as described (77,79). As explained in the text, by gating analysis the cells expressing γ H2AX were colored red. The regions in Panel F represent the areas on the scatterplot locating the cells that are ATM-S1981^P positive and γ H2AX negative (*a*), ATM-S1981^P positive and γ H2AX positive (*b*), ATM-S1981^P negative and γ H2AX positive (*c*), and ATM-S1981^P negative and γ H2AX negative (*d*).

Figure 5.

Effect of treatment of TK6 cells with NO-ASA in the absence and presence of NAC, and with NAC alone, on ATM activation and phosphorylation of H2AX. Bivariate distributions representing cellular DNA content versus γ H2AX IF or versus ATM-S1981^P IF of TK6 cells untreated (Ctrl), treated with 5 μ M of NO-ASA alone, or with 5 μ M of NO-ASA in the presence of 50 mM NAC. NAC was included 20 min prior to administration of NO-ASA; exposure to NO-ASA or NO-ASA plus NAC was for 1 h. The skewed dashed line represents the upper level of γ H2AX expression for 97% G₁ and S-phase cells from the untreated (Ctrl) culture, representing constitutive H2AX phosphorylation (47,48,63). Mitotic cells (M) express ATM-1981^P and γ H2AX constitutively and they are marked by the rectangular dashed line boundaries. The DNA content frequency histogram of the cells is shown in the inset in the left (Ctrl) panel. Note an induction of ATM-S1981^P and γ H2AX primarily in S-phase cells after treatment with NO-ASA, and attenuation of this effect in the presence of NAC.

Figure 6.

Effect of treatment of HL-60 cells with aphidicolin (Aph) or hydroxyurea (Hxu) on ATM activation and H2AX phosphorylation. Bivariate (ATM-S1981^P IF or γ H2AX IF vs. DNA content) distributions representing untreated cells (Ctrl) and cells treated with 0.5 mM Hxu for 2 h, or with 4 μ M Aph for 2 h (23). Subpopulations of cells in G₁, S, and G₂M can be distinguished based on differences in DNA content, as shown in the respective left panels. Apoptotic cells (Ap), identified by fluorescence microscopy (23), are characterized by markedly increased ATM-S1981^P as well as γ H2AX IF; they are located within the oval dashed gates. The dashed-line thresholds show the upper limit of ATM-S1981^P or H2AX IF for 97% of the cells from the untreated (Ctrl) cultures, respectively. A DNA content histogram of the untreated cells is shown in the Ctrl γ H2AX versus DNA content panel as an inset.

Figure 7.

Induction of ATM activation and H2AX phosphorylation by exposure of cells to cigarette smoke. A549 cells were “mock” treated (Ctrl) or exposed to cigarette smoke for 5 or 20 min, as described (35,90). The cells were then incubated in culture for 1 h, fixed and immunostained for ATM-S1981^P or γH2AX; DNA was counterstained with DAPI, cellular fluorescence was measured by laser scanning cytometry (LSC). The dashed-line thresholds show the upper limit of ATM-S1981^P or γH2AX IF for 97% of the cells from the mock-treated (Ctrl) cultures, respectively. The inset in the left panel shows the DNA content histogram of the untreated cells prior to exposure; cell treatment with smoke followed by 1 h incubation did not significantly affect the cell cycle distribution (not shown).

Figure 8.

Kinetics of cell cycle progression revealed by UV-induced photolysis of DNA containing incorporated BrdU and reported by H2AX phosphorylation in relation to cell cycle phase. Expression of γ H2AX in HeLa cells pulse-labeled with BrdU and then incubated for 2, 8, or 12 h prior to UV irradiation; left panel shows BrdU labeled but not irradiated cells. Following UV irradiation, the cells were cultured for an additional 1 h, then fixed, their γ H2AX was detected immunocytochemically, the DNA was counterstained with DAPI, and cellular fluorescence was measured by laser scanning cytometer, as described (102). Insets show DNA content frequency histograms from the respective cultures. Note that after 8 h the BrdU-labeled cells are predominantly in G₂M and the very first labeled cells already appear in G₁. The frequency of BrdU-labeled cells in G₁ is distinctly higher 12 h after pulse-labeling with BrdU compared with 8 h, while fewer labeled cells remain in G₂M.

Figure 9.

Different levels of H2AX phosphorylation and ATM activation in non-apoptotic and apoptotic HL-60 cells treated with the DNA polymerase- α inhibitor aphidicolin. HL-60 cells were untreated (Ctrl) or treated with 4 μ M aphidicolin (Aph) for 2 h in culture. The bivariate distributions show expression of γ H2AX or ATM-S1981^P versus DNA content, respectively. The replication stress caused by Aph results in H2AX phosphorylation in the absence of ATM activation (23). The Aph-induced γ H2AX expression is primarily limited to the S-phase cells, with the early-S cells most affected. The cells marked *enS* most likely were entering S phase (initiating DNA replication) during the 2-h interval after administration of Aph. The dashed-line thresholds show the upper limit of γ H2AX or ATM-S1981^P IF for 95% of the cells from the untreated (Ctrl) cultures, respectively. Treatment with Aph induces apoptosis. Apoptotic cells (Ap) are characterized by markedly higher γ H2AX and ATM-S1981^P IF compared with nonapoptotic cells. The inset shows a DNA content frequency histogram from the untreated culture; the 2-h treatment with Aph had minimal effect on the DNA content distribution (not shown; see Ref. 23).

Figure 10.

Correlation between induction of γ H2AX, caspase-3 activation and cell cycle position in HL-60 cells treated with Tpt for 1 or 3h. The respective scatterplots show the bivariate distributions of γ H2AX IF versus cellular DNA content, or versus expression of activated caspase-3 (caspase-3*). Each of these parameters was measured using fluorochromes of different color (γ H2AX, long-red; DNA, blue; caspase-3*, green) excited by different lasers; fluorescence was measured by LSC, as described (32,109). IF of γ H2AX and of caspase-3* is presented as maximal pixel (MP). The thresholds (dashed skewed lines) represent the upper level of γ H2AX IF for 95% of the untreated (Ctrl) cells. Note that apoptotic cells (Ap) seen after treatment with Tpt for 3 h are characterized by very high expression of γ H2AX and also by expression of activated caspase. The cells with primary DSBs induced by Tpt (prDSBs) show elevated expression of γ H2AX but no caspase-3 activation.