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The role of histone acetylation *versus* DNA damage in drug-induced senescence and apoptosis

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Abstract

The present study was undertaken to determine the significance of histone acetylation *versus* DNA damage in drug-induced irreversible growth arrest (senescence) and apoptosis. Cellular treatment with the DNA-damaging drugs doxorubicin and cisplatin or with the histone deacetylase inhibitor trichostatin A, led to the finding that all the three drugs induced senescence at concentrations significantly lower than those required for apoptosis. However, only doxorubicin and cisplatin induced activation of H2AX, a marker for double-strand break formation. Interestingly, this occurred mainly at apoptosis and not senescence-inducing drug concentrations, suggesting that non-DNA-damage pathways may be implicated in induction of senescence by these drugs. In agreement with this, chromatin immunoprecipitation experiments indicated that doxorubicin was able to induce acetylation of histone H3 at the promoter of p21/WAF1 only at senescence-inducing concentrations. Collectively, these findings suggest that alteration of chromatin structure by cytotoxic drugs may represent a key mediator of senescence.
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Abbreviations: DSBs, DNA double-strand breaks; SA-β-Gal, senescence-associated beta galactosidase; γ-H2AX, histone H2AX

Introduction

Proliferation arrest and cell death are the most described biological responses to anti-cancer drugs. During the last decade, the major focus of basic research was directed towards defining the molecular pathways of apoptosis and determination of their role in chemotherapy outcome, particularly since this form of cellular demise was believed to be a key factor in the development of drug resistance.[1] However, recent evidence indicates that the inhibition of apoptosis alone may not be sufficient for acquisition of the drug resistance phenotype[2–4] and that cancer cells not only have to be viable, but must be able to proliferate in order for the tumor to progress and develop a drug-resistant phenotype. Considering that cancer cell lines with deficiencies in the apoptotic pathway can still undergo proliferation arrest as a generalized response to drugs, achieving irreversible growth arrest would be sufficient for the control of cancer progression and the prevention of drug resistance. Therefore, adequate understanding of the molecular mechanism(s) responsible for chemotherapeutic agent-induced senescence could facilitate the development of novel strategies to prevent and/or to reverse drug resistance in cancer.

Earlier investigations stressed the concept that damage to DNA constituted the primary cause of proliferation arrest and senescence in response to various stimuli.[5] Upon exposure to cytotoxic drugs, cells are believed to slow down their proliferation to allow repair of damaged DNA. With the recent discovery that phosphorylation of the histone H2AX (γ-H2AX) is associated with drug and irradiation-induced DNA double-strand breaks (DSBs), this molecule has been proposed as a mediator of DNA-damage-induced proliferation arrest.[6–10] Although this association has been observed in various cases, it does not exclude the possibility that proliferation arrest can occur independently of DNA damage. Since non-DNA targeting drugs can also inhibit proliferation,[11–13] it is plausible that cellular processes other than DNA damage may activate signaling pathways leading to proliferation arrest.

Epigenetic alterations due to chromatin remodeling have been shown to play a key role in the control of cellular proliferation and response to chemotherapeutic agents.[14,15] As changes in chromatin conformation may affect the binding of transcription factors and repair enzymes to DNA, the possibility exists that, whether DNA is damaged or not, gene expression may not be allowed unless chromatin is in its active state.[16] Histone acetylation at the promoter region of tumor suppressor genes has been shown to induce their expression leading to the inhibition of cellular proliferation[17] often through the induction of the same antiproliferative pathways that are activated by DNA damage.[18] However, the question of whether chromatin remodeling and DNA damage occur concurrently or in response to different stress levels has not yet been adequately addressed.

In the present study, the relationship between DSB formation and drug-induced irreversible growth arrest and apoptosis has been investigated. Special emphasis was placed on the determination of the stress level required for the induction of each one of these cellular responses. The results demonstrated that drug-induced DSBs correlated with apoptosis and not cell proliferation arrest. In contrast, stress levels required for induction of histone acetylation correlated with the induction of cellular senescence. Our findings indicate

that drug concentrations that induce chromatin remodeling may be sufficient for the induction of senescence.

Results

Correlation between doxorubicin concentrations and the onset of proliferation arrest or cell death

The topoisomerase inhibitor, doxorubicin, also known to act by inducing DNA double-strand breaks[19,20] was utilized to define the relationship between DSB formation and the cellular responses to this drug. Incubation of the human neuroblastoma cell line SKN-SH cells with doxorubicin for up to 5 days resulted in proliferation arrest and/or cell death depending on the concentration used (Figure 1a). At concentrations less than 10^{-7} M, doxorubicin delayed cellular proliferation, and at 10^{-7} M, the cell number remained constant during the entire period of incubation suggesting irreversible growth arrest (senescence). In contrast, greater doxorubicin concentrations induced cell death.

Drug effect on the cell cycle was determined by flow cytometry (Figure 1b). The population of cells in the S-phase decreased, whereas those in the G2-M increased and reached a peak at 10^{-7} M doxorubicin. No further accumulation of cells in the G2-M phase was observed at higher concentrations, suggesting that proliferation of most, if not all cells, was suppressed at 10^{-7} M doxorubicin. This was associated with an increased activity of the senescence-associated beta galactosidase (SA-β-Gal) (Figure 1c), confirming that the cells were in senescence. At a higher doxorubicin concentration (10^{-6} M), the cytotoxic response was apoptotic in nature as demonstrated by DNA fragmentation (Figure 1d). These findings and others from our laboratory[3] provide evidence that senescence and apoptosis are induced at distinct stress levels.

Molecular determinants associated with doxorubicin-induced senescence and apoptosis in SKN-SH cells

The expression of molecular markers associated with doxorubicin-induced senescence (p21/WAF1) and apoptosis (activated caspase-3) were compared with the induction of DSBs detected by activation of H2AX (γ-H2AX). As shown in Figure 2a, expression of p21/WAF1 was maximal at 10^{-7} M doxorubicin followed by a sharp decrease starting at 5×10^{-7} M. Expression of p21/WAF1 correlated with activation of p53 at low drug concentrations but not at higher ones. However, in contrast to p21/WAF1, caspase-3 activation was detected at drug concentrations starting at 5×10^{-7} M and continued through 10^{-6} M. Of note, p53 was also detected at

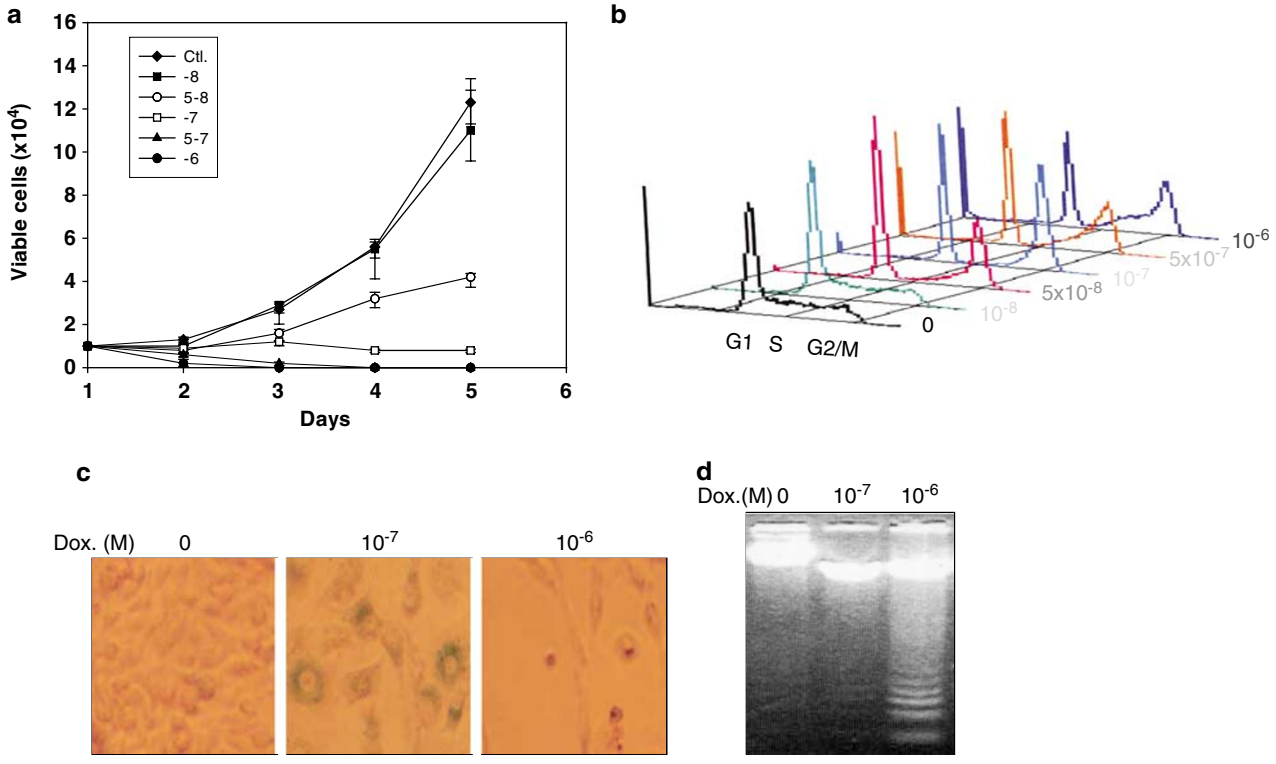

Figure 1 Induction of senescence and apoptosis by doxorubicin in SKN-SH cells. (**a**) Cell viability assay in response to the indicated doxorubicin concentrations was measured by MTT as described in the methods section. Data represent means of six determinations $\pm$S.E. (**b**) Flow cytometry analysis of doxorubicin-induced cell-cycle arrest. Cells were subjected to treatment with doxorubicin at the indicated concentrations (M) for 24 h, then fixed and stained with propidium iodide, and analyzed for DNA content. Cells in the G1, S, and G2-M phases of the cell cycle are represented in the three major peaks of this diagram. (**c**) Cytotoxic responses associated with low and high drug concentrations. Cells incubated with 10^{-7} and 10^{-6} M doxorubicin for 5 days were stained for SA-b-Gal activity to detect senescence. (**d**) DNA fragmentation indicative of apoptosis was also determined after 24 h of treatment with these two drug concentrations. Data are representative of three independent experiments

these later drug concentrations supporting its implication in both senescence and apoptosis. These data suggested that expression of p21/WAF1 and activation of caspase-3 are elements of two independent response pathways. A possible explanation for the inverse relationship between these two events could be that p21/WAF1 is a putative substrate for caspase-3, as has been demonstrated previously.[21] However, examination of p21/WAF1 expression by RT-PCR (Figure 2b) indicated that although this may be the case at intermediate drug concentrations (such as 5×10^{-7} M doxorubicin), high drug concentrations seemed to exert an inhibitory effect p21/WAF1expression at the message level. Overall, the data shown in Figure 2a concur with those presented in Figure 1 indicating that doxorubicin induces primarily proliferation arrest up to 10^{-7} M and cell death at higher concentrations.

Expression of γ-H2AX increased in response to the treatment with doxorubicin (Figure 2a) confirming that this drug was able to induce DSBs. However, this marker was detected mainly at concentrations that correlated with caspase-3 activation suggesting that DSB formation was more closely associated with cell death than with proliferation arrest. Immunohistochemical analyses carried out to detect γ-H2AX in intact cells (Figure 2c) confirmed this observation and suggests that doxorubicin-induced DSBs may not play a major role for the induction of p21/WAF1 and proliferation arrest by this drug.

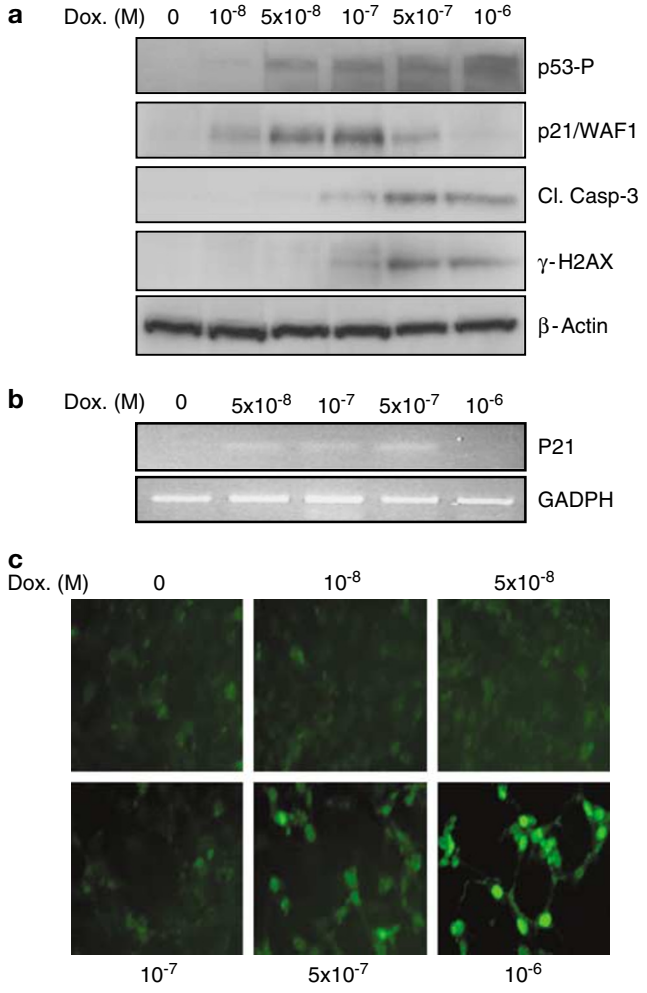

Figure 2 Doxorubicin-induced expression of p21/WAF1 and activation of H2AX and caspase-3. (**a**) SKN-SH cells were treated with doxorubicin at the indicated concentrations for 24 h. Expression of phosphorylated p53, p21/WAF1, activated (cleaved) caspase-3 (Cl. Casp-3), γ-H2AX and β-Actin was detected by Western blot. (**b**) RT-PCR expression of p21/WAF1 upon exposure of cells to increasing amounts of doxorubicin using GGC AGA CCA GCA TGA CAG ATT T and GGC GGA TTA GGG CTT CCT CT as forward and reverse primers. (**c**) Expression of γ-H2AX in intact cells. Cells grown on coverslips were treated for 24 h with doxorubicin and γ-H2AX was detected on fixed cells using a specific antibody. Data are representative of at least three independent experiments

Effect of doxorubicin on DNA integrity determined by the comet assay

The apparent lack of association between expression of p21/WAF1 and activation of H2AX prompted the utilization of another analytical procedure to determine the effect of doxorubicin on DNA damage. The comet assay has been used to measure both single-strand breaks (SSBs) and DSBs.[22] In this assay, doxorubicin-treated cells were electrophoresed in agarose gel and the damaged DNA that leaked from the nucleus can be visualized with the DNA intercalating agent, SyberGreen, as a fluorescent comet. Data presented in Figure 3a indicate that cells treated with up to 10^{-7} M

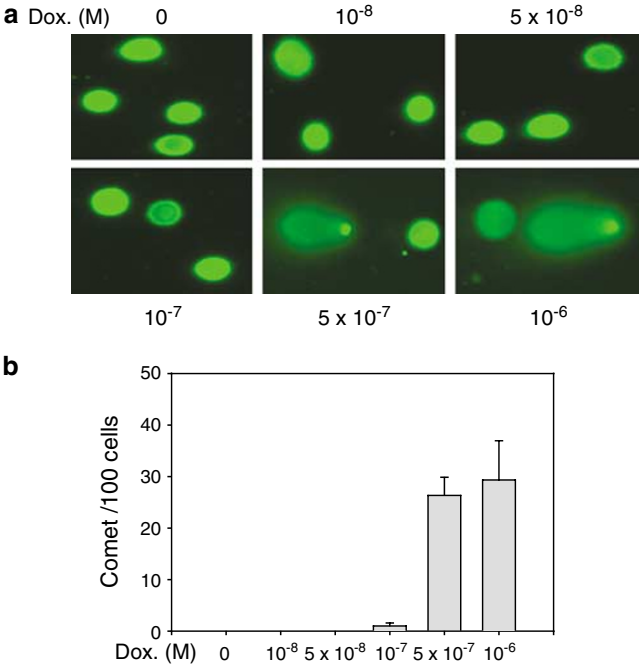

Figure 3 Measure of doxorubicin-induced DNA damage by comet assay. (**a**) SKN S-H cells were treated with doxorubicin for 24 h. Single cells were then electrophoresed in agarose gel on a coverslip and stained with SyberGreen as described in the Methods section. Labeled DNA was visualized under fluorescence microscope. Cells with damaged DNA displayed a comet. (**b**) The number of comets per 100 cells were counted and represented as a function of drug concentration. Data represent means of three independent determinations $\pm$ S.E.

doxorubicin displayed a round and well-defined staining of DNA contained within the nucleus. In the presence of 5×10^{-7} M doxorubicin or higher concentrations, treated cells had a comet-like staining indicating that DNA has been damaged. Interestingly, not more than 30% of cells treated with high doxorubicin concentrations displayed the comet pattern (Figure 3b). The later finding suggests that these types of DNA damage may not be the only cause of cell death and that induction of apoptosis in a DNA damage-independent manner may also account for cell killing. Nevertheless, the results presented demonstrated that doxorubicin-induced SSBs and/or DSBs were detected only at drug concentrations greater than those required for proliferation arrest.

Validation for other drugs and cell types

Cisplatin, a widely known DNA alkylating drug and inducer of DSBs,[23] was used to verify whether the findings described above, applied to other DNA-damaging drugs. As shown in Figure 4a, expression profiles of p21/WAF1, cleaved caspase-3 and activated H2AX in response to cisplatin in SKN-SH cells were very similar to those induced by doxorubicin in the same cells (Figure 2a). Moreover, expression profiles of these markers in the human glioma cell line U251 with either doxorubicin (Figure 4b) or cisplatin (Figure 4c) were also identical to those obtained with SKN-SH in response to these drugs (Figures 2a and 4a) suggesting that this type of cellular response may occur with other drugs and in other cell lines.

Relationship between stress level and incubation time required for the induction of senescence DSBs, and apoptosis

Experiments were undertaken to determine whether prolonged incubation with a senescence-inducing doxorubicin concentration (10^{-7} M) could ultimately result in DSB formation. As shown in Figure 5a, in response to such treatment, the level of p21/WAF1 was sustained for up to 5 days and there was no detectable activation of H2AX during this incubation period. When the cells were subjected to an apoptosis-inducing doxorubicin concentration (10^{-6} M), a rapid activation of H2AX was observed, as early as 1 h, whereas that of caspase-3 was initiated between 6 and 24 h (Figure 5b). This finding suggested that activated H2AX may be an early event in the apoptotic pathway initiated by exposure to DNA-damaging drugs. Overall, these data further confirm the lack of association between DSBs and proliferation arrest and suggest that this type of damage may signal primarily for apoptotic cell death.

Effect of the non-DNA damaging drug Trichostatin A on proliferation arrest and apoptosis

As DSBs appeared to be unlikely mediators of doxorubicin or cisplatin induction of p21/WAF1 and cellular senescence at least in the cellular models used in this study, we asked the question of what could be the cause. Recent reports have shown that histone deacetylase inhibitors (HDACIs) induce senescence via activation of cell-cycle inhibitors such as p16/INK4.[24] We used a deacetylase inhibitor, trichostatin A (TSA), to determine its effect on cellular proliferation, the expression of p21WAF1 and the activation of caspase-3. As shown in Figure 6a, depending on the concentration used, TSA caused either proliferation arrest or cell death. This drug appeared to induce a G2/M cell-cycle arrest (Figure 6b) with features of senescence as shown by changes in cell morphology and activity of the SA-β-Gal (Figure 6b, insert). As TSA affects primarily the chromatin structure, no DNA double-strand breaks, represented by γ-H2AX, were detected even at high drug concentrations (Figure 6c). However, similar to doxorubicin and cisplatin, cellular treatment with TSA led to the activation of p53 and induction of p21/WAF1 followed by activation of caspase-3, suggesting that changes in chromatin structure induced by this drug may act as a signal for both cellular responses.

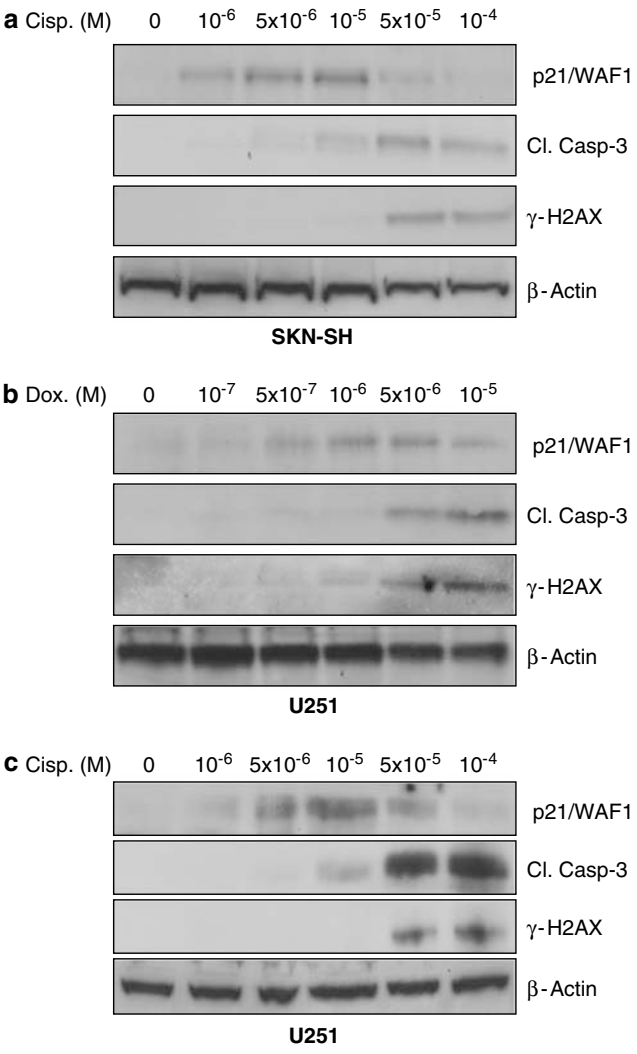

Figure 4 Effect of doxorubicin and cisplatin on expression of p21/WAF1, cleaved caspase-3 and γ-H2AX in neuroblastoma (SKN-SH) and glioma (U251) cell lines. SKN-SH cells were treated with cisplatin (**a**) and U251 cells were incubated with either doxorubicin (**b**) or cisplatin (**c**) for 24 h. Expression of the above molecular markers was detected by Western blot. Expression of β-Actin was used as gel-loading control

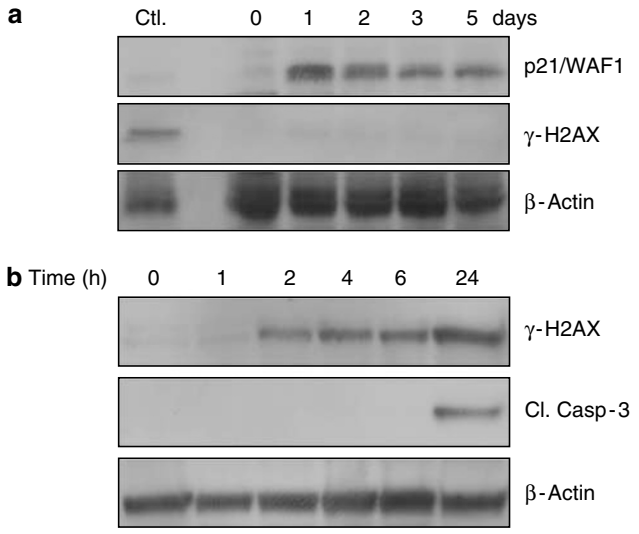

Figure 5 Kinetics of doxorubicin-induced expression of p21/WAF1 and activation of γ-H2AX and caspase-3. (**a**) SKN-SH cells were incubated with doxorubicin concentration (10^{-7} M) for up to 5 days. Expression of p21/WAF1 was compared to activation of H2AX by Western blot using specific antibodies. Ctl. Represents a positive control for activated H2AX. Expression of β-Actin was used as a loading control. (**b**) The cells were exposed to apoptosis-inducing drug concentration (10^{-6} M) for up to 24 h and expression of the above molecules was detected as in (a). Data are representative of three independent experiments

Correlation between doxorubicin-induced histone acetylation and p21/WAF1 expression

To determine whether low concentrations of doxorubicin that induce p21/WAF1 expression also affect chromatin structure, we examined the status of histone acetylation by using a polyclonal antibody that recognizes acetylated histone H3 at lysine 9. As shown in Figure 7a, there was a strong association between acetylation of this histone and p21/WAF1 expression. In contrast, these events were inversely correlated with activation of H2AX. This could be explained by the fact that if DSBs signal for caspase activation and apoptosis, histone acetylases may represent potential substrates for caspases.

To determine whether histone acetylation was altered at the promoter region of p21/WAF1, we utilized the chromatin immunoprecipitation assay (ChIp).[25] This procedure revealed that histone acetylation occurred essentially at drug concentrations affecting cellular proliferation but not cell death (Figure 7b). Even within the same promoter, the sequence surrounding -105 bp site appeared to be more sensitive to changes in stress levels than that surrounding the -2760 site. This supported other findings in this study (See Figure 7a) and suggested that doxorubicin-induced chromatin acetylation at the promoter region of p21/WAF1 may act to increase its expression and facilitate proliferation arrest.

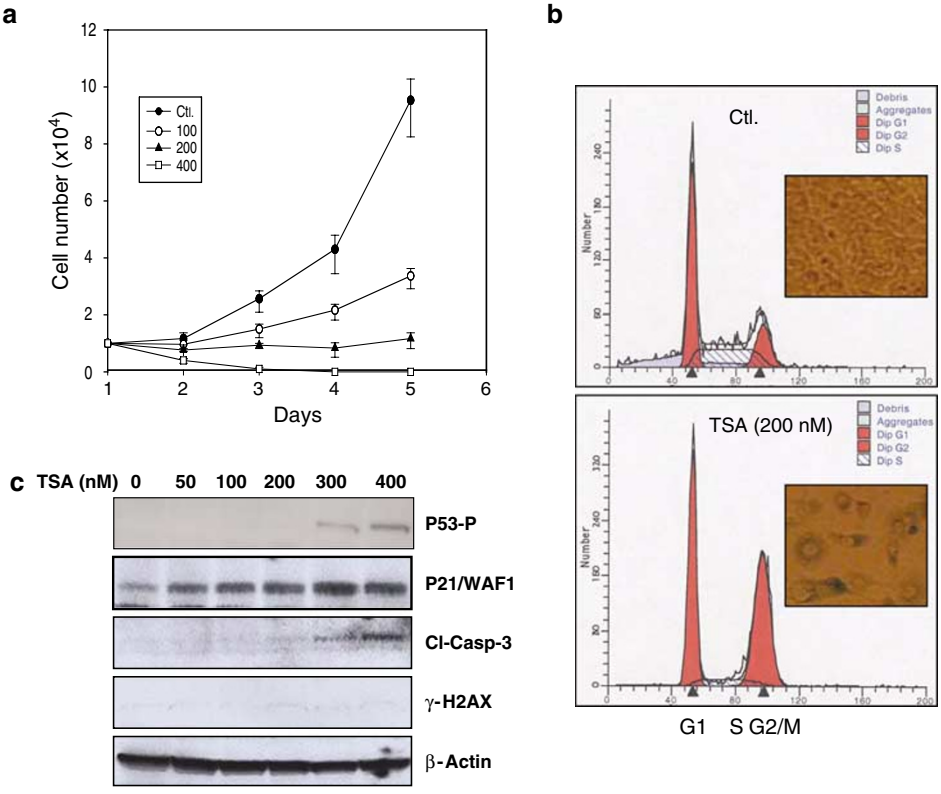

Figure 6 Induction of proliferation arrest and cell death by trichostatin A (TSA). (**a**) SKN-SH cells were treated with the indicated concentrations of TSA (nM) and cell number was counted for up to 5 days. (**b**) FACS analysis of the effect of TSA on cellular distribution in the cell cycle after 24 h incubation. The inserts show a representative senescence-associated beta galactosidase (SA-β-Gal) staining upon cellular incubation with or without TSA for 5 days. (**c**) Expression of activated P53 (p53-P), p21/WAF1, Cleaved caspase-3 (Cl-casp3) and γH2AX were determined by Western blot after treatment with doxorubicin for 24 h. The data is representative of three independent experiments

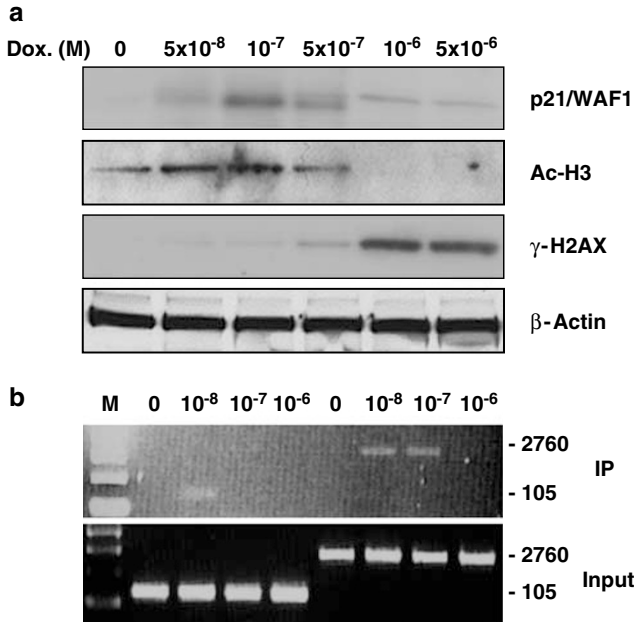

Figure 7 Effect of doxorubicin on histone acetylation. (**a**) SKN-SH cells were exposed to doxorubicin at the indicated concentrations for 24 h and expression of p21/WAF1, acetylated histone H3 (Ac-H3), γH2AX and β-actin were determined by Western blot. (**b**) Chromatin immunoprecipitation assay showing enhanced histone acetylation at the p21/WAF1 promoter region. After treatment with doxorubicin for 24 h, chromatin was immunoprecipitated and the associated DNA extracted and amplified with PCR primers corresponding to two different segments of the p21/WAF1 promoter (-105 and -2760 bp). The 'Input' indicates amplified DNA sequences before chromatin immunoprecipitation

Discussion

The role of senescence in cancer chemotherapy and particularly in the development of drug resistance has become increasingly evident, leading to interest in defining the mechanism(s) by which chemotherapeutic agents induce this response. DNA damage in response to cytotoxic drugs has been frequently described as the principal regulator of proliferation arrest, cellular senescence and apoptosis. However, recently, it has become recognized that changes in chromatin remodeling can induce similar responses in a DNA-damage independent manner. In the present study we have investigated the role of DNA damage *versus* histone acetylation in induction of senescence and the effect of drug dosage in targeting either histones or DNA. This has been addressed by comparing the occurrence of DNA damage, expression of markers of proliferation arrest and extent of cell death in response to DNA-damaging and non-damaging drugs.

The ability to distinguish between the stress levels required for senescence and apoptosis (Figures 1 and 2),[3,4] makes it possible to investigate whether DNA damage was part of the signaling pathway leading to one or the other of these cellular demises. If it associated with senescence, H2AX activation by a DNA-damaging drug must occur with lower or at least equivalent drug amounts to that required for the induction of p21/WAF1. The data presented in Figures 2 and 3 revealed that this was not the case and that activation of H2AX required much higher drug concentrations than those needed for the induction of p21/WAF1. Instead, activation of H2AX appeared to be associated with that of caspase-3 (Figures 2 and 4) suggesting that this molecule may represent an essential signal for apoptosis.[26] The rapid activation of H2AX, compared to caspase-3 in response to doxorubicin (Figure 5b) further confirmed this hypothesis and suggested that DSBs may represent an early event in drug-mediated apoptosis.

A significant finding of this investigation was that histone acetylation at the promoter region of p21/WAF1 may mediate doxorubicin-induced expression of this cell-cycle inhibitor and cellular senescence. In addition, as the histone deacetylase inhibitor TSA was able to induce senescence despite the absence of DNA damage (Figure 6), it is suggested that DNA-damaging drugs may induce senescence in a DNA damage-independent manner. Data presented in Figure 7a appeared to validate this hypothesis and indicated that histones were more sensitive to stress than DNA. Chromatin acetylation is known to result in its decondensation, thus facilitating access of transcription machinery to the promoter regions of tumor suppressor genes.[27] Chromatin immunoprecipitation results (Figure 7b) demonstrated that depending on the stress level, DNA damaging drugs can induce histone acetylation and induction of the tumor suppressor p21/WAF1 without significant induction of DNA damage.

In conclusion, the present study suggests that although treatment of cancer cells with chemotherapeutic agents elicited the generation of DSBs at higher concentrations, alteration in histone acetylation appeared to be the key mediator of proliferation arrest at low stress levels. We propose that depending on concentrations, the nature of drug targets may change and that histones may be more sensitive to low stress levels than DNA.

Materials and Methods

Reagents

Human neuroblastoma SKN-SH and glioma U251 cell lines were purchased from ATCC (ATCC, Rockville MA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from BioWhittaker (Walkersville, MD, USA). Doxorubicin, 3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl tetrazolium bromide (MTT), cisplatin, and 5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside (X-Gal), and protein A agarose were purchased from Sigma (St. Louis, MO, USA). Antibodies to p21/WAF1 and to β-actin were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA), antibodies to phosphorylated p53, cleaved caspase-3 and secondary antibodies conjugated to horseradish peroxidase from Cell Signaling Technologies (Beverly, MA, USA). Antibody to γ-H2AX and to acetylated histone H3 (K9) were purchased from Upstate Cell signaling Solutions (Lake Placid, NY). Enhanced chemiluminescence reagents (ECL) were from Amersham (Arlington Heights, IL). Immobilon-P transfer membrane for Western blots was purchased from Millipore (Bedford, MA, USA).

Cell culture, drug treatment and cytotoxicity assay

Cells were cultured in DMEM supplemented with 10% FBS at 37°C in a 95% Air/5% CO_2 atmosphere. Cytotoxic activity of doxorubicin was quantitatively determined by a colorimetric assay utilizing 3-(4,5-dimethyl-2-thiazolyl)

2,5-diphenyl tetrazolium bromide (MTT), as described previously.[28] Briefly, cells were seeded at 10^4 cells/well in 96-well plates and maintained in culture for 24 h at 37°C in DMEM supplemented with 10% FBS. The drug was added to designated wells and incubated for 72 h, following which MTT (10 μl of 5 mg/ml solution) was added to each well (100 μl) and incubated for 4 h at 37°C. The cells were solubilized by incubation with 100 μl of HCl 0.5 N/isopropanol for 15 h at 37°C. The optical density of this solution was measured at 570 nm and the percentage of viable cells estimated by comparison with untreated control cells.

Western-blot analysis

Cells were seeded in 75 cm^2 flasks in DMEM containing 10% FBS and cultivated for 24 h before addition of the drug. After incubation for an additional 24 h, cells were pelleted and washed with TBS and re-suspended in buffer A (10 mM HEPES pH 7.9, 0.1 mM EDTA, 10 mM KCL, 0.1 EGTA, 1 mM DTT, 0.5 mM PMSF). The mixture was incubated for 30 min on ice then 25 μl of 10% Nonidet P-40 was added and the homogenate centrifuged for 30 s. The nuclear pellet was re-suspended in 50 μl of ice-cold buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). The samples were then incubated at 4°C for 30 min and centrifuged at 12 000 $\times$ *g* for 5 min. The supernatant containing the nuclear extract was processed by Western blot for detection of p21/WAF1, and activated H2AX. The cytoplasmic fractions were processed for detection of cleaved caspase-3 and β actin. Equal quantities of protein were separated by electrophoresis on a 12% SDS-PAGE gel and transferred to Immobilon-P membranes. Proteins of interest were identified by reaction with specific primary and secondary antibodies linked to horseradish peroxidase. Reactive bands were detected by chemi-luminescence.[3]

Flow cytometry

SKN-SH cells (about 10^6) were incubated with various concentrations of doxorubicin for 24 h, then collected by trypsinization and washed twice with PBS containing 5 mM EDTA. Cells were fixed in 70% ethanol and incubated for 10 min on ice. Before flow cytometry analysis, cells were washed twice with PBS/5 mM EDTA and subjected to treatment with RNAse A (2 μg/ml of PBS) for 30 min at 37°C. The cells were then incubated with propidium iodide (10 μg/ml) for 1 h at room temperature, filtered and DNA content analyzed by flow cytometry.

Senescence associated-β-Galactosidase (SA-β-Gal) staining

Cells were seeded into 24-well plates in DMEM culture medium and after 24 h, doxorubicin or TSA were added and the cells incubated for 5 days. SA-β-Gal staining was performed as previously described.[29] In brief, cells were fixed for 5 min in 3% formaldehyde, washed and incubated at 37°C with X-gal (1 mg/ml), dissolved in a solution containing 40 mM citric acid pH 6.5, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$. After 24 h incubation, photographs were taken under a phase microscope.

Measure of DNA fragmentation

Cells were incubated with doxorubicin at various concentrations for 24 h at 37°C and DNA prepared from Triton X-100 lysates for analysis of fragmentation. Briefly, cells were lysed in a hypotonic solution containing 10 mM Tris HCl (pH 7.4), 1 mM EDTA, and 0.2% Triton X-100, and centrifuged at 11 000 $\times$ *g* for 5 min. Supernatants were electrophoresed on a 1% agarose gel and DNA fragments visualized under UV light after staining with ethidium bromide.

Comet assay

This single-cell electrophoresis assay is used for evaluating the formation of both single-strand and double-strand breaks following cell exposure to DNA damaging agents.[22] After drug treatment, cells (10^5 cell/ml) were washed twice with PBS and mixed at a ratio 1 : 10 (v/v) with LMA agarose (Trevigen, Gaithensburg, MD, USA) at 37°C. A 75 μl aliquot of the cell suspension was spotted on a microscope slide and maintained at 4°C for 30 min to allow polymerization of the gel. The cells were then lysed in a solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10) and 1% Triton-X100, followed by incubation in alkali conditions (300 mM NaOH, 1 mM EDTA) for 1 h at room temperature to allow DNA unwinding and expression of SSBs and DSBs. The slides were then placed in a horizontal electrophoresis system and subjected to a 1 V/cm current for 15 min. They were then exposed for 5 min to absolute ethanol and subsequently air-dried. Just before comet scoring, the DNA was stained with SyberGreen (1 $\mu\gamma$/ml 50 μl/slide), and viewed by fluorescence microscopy (excitation 494 nm, emission 521 nm).

ChIp assay

Cells were seeded in 25 cm^2 flasks and when they had reached 60% confluency, doxorubicin was added and incubated for times varying from 1 to 24 h. Chromatin immunoprecipitation was carried out by using the ChIp Assay kit #17-295 (Upstate Cell Signaling Solutions, Lake Placid, NY, USA). The cells were lysed in SDS lysis buffer provided in the kit and cell lysate sonicated to shear DNA into 100–200 base pairs. Anti-acetyl histone H3 (K9) and Salmon sperm DNA/ProteinA agarose were utilized to immunoprecipitate chromatin fragments. DNA was then extracted from the immunoprecipitate and amplified by PCR using the following:[25] region $-105/+25$ of the p21/WAF1 promoter spanning the TATA box of the promoter 5′-GCGGCGCGGTGG GCCGAGCGCGGG-3′ (Forward primer), 5′-GGCTCCACAAGGAACTGACT-3′ (Backward primer). Region $-2760/-2486$ of the p21/WAF1 promoter 5′TTGTGCCACTGCTGA CTTTGTC-3′ (Forward primer), 5′-AGCCTGAAGAAGGAGGATGT GAGG-3′ (Backward primer). Amplification was carried out for 35 cycles under the cycling conditions described previously.[25]

Acknowledgements

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