DNA damage detected with γH2AX in endometrioid adenocarcinoma cell lines

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Abstract. Phosphorylation of histone H2AX (γH2AX) is a sensitive marker of DNA damage, particularly induction of DNA double-strand breaks. Using multiparameter cytometry we explored the effects of doxorubicin (DOX), cisplatin (CDDP) and 5-fluorouracil (5-FU) on four types of endometrioid adenocarcinoma cell lines (HEC-1A, HEC-1B, Ishikawa, KLE) correlating the drug-induced increases in phosphorylated H2AX (γH2AX) with cell cycle phase, induction of apoptosis and induction of cell senescence, the latter detected by analysis of β-galactosidase. The study revealed significant differences among the cell lines in the effects of DNA damage vis-à-vis cell cycle phase specificity, induction of apoptosis or senescence following drug treatment. DOX treatment showed little cell cycle specificity in terms of induction of γH2AX, and its mechanism, which is similar to another anthracycline DNA topoisomerase II inhibitor mitoxantrone, may involve oxidative DNA damage modulated by other factors. Treatment with CDDP and 5-FU led to phosphorylation of H2AX preferentially in S-phase cells, consistent with the induction of replication stress. The response of Ishikawa cells expressing wt p53 was different compared to other cell lines. The data suggest that the treatment of endometrioid adenocarcinoma with these drugs may have to be customized to individual patients. The flow cytometric bivariate analysis of γH2AX and DNA content is a useful technique for better understanding the effects of antitumor agents and may contribute to customized patient treatments.

Introduction

The incidence of endometrioid adenocarcinoma, one of the common malignant tumors in gynecology, has significantly increased in parallel with the increasing number of women who are obese or nulliparous. In Japan, the current incidence of this cancer is over 4-fold higher than it was 20 years ago (1). Depending on the clinical stage of endometrioid adenocarcinoma, treatment involves either surgery alone or surgery followed by chemo- or radiation therapy. Chemotherapy, which is performed on 33.7% of all cases post-surgery in Japan (2), plays an important role, and many anticancer drugs found to be effective target DNA and induce DNA damage. However, the detailed mechanism of DNA damage and DNA damage response induced by these drugs remains to be elucidated.

DNA damage in individual cells has been detected by a single-cell DNA gel electrophoresis technique (comet assay), in which the extent and length of the comet's tail reports the severity of DNA damage (3). Recently, however, it has become apparent that phosphorylation of histone H2AX, one of the variants of the nucleosome core histone H2A, can provide a sensitive and reliable marker of DNA damage. Namely, DNA damage, particularly when it involves formation of DNA double-strand breaks (DSBs), induces phosphorylation of histone H2AX on Ser-139; phosphorylated H2AX is defined as γH2AX (4). The phosphorylation takes place on H2AX molecules on both sides of DSBs along a megabase length of DNA. (3) Although DSBs generated during DNA fragmentation in the course of apoptosis also induce γH2AX, the degree of γH2AX induction in apoptotic cells is much greater compared to the primary DSBs induced by antitumor drugs or radiation (1,5,6). The presence of γH2AX in the cells can be detected immunocytochemically in the form of distinct nuclear γH2AX immunofluorescent foci and each focus is considered to correspond to a single DSB (7,8). This immunocytochemical approach made it possible to assay DNA damage and repair in situ, in the chromatin of individual cells (9). Compared to the comet assay, the immunocytochemical approach is significantly more sensitive (6). The use of multiparameter flow cytometry in measurements of γH2AX immunofluorescence enables one to correlate DNA...
damage with cellular DNA content and, therefore, cell cycle phase. Determination of the cell cycle phase targeted by the drug is of importance in elucidation of the mechanism of the anticancer drug activity.

In the present study, we examined the effects of doxorubicin (DOX), cisplatin (CDDP) and 5-fluorouracil (5-FU), drugs commonly used to treat endometrioid adenocarcinoma (10,11), on four different cell lines of this cancer. Each of these drugs has been found to be effective in >20% of endometrioid adenocarcinoma cases (2).

Material and methods

Cell culture and drug treatments. We used four cell lines of endometrioid adenocarcinoma. HEC-1A and HEC-1B were obtained from Health Science Research Resources Bank, Osaka, Japan, and were grown in dishes (Becton Drive, Franklin Lakes, NJ) in Dulbecco’s minimum essential medium supplemented with 10% fetal bovine serum (Cambrex Bio Science Walkersville Inc., Walkersville, MD). Ishikawa cells was obtained from the European Collection of Cell Cultures and grown in minimal essential medium (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM glutamine (Sigma), 1% non-essential amino acids (Sigma) and 5% fetal bovine serum. KLE was obtained from DS-Pharma Biomedical Co., Osaka, Japan, and grown in 45% F12 and 45% Dulbecco’s modified Eagle’s media (Sigma) supplemented with 10% fetal bovine serum. The media for the four cell lines were supplemented with 100 U/ml penicillin (Meiji Seika Kaisha, Ltd., Tokyo, Japan) and 100 μg/ml streptomycin (Meiji Seika Kaisha Ltd., Tokyo, Japan). All the cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. The cultures were treated with DOX (Sigma), CDDP (Sigma) or 5-FU (Sigma) for different time intervals. DOX, CDDP and 5-FU were dissolved in PBS and resuspended in 100 μl of 1:20 diluted FITC-conjugated F(ab’₂)² fragment of goat anti-mouse immunoglobulin (Dako, Glostrup, Denmark) for 30 min at room temperature in the dark. The cells were then counterstained with 5 μg/ml propidium iodide (PI) (Sigma) in the presence of 100 μg/ml of RNaseA (Sigma) for 30 min. The expression of p53 (DO-7, Dako, 1:100) and p21WAF1/CIP1 (DCS60, Cell Signaling Technology, Inc., Danvers, MA, 1:100) were determined in the same way as γH2AX immunostaining.

Fluorescence measurements by flow cytometry. The FITC (green) and PI (red) fluorescence of individual cells in suspension induced by excitation with a 488 nm argon ion laser was measured using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The green and red fluorescence from each cell was separated and quantified using standard optics and Cell Quest software (Becton-Dickinson). Ten thousand cells were measured per sample. All experiments were repeated at least three times.

Senescence-associated β-galactosidase staining. Senescence-associated ß-galactosidase staining was performed at pH 6 in cultured cells with the senescence staining kit (Cell Signaling Technology Inc.) following the manufacturer’s recommendations (12). The kit detects ß-galactosidase activity at pH 6, which is present only in senescent cells. Percentages of senescence-associated ß-galactosidase-positive cells, which show development of blue color in their cytoplasm, were determined for each sample using a bright-field microscope.

Results

Induction of γH2AX, apoptosis and cell cycle effects after treatment with DOX. As is evident from the bivariate (DNA content vs. γH2AX) distributions and DNA content frequency histograms (Fig. 1), exposure to 0.1 μg/ml DOX led to induction of H2AX phosphorylation, perturbation of cell cycle progression and apoptosis of cells in all four endometrioid adenocarcinoma cell lines. The increase in H2AX phosphorylation was apparent by the presence of cells with the γH2AX level above that of the control cells. Apoptotic cells could be discriminated on the respective scattergrams or DNA content frequency histograms as cells with decreased DNA content (sub-G₁ cell population). Cell cycle perturbations were reflected by the characteristic changes in cellular DNA content frequency histograms. The DOX-induced increase in γH2AX was not cell cycle phase-specific since it affected, to an approximately similar degree, G₁, S, and G₂M cells. Among the endometrioid cell lines the most sensitive appeared to be HEC-1A, which showed elevated expression of γH2AX, distinctly above that of the maximal control level, after 3 h of treatment with DOX. Apoptosis was also most pronounced in HEC-1A cultures, with the distinct presence of the sub-G₁ cell population at 72, 96 and 120 h.

Perturbation of the cell cycle progression in DOX-treated cultures manifested as accumulation of cells in S and G₂M phases of the cell cycle, maximally expressed in the HEC-1A cell line after 24 h. Prolonged treatment with DOX (48 h) led to prominent arrest of cells in G₂/M. Although a portion of the HEC-1B and Ishikawa cells gradually underwent apoptosis in G₂/M phase, a distinct G₂/M phase population with marked increase in γH2AX was seen in each histogram.

We also monitored the morphological changes of the cells after treatment with DOX. Fig. 2 demonstrates the changes in HEC-1B. There was a gradual increase in the frequency of distinct γH2AX immunofluorescent foci and progressive enlargement of the nuclei. After 120 h, nuclear fragments with intense γH2AX immunofluorescence and apoptotic bodies
were intermingled with enlarged nuclei containing densely stained γH2AX foci. These findings indicated on the presence of large apoptotic cells population mixed with a minor surviving population of cells having significantly damaged DNA.

**Induction of γH2AX, apoptosis and cell cycle effects after treatment with CDDP.** All four types of the endometrioid adenocarcinoma cell lines responded to treatment with 10 μg/ml CDDP by significant increases in the level of phosphorylated H2AX, perturbation of cell cycle progression, and apoptosis. Interestingly, however, unlike following treatment with DOX which induced H2AX phosphorylation to a similar degree in all phases of the cell cycle (Fig. 1), the treatment with CDDP preferentially affected S-phase cells. This is clearly evident from the horseshoe shape of the γH2AX distribution which was evident in all four cell lines (Fig. 3). The timing and intensity of H2AX phosphorylation varied between the cell lines, with KLE cells responding most rapidly, showing distinct elevation of γH2AX level following only 3 h of treatment. The maximal intensity of H2AX phosphorylation was seen in Ishikawa cells at 72-120 h.

The time of induction of apoptosis also varied between the cell lines. A distinct subpopulation of apoptotic cells was already apparent after 24 h of treatment of HEC-1A cells, after 48 h treatment of KLE cells and after 96 h treatment of HEC-1B and Ishikawa cells. Essentially no live cells were seen after 120 h of treatment among the cultures of all cell lines.
Figure 3. Bivariate distributions (DNA content vs. γH2AX) of endometrioid adenocarcinoma cell lines, HEC-1A, HEC-1B, Ishikawa and KLE treated with 10 μg/ml CDDP for time intervals as indicated. The solid-skewed lines indicate the upper level of γH2AX immunofluorescence for 95% of cells in the untreated (control) culture. All four types of cells demonstrated elevation of γH2AX through the cell cycle 3 h after the start of treatment. While HEC-1A, B and KLE showed increased γH2AX followed by increase levels of apoptosis, Ishikawa cells exhibited S-phase progression with significantly increased γH2AX and followed by apoptosis. Ishikawa culture also contained a viable population with modest increase in γH2AX in early S phase after 72 h.

Figure 4. Bivariate distributions (DNA content vs. γH2AX) of endometrioid adenocarcinoma cell lines, HEC-1A, HEC-1B, Ishikawa and KLE treated with 3 μg/ml 5-FU for time intervals as indicated. The solid-skewed lines indicate the upper level of γH2AX immunofluorescence for 95% of cells in the untreated (control) culture. 5-FU showed a significant increase in γH2AX followed by apoptosis only in Ishikawa cultures: the other three cell lines do not show significant changes except for mild elevation of γH2AX.
Changes in the cell cycle progression manifested as an accumulation of cells in S phase, and were most pronounced in KLE cells after 24 h and in HEC-1B and Ishikawa cells after 48 h. In contrast, there was no evidence of S-phase arrest in the HEC-1A cell line as these cells more rapidly underwent apoptosis.

Induction of γH2AX, apoptosis and cell cycle effects after treatment with 5-FU. Treatment with 3 μg/ml of 5-FU induced modest increases in γH2AX in late-S to G2M phases in HEC-1A, S phase in HEC-1B and early-S phase in KLE cells, respectively (Fig. 4). Extensive apoptosis that can be recognized by the presence of sub-G1 cells did not occur in these three cell lines. However, Ishikawa cells showed dramatic increases in γH2AX throughout the cell cycle at 24 h. After 72 h, although a part of Ishikawa cell population underwent apoptosis, the majority expressed a marked increase in γH2AX (Fig. 4). It is likely that cells with such high levels of expression of γH2AX were undergoing apoptosis and additional phosphorylation of H2AX was induced by apoptosis-associated DNA fragmentation. The appearance of the sub-G1 cells in Ishikawa cell culture was apparent following 96 and 120 h of treatment with 5-FU (Fig. 4).

Detection of senescence-associated β-galactosidase after treatment with DOX. Since viable cell populations that showed arrests in the cell cycle with marked increase in γH2AX were seen in HEC-1B and Ishikawa cell cultures treated with DOX for 120 h, we explored whether those cells were undergoing senescence (Fig. 5). In the untreated HEC-1B and Ishikawa culture cells 30% and 0% cells were β-galactosidase-positive, respectively. After the treatment with 0.1 μg/ml DOX for 120 h, among the cells that remained in the HEC-1B and Ishikawa culture cells 30% and 0% cells were β-galactosidase-positive, respectively. After the treatment with 0.1 μg/ml DOX for 120 h, among the cells that remained in the HEC-1B and Ishikawa cultures, 100% cells were β-galactosidase-positive. These data thus confirmed that treatment of cells of HEC-1B and Ishikawa lines with DOX for 120 h induces cell senescence in at least a portion of the population.

Expression of the cell cycle-related proteins after treatment with CDDP or 5-FU. Since the presence of wild-type p53 may have affected the response of Ishikawa cells to CDDP or 5-FU,
we studied changes of expression of p53 and p21WAF1/CIP1 before and after drug treatment. Following exposure to 10 μg/ml CDDP for 72 h, expression of p53 was slightly increased while expression of p21WAF1/CIP1 increased significantly (data not shown). Treatment of Ishikawa cells with 3 mg/ml 5-FU for 24 h, resulted in increased expression of both p53, and p21WAF1/CIP1 compared to that seen in untreated cells (Fig. 6). The other three cell lines showed no changes in p53 or p21WAF1/CIP1 after treatment with 5-FU (data not shown).

Discussion

The induction of γH2AX by DOX, CDDP or 5-FU, which was measured in relation to the cell cycle phase by cytometry, and visualized on cell images as the presence of immunofluorescent foci, very likely represents DNA damage that involves formation of DSBs caused by these drugs. Each γH2AX focus is considered to correspond to a single DSB (7,13). The DOX-induced morphological changes were characterized by enlargement of HEC-1B cell nuclei and an increase in the frequency of γH2AX foci, as shown in Fig. 2. Nuclear chromatin present in apoptotic bodies or in fragmented nuclei of apoptotic cells was very distinctive due to the highly intense immunofluorescence of γH2AX reflecting coalescence of the foci. This is consistent with our earlier observations that DNA fragmentation during apoptosis leads to very extensive phosphorylation of H2AX (5.6).

DOX is DNA topoisomerase II (topo2) inhibitor of the anthracycline family. The mechanism of its cytotoxicity is considered to involve generation of reactive oxidative species (ROS) mediated by poly(ADP-ribose)polymerase and NAD(P)H oxidase activation rather than inhibition of topo2 (14). The site-specific oxidative DNA damage induced by DOX in the presence of copper (II) appears to be the type of lesion responsible for inducing apoptosis (14). The present data demonstrated that exposure of cells to DOX led to the increase in γH2AX in all phases of the cell cycle, in each of the four cell lines studied. These results agree, with our prior findings that another topo2 inhibitor of the anthracycline family, mitoxantrone also induced H2AX phosphorylation indiscriminately, in all phases of the cell cycle (5.6). Involvement of the oxidative mechanism in DNA damage induced by mitoxantrone was confirmed by the observation that H2AX phosphorylation induced by mitoxantrone was distinctly attenuated by the ROS scavenger N-acetyl-L-cysteine (15). This is in contrast to the top1 inhibitor topotecan which was shown to selectively induce H2AX phosphorylation in S-phase cells, having little effect on cells in G1 or G2/M phase (5.6). It should be noted however, that exposure of cells to exogenous oxidants such as H2O2 preferentially induced H2AX phosphorylation and ATM activation in S-phase cells (16). Thus, it appears that in addition to the mechanism of oxidative DNA damage caused by topo2 inhibitors there are other factors modulating the DNA damage response. Several groups reported that inhibiting DOX-induced intracellular oxidative stress by the overexpression of antioxidant enzymes prevented apoptosis in tumor cells (17), and that depleting endogenous antioxidants (e.g. glutathione) made tumor cells more susceptible to DOX (18,19). However, there are other studies that did not support the role of oxidative stress as the sole factor responsible for tumor cell apoptosis induced by DOX (20,21).

A distinct population with marked increase in γH2AX in G2M phase seen in HEC-1B and Ishikawa cells appeared to have undergone senescence. These cells had markedly enlarged nuclei and expressed β-galactosidase. It is likely that these cells were reproductively dead and unable to proliferate. Thus, it appears that, depending on cell type, treatment with DOX either led to induction of apoptosis and/or cell senescence.

CDDP is a well-known DNA-damaging agent, and it is currently thought that DNA platination is an essential first step in its cytotoxic activity. It is generally accepted that cytotoxic effects of CDDP can be ascribed to its interaction with nucleophilic N7-sites of purine bases in DNA to form DNA-DNA intra-strand and inter-strand cross-links and DNA-protein bonds. The most common adducts are 1,2-intra-strand cross-links between adjacent guanines. Eventual apoptosis has been shown to be the key cellular event responsible for the anticancer activity of CDDP (22,23). An important mediator of CDDP-provoked apoptosis is the tumor suppressor protein p53, which possess a dual role in stress response. p53 has been reported to be capable of arresting cells at the G1/S- and possibly G2/M-checkpoints after which it is involved in mediating their DNA repair, or initiating apoptosis if damage is irreparable (24,25). However, inactivation of p53 may contribute to hypersensitivity as well as chemotherapeutical resistance toward CDDP depending on the type of tumor cell type involved; p53 is not always required for apoptosis (26). In the present study, all four cell lines treated with CDDP showed DNA damage initially in S-phase cells (Fig. 3), which were also evident as distinct γH2AX foci seen by immunocytochemical staining (data not shown). The alteration of DNA structures induced by CDDP is considered to cause replication stress and stalling of DNA replication forks (27). Such DNA lesions are expected to induce apoptosis. Indeed extensive apoptosis was observed, manifesting in the case of HEC-1A and KLE cells as the presence of sub-G1 cells after 48 h of treatment, and in the case of HEC-1B and Ishikawa cells as cells with very high expression of γH2AX (Fig. 3). As mentioned before, these high levels of γH2AX most likely represent increased sites of DNA DSBs created during the process of apoptosis subsequent to the primary DNA damage caused by the anti-tumor agent (5.6).

Since Ishikawa cells possess wild-type p53, their response to CDDP may be mediated by this transcriptional factor. This prompted us to study possible downstream p53 effects, namely expression of p21WAF1/CIP1. Although the expression of p53 was slightly increased after the treatment with CDDP for 72 h, that of p21WAF1/CIP1 was significantly increased (data not shown). p21WAF1/CIP1, a CDK inhibitor, is known to negatively regulate cell cycle progression and is usually induced by p53. However transforming growth factor-β also induces p21WAF1/CIP1, and the induction of p21WAF1/CIP1 in many malignancies has been shown to be p53-independent (28-30). The response seen in Ishikawa might at least in part be mediated by p21WAF1/CIP1.

A potent antitumor agent, 5-FU affects pyrimidine synthesis by inhibiting thymidylate synthetase thus depleting intracellular dTTP pools. It is metabolized to ribonucleotides
and deoxyribonucleotides, which can be incorporated into RNA and DNA. Treatment of cells with 5-FU leads to an accumulation of cells in S phase and has been shown to induce p53-dependent apoptosis in a colon cancer cell line (31). This drug also induced phosphorylation of H2AX preferentially in S-phase cells, consistent with the DNA damage response caused by replication stress (27).

Interestingly, whereas the 5-FU-induced increase in γH2AX in HEC-1A, HEC-1B and KLE cells was relatively modest, it was very high in Ishikawa cells. Also while Ishikawa cells were undergoing apoptosis, seen as both the dramatic rise in γH2AX and appearance of sub-G 1 cells, the other three cell lines showed little evidence of apoptosis. To ascertain whether the response of Ishikawa cells to 5-FU was mediated by wild-type p53 we also measured the effect of this drug on expression of p53 and its downstream effector p21WAF1/CIP1 (Fig. 6). The data show activation of p53 and induction of p21WAF1/CIP1, which is consistent with the notion that 5-FU is effective on uterine endometrioid adenocarcinoma through the p53 pathway and apoptosis is the mode of cell death.

In the present study, we conducted flow cytometric bivariate analysis of γH2AX and DNA content after treatment of four endometrioid adenocarcinoma cell lines with CDDP, DOX and 5-FU. This is the first report presenting analysis of DSBs induction vis-à-vis cell cycle phase in endometrioid adenocarcinoma cell lines following treatment with these common drugs. Since various pathways that effect cell cycle progression might be activated when DNA damage occurs (32,33), expression of p53 and p21WAF1/CIP1 were examined. We also examined the induction of senesence markers, because severe DNA damage might lead to cellular senescence. We demonstrated significant differences in the effects on DNA damage, cell cycle arrest and induction of apoptosis or senescence by CDDP, DOX and 5-FU, which are commonly used in treatments of endometrioid adenocarcinoma. These results suggest that the treatment may benefit by being customized to individual patients, particularly with respect to whether the tumor has mutated p53. Thus, the flow cytometric bivariate analysis of γH2AX and DNA content is a useful technique for better understanding the effects of antitumor agents that target DNA and may contribute to the possibility of customized treatments.

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References


