

A decrease in cyclin B1 levels leads to polyploidization in DNA damage-induced senescence

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Abstract

Adriamycin, an anthracycline antibiotic, has been used for the treatment of various types of tumours. Adriamycin induces at least two distinct types of growth repression, such as senescence and apoptosis, in a concentration-dependent manner. Cellular senescence is a condition in which cells are unable to proliferate further, and senescent cells frequently show polyploidy. Although abrogation of cell division is thought to correlate with polyploidization, the mechanisms underlying induction of polyploidization in senescent cells are largely unclear. We wished, therefore, to explore the role of cyclin B1 level in polyploidization of Adriamycin-induced senescent cells. A subcytotoxic concentration of Adriamycin induced polyploid cells having the features of senescence, such as flattened and enlarged cell shape and activated β -galactosidase activity. In DNA damage-induced senescent cells, the levels of cyclin B1 were transiently increased and subsequently decreased. The decrease in cyclin B1 levels occurred in G2 cells during polyploidization upon treatment with a subcytotoxic concentration of Adriamycin. In contrast, neither polyploidy nor a decrease in cyclin B1 levels was induced by treatment with a cytotoxic concentration of Adriamycin. These results suggest that a decrease in cyclin B1 levels is induced by DNA damage, resulting in polyploidization in DNA damage-induced senescence.

Keywords: Adriamycin; cyclin B1; fluorescein- β -D-galactopyranoside; polyploidy; senescence; β -galactosidase

1. Introduction

Adriamycin, an anthracycline antibiotic, has been used for the treatment of various types of tumours. Adriamycin is thought to act via a number of different mechanisms, including intercalation into DNA, free radical formation, lipid peroxidation, DNA binding and alkylation, and the initiation of DNA damage through inhibition of topoisomerase II (Gewirtz, 1999). It has been reported that Adriamycin induces at least two distinct types of growth repression, senescence and apoptosis, in a concentration-dependent manner (Rebbaa et al., 2003; Eom et al., 2005; Park et al., 2005; Sliwinska et al., 2009). However, the molecular mechanisms governing induction of senescence are still unclear.

Cellular senescence is a condition in which cells are unable to proliferate further. This is a stress response and can be induced by multiple mechanisms including telomere shortening and DNA damage (Collado et al., 2007; d'Adda di Fagagna, 2008). Senescence induced by DNA-damaging anticancer drugs was one of the key determinants of tumour response to chemotherapy (Roninson, 2003; Kahlem et al., 2004), since the ability of cancer cells to undergo apoptosis may not play a significant role in response to DNA-damaging drugs (Brown and Wauters, 1999). Senescent cells generally show flattened and enlarged cell shape, and an increase in SA- β -gal (senescence-associated β -galactosidase) activity (Dimri et al., 1995). In addition, senescent cells frequently show polyploidy (Wagner et al., 2001; Park et al., 2005; Yang et al., 2007; McCrann et al., 2008; Sliwinska et al., 2009). Abrogation of cell division accompanying over-replication of DNA is thought to result in polyploidization. In some polyploid cell

types, such as megakaryocytes and trophoblasts, polyploidization involves a bypass of mitosis owing to subnormal levels of the mitotic regulatory kinase, cyclin B1/Cdk1, suggesting that the decreased activities of cyclin B/Cdk1 kinase are important for polyploidization through abrogation of mitosis in these cell types (Edgar and Orr-Weaver, 2001). However, the mechanisms underlying induction of polyploidy in DNA damage-induced senescent cells are largely unclear. We wished, therefore, to explore a role of cyclin B1 level in polyploidization of DNA damage-induced senescent cells.

In this study, we show that treatment with Adriamycin at a subcytotoxic dose decreases the level of cyclin B1 during polyploidization of senescent cells. Our findings suggest the possibility that a decrease in cyclin B1 levels is important for induction of polyploidization in DNA damage-induced senescence.

2. Materials and methods

2.1. Cells

HeLa cells (Japanese Collection of Research Bioresources) were cultured in IMDM (Iscove's modified Dulbecco's medium) containing 5% FBS (fetal bovine serum).

2.2. Antibodies

Mouse monoclonal anti-CDK1 (17, Santa Cruz Biotechnology), anti-cyclin A (CY-A1, Sigma), anti-actin (C4, Millipore) antibodies

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Abbreviations: FBS, fetal bovine serum; FDG, fluorescein- β -D-galactopyranoside; HRP, horseradish peroxidase; SA- β -gal, senescence-associated β -galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

and affinity-purified rabbit polyclonal anti-phospho-histone H2A.X (Ser139; #2577S, Cell Signaling Technology), and anti-cyclin B1 (H-433, Santa Cruz Biotechnology) antibodies were used. HRP (horseradish peroxidase)-conjugated F(ab')₂ fragments of anti-mouse IgG antibody (GE Healthcare) and HRP-conjugated anti-rabbit IgG antibody (Beckman Coulter) were used. FITC-conjugated F(ab')₂ fragments of anti-rabbit IgG antibody were obtained from Sigma.

2.3. Flow cytometry

For cell cycle analysis, cells detached by trypsinization were fixed in 1.25% paraformaldehyde on ice for 1 h, then permeabilized with 70% ethanol for at least 1 h at -30°C (Nakayama and Yamaguchi, 2005). After washing twice with PBS containing 3% FBS, cells were incubated with anti-cyclin B1 on ice for 1 h and then stained with FITC-conjugated anti-rabbit antibody for 1 h. Subsequently, cells were treated with 200 µg/ml RNaseA and 50 µg/ml PI (propidium iodide) at 37°C for 30 min to stain DNA. Protein levels and DNA profiles were analysed by flow cytometry using a MoFlo cell sorter (Beckman Coulter) equipped with a 488-nm argon laser. Dead cells and debris were excluded by gating on forward scatter and pulse-width profiles.

2.4. Western blotting

Whole cell lysates prepared by the addition of SDS-sample buffer were separated by SDS/PAGE and electrotransferred on to PVDF membrane (Millipore). Immunodetection was performed as reported previously (Kasahara et al., 2004; Matsuda et al., 2006; Nakayama et al., 2006).

2.5. Senescence-associated β -galactosidase activity

SA- β -gal activity was detected as described previously (Dimri et al., 1995). In brief, HeLa cells treated with 20 ng/ml Adriamycin for 3 days were fixed in 3% paraformaldehyde at room temperature for 4 min. Fixed cells were washed with PBS and then incubated with 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) at 37°C for 20 h in staining solution containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂ and 40 mM sodium phosphate buffer (pH 6.0). When SA- β -gal activity was detected by flow cytometry or fluorescence microscopy, detached cells were fixed with 3% formaldehyde for 4 min and then incubated at 37°C for 2 h with 20 µM fluorescein- β -D-galactopyranoside (FDG, Marker Gene Technologies, Inc.), a hydrolysable substrate for SA- β -gal (Nolan et al., 1988).

2.6. Semiquantitative RT-PCR

Total RNAs were isolated from HeLa cells with the ISOGEN reagent (Nippon gene), and cDNAs were synthesized from 1 µg of each RNA preparation using the PrimeScript RT reagent Kit (Takara). To avoid the saturation of PCR products, conditions of PCR were optimized before semiquantitative RT-PCR was carried out. The primers used for PCR are as follows: cyclin B1,

5'-TCTACCTTTGCACTTCCTTCGG-3' (forward) and 5'-TACACCTTTGCCACAGCCTTGG-3' (reverse) (Badie et al., 2000); GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse) (Biton et al., 2006). The sizes of PCR products are 402 and 452 bp, respectively. Amplification was carried out using an MJ mini thermal cycler (Biorad) with Ex Taq (Takara) under the following conditions: initial heating at 94°C for 2 min, followed by 26 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 1 min. The products of RT-PCR were electrophoresed on a 1.8–2.0% agarose gel containing ethidium bromide. The density of each amplified fragment was quantified with ChemiDoc XRS plus and Quantity one software (Biorad).

2.7. Immunofluorescence microscopy

Cells were fixed in PBS containing 4% paraformaldehyde and 20% methanol for 20 min and permeabilized in 100% methanol at -20°C for 1 min. These cells were sequentially incubated with anti-phospho-histone H2A.X (Cell Signaling Technology) and FITC-anti-rabbit IgG antibody for 1 h each. DNA was stained with 40 µg/ml propidium iodide for 20 min after treatment with 200 µg/ml RNase A.

3. Results

3.1. Induction of polyploidization in DNA-damaged senescent cells

Cytotoxic agents often induce terminal growth arrest with features of senescence at subcytotoxic concentrations (Han et al., 2002; Rebbaa et al., 2003; Eom et al., 2005). To examine whether treatment with Adriamycin, a DNA-damaging anticancer agent, induced senescence, HeLa cells treated with Adriamycin were analysed for viability and SA- β -gal activity. Dose-dependent cytotoxicity was observed after a 3-day treatment with Adriamycin (Figure 1A). When HeLa cells were treated with a subcytotoxic concentration of Adriamycin (20 ng/ml), the phosphorylation of H2AX, a marker of the DNA damage response (Bonner et al., 2008) was observed (Figure 1B), suggesting that a subcytotoxic concentration of Adriamycin induced the DNA damage. Microscopic analysis of cells incubated with X-gal, a substrate for SA- β -gal, showed that SA- β -gal activity was augmented upon Adriamycin treatment in agreement with previous observations (Rebbaa et al., 2003; Eom et al., 2005; Park et al., 2005; Sliwinska et al., 2009) (Figure 1C). To quantitatively analyse the SA- β -gal activity, Adriamycin-treated cells were incubated with FDG, a substrate for β -galactosidase. FDG gives rise to fluorescence after being cleaved by β -galactosidase, enabling us to detect SA- β -gal activity by a fluorescent microscope and a flow cytometer. Under a laser-scanning microscope, an accumulation of fluorescence was observed in flattened cells induced by a subcytotoxic concentration of Adriamycin (Figure 1D), consistent with results using X-gal (Figure 1C). Flow cytometry analysis showed that mean fluorescence intensity was increased upon

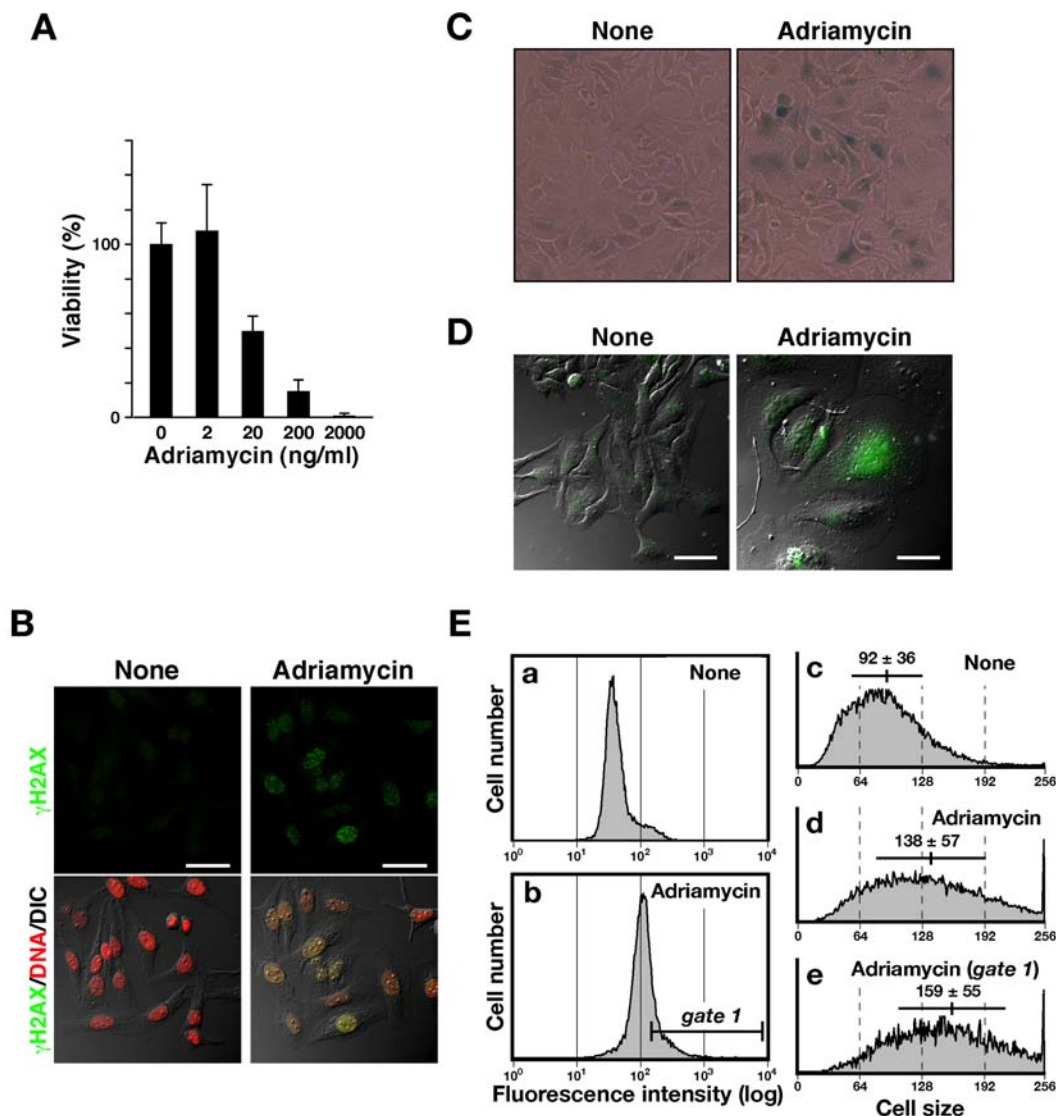


Figure 1 Induction of senescence by a subcytotoxic concentration of Adriamycin

(A) HeLa cells were treated with the indicated concentrations of Adriamycin for 3 days, and the number of viable cells was counted. The number of untreated cells is set to 100%, and numbers of Adriamycin-treated cells as a percentage of control are shown. The data presented are average values \pm S.D. of triplicate. (B) HeLa cells treated with 20 ng/ml Adriamycin for 1 day were fixed and stained for γ H2AX and DNA. Scale bars, 20 μ m. (C, D) HeLa cells treated with 20 ng/ml Adriamycin for 3 days were fixed and examined for senescence-associated β -galactosidase activity. Fixed cells were incubated with X-gal (C) or FDG (D) prior to analysis with an optical microscope or a laser-scanning microscope, respectively. Scale bars, 50 μ m. (E) HeLa cells treated with 20 ng/ml Adriamycin for 3 days were fixed and incubated with 20 μ M FDG. The fluorescence intensity was measured by flow cytometry. Histograms of fluorescence intensity (a, b) and forward scatter histograms (c–d) are shown. In E(b), a gate (*gate 1*) was drawn to include cells having higher fluorescent intensities, and the gated events were plotted as a forward scatter histogram to evaluate cell size (e). Average value \pm S.D. is shown.

treatment with Adriamycin, indicating that the SA- β -gal activity was increased in most of the cells (Figures 1E-a, 1E-b). Gated events having higher fluorescence intensities (*gate 1*) showed the increase in cell sizes determined by analysing the forward scatter profiles [Figures 1E(c)–1E(e)]. These results suggest that a subcytotoxic concentration of Adriamycin induces senescent cells showing the typical features, such as flattened and enlarged cell shape and activated β -gal activity.

To investigate whether polyploidization is induced in senescent cells, cells were treated with Adriamycin at a subcytotoxic concentration for 3 days. Microscopic and flow cytometry

analyses showed that polyploid cells with large cell sizes were induced upon treatment with a subcytotoxic concentration of Adriamycin (Figures 2A and 2B). These results suggest that treatment with a subcytotoxic concentration of Adriamycin induces polyploidization in Adriamycin-induced senescent cells.

3.2. Decrease in cyclin B1 levels during polyploidization in senescent cells

Since the polyploid trophoblasts and megakaryocytes show low levels of mitotic regulators (Edgar and Orr-Weaver, 2001), we

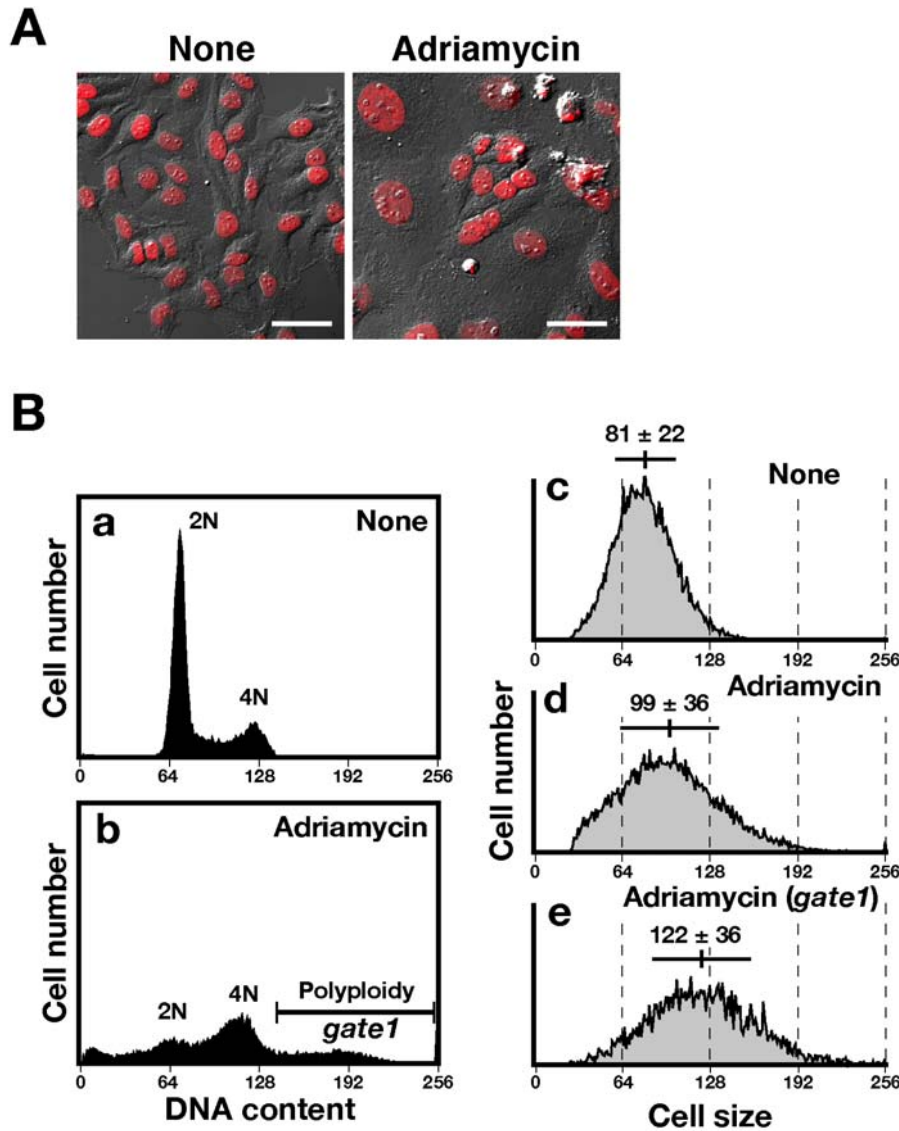


Figure 2 Induction of polyplodization by a subcytotoxic concentration of Adriamycin

(A) HeLa cells were treated with 20 ng/ml Adriamycin for 3 days. The cells were fixed and stained for DNA. Scale bars, 50 μ m. (B) HeLa cells were treated with 20 ng/ml Adriamycin for 3 days, and DNA contents (a, b) and cell sizes (c–e) were measured by flow cytometry. (a, b) Peak haploid genome equivalents (2N, 4N) and polyplody are indicated. Each curve represents 20000 cells. In B(b), a gate (*gate 1*) was drawn to include polyplody cells, and the gated events were plotted as a forward scatter histogram to evaluate cell size (e). Average value \pm S.D. is shown.

examined the levels of cyclin B1, cyclin A and CDK1 in senescent cells. Although untreated cells showed a low level of cyclin B1 owing to the asynchronous cell cycle (Figure 3A, day 0), upon treatment with Adriamycin, the levels of cyclin B1 were increased at day 1 together with an increase in the numbers of G2-arrested cells (see Figure 4A). Then, the levels of cyclin B1 gradually decreased to a level similar to the levels at day 0 (Figure 3A, days 1–4), despite no significant change in the transcriptional level being observed (Figure 3B). The decrease in cyclin B1 levels was more rapid with a subcytotoxic concentration of Adriamycin (20 ng/ml) than a cytotoxic concentration (200 ng/ml). A similar pattern of change in the protein levels was found for cyclin A but not for CDK1 (Figure 3A). To examine the relationship between the

levels of cyclin B1 and senescence, cells were treated with Adriamycin for the periods indicated in Figure 3(C) and examined for SA- β -gal activity. Quantitative analysis with FDG showed that SA- β -gal activity gradually increased over the period from days 1 to 3, and the high SA- β -gal activity was sustained at least until day 4 (Figure 3C). These results suggest that a decrease in cyclin B1 levels is induced in DNA-damaged senescent cells.

To examine the relationship between the decrease in cyclin B1 levels and polyplodization in DNA-damaged senescent cells, cyclin B1 levels and DNA contents were analysed by dual-colour flow cytometry. Flow cytometry analysis showed that cyclin B1 was highly expressed in untreated cells with 4 N DNA content (4 N cells) (Figures 4A and 4B, day 0), consistent with

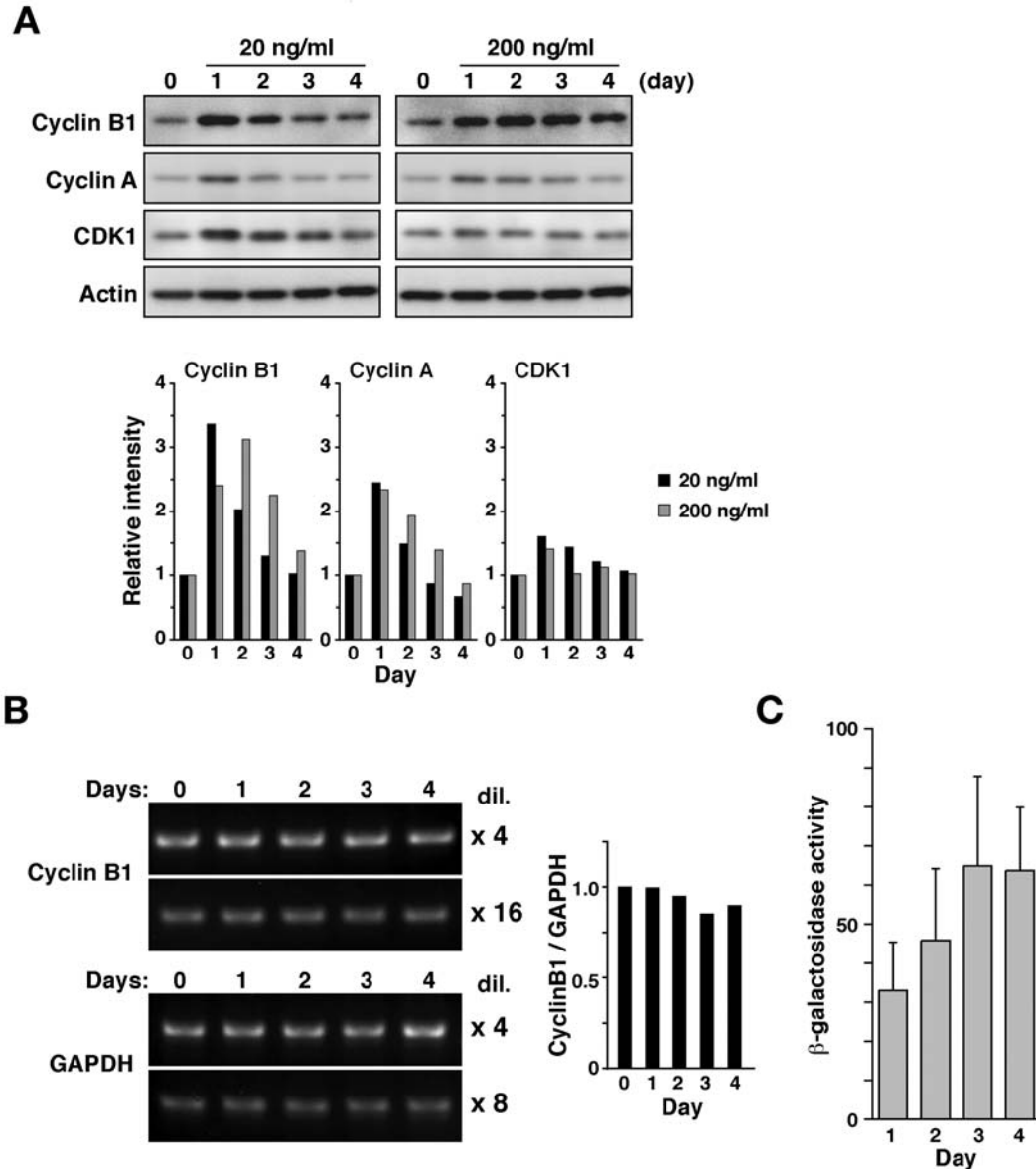


Figure 3 Decrease in the levels of cyclin B1 upon Adriamycin treatment

(A) HeLa cells were treated with 20 or 200 ng/ml Adriamycin for 4 days. Total cell lysates were obtained at the indicated periods of incubation and subjected to Western blotting, probed with anti-cyclin B1, anti-cyclin A, anti-CDK1 and anti-actin antibodies. The amounts of proteins were quantified by measuring signal intensities and normalized to the levels of actin. (B) RT-PCR analysis of cyclin B1 mRNA (predicted size 402 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (predicted size 452 bp). HeLa cells treated with 20 ng/ml Adriamycin were harvested for RNA preparation at the indicated periods of incubation. Reverse transcription products were used for PCR over a range of dilutions (dil.). The amounts of cyclin B1 products were quantified by measuring signal intensities and normalizing to the levels of GAPDH. The data presented represent two similar experiments. (C) HeLa cells treated with 20 ng/ml Adriamycin for 4 days were fixed at the indicated periods of incubation. The fixed cells were incubated with 20 μ M FDG, and the levels of fluorescent product were measured by flow cytometry. The mean fluorescence intensity was subtracted from that for untreated cells.

previous studies (Ohi and Gould, 1999). Treatment with a subcytotoxic concentration of Adriamycin (20 ng/ml) increased the number of 4 N cells with high levels of cyclin B1 a day after treatment (day 1), suggesting that cells were arrested at G2 phase. Then, along with the increase in the number of polyploid cells (days 2–4), cyclin B1 levels were decreased despite 4 N DNA content (Figures 4A, 4C). We compared the effects of a subcytotoxic concentration of Adriamycin (20 ng/ml) to those of a cytotoxic concentration (200 ng/ml) on the polyploidization

and the cyclin B1 levels. Flow cytometry analysis showed that polyploid cells were less induced by treatment with 200 ng/ml Adriamycin than those treated with 20 ng/ml Adriamycin (Figures 4B and 4C). 4 N cells still expressed high levels of cyclin B1 until 3 days of treatment with 200 ng/ml Adriamycin (Figure 4B). These results suggest that a decrease in cyclin B1 levels is induced by treatment with a subcytotoxic concentration of Adriamycin, resulting in polyploidization in DNA-damage-induced senescent cells.

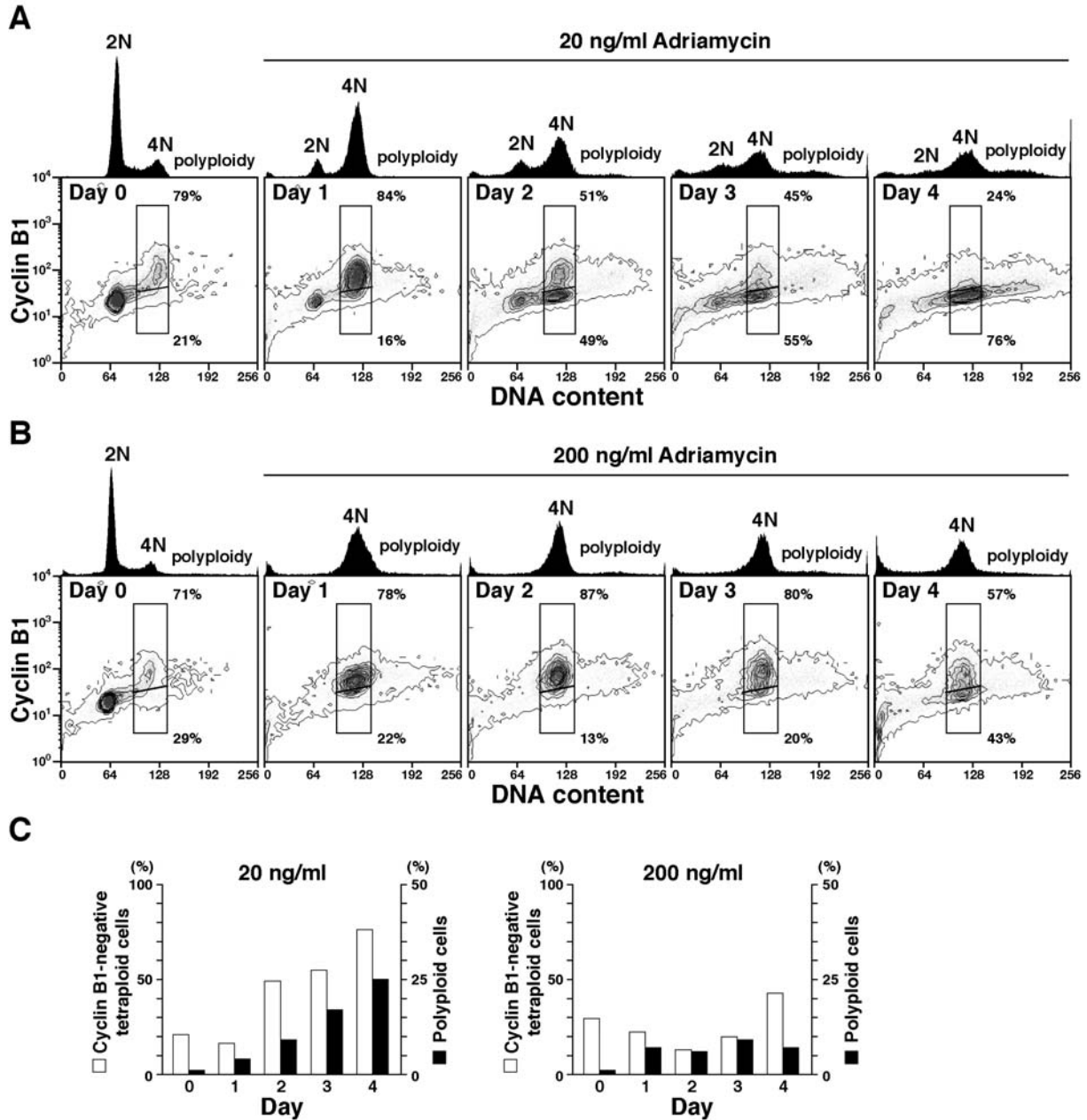


Figure 4 Decrease in the levels of cyclin B1 in cells undergoing senescence-associated polyploidization. HeLa cells were treated with Adriamycin at 20 ng/ml (A) or 200 ng/ml (B) and fixed at the indicated periods of treatment. The bivariate contour plots are shown together with dot plots for each individual plot. DNA content is shown on the x-axis, while cyclin B1 protein level is shown on the y-axis (log scale). The regions designated by a line include cells having 4N DNA content: lower and upper regions indicate cyclin B1-negative and -positive cell populations, respectively. The percentage of cells with cyclin B1 or without cyclin B1 to 4N cells is shown. DNA histograms above each bivariate plot. Peak haploid genome equivalents (2N, 4N, polyploidy) are indicated. Each plot represents 20 000 cells. The percentages of cyclin B1-negative tetraploid cells and polyploid cells are plotted (C).

4. Discussion

In the present study, we show that a subcytotoxic but not cytotoxic concentration of Adriamycin induces polyploid cells having some features of senescence, such as flattened and enlarged cell shape and an increase in SA- β -gal activity. The

levels of cyclin B1 were decreased during polyploidization in Adriamycin-induced senescent cells. Our results suggest that a decrease in cyclin B1 levels is involved in polyploidization of DNA-damage-induced senescent cells.

It has been reported that DNA-damaging anticancer drugs induce down-regulation of mitotic regulators, such as cyclin B1

and Cdk1 in cells undergoing senescence (Han et al., 2002; Park et al., 2005; Jackson and Pereira-Smith, 2006). We found that even at low concentrations, Adriamycin caused DNA damage (Figure 1B) and decreased the level of cyclin B1 (Figures 3A and 4A). The appearance of polyploid cells was preceded by accumulation of cyclin B1-negative tetraploid cells (Figure 4), suggesting that the decrease in cyclin B1 levels is involved in polyploidization. The decrease in cyclin B1 levels leads to inhibition of cell division owing to low CDK1 activity (Nurse, 1990). Therefore, a subcytotoxic concentration of Adriamycin is likely to inhibit CDK1 activity through a decrease in the levels of cyclin B1. Suppression of CDK1 activity not only inhibits cell division, but also allows the assembly of PreRCs (prereplication complexes) for licensing the DNA for another round of replication (Diffley, 2004). Suppression of CDK1 activity is involved in the polyploidization of megakaryocyte and trophoblast cells, supporting the idea that polyploid cells are generated through inhibition of cell division and repeated replication of DNA (Edgar and Orr-Weaver, 2001). Taken together, a decrease in cyclin B1 levels, induced by DNA damage, may be critical for polyploidization through the sustained inhibition of cell division and possibly through licensing DNA replication in cells undergoing Adriamycin-induced senescence.

The protein levels of cyclin B1 are tightly regulated by transcription and degradation. Cyclin B1 is stably accumulated during G2 phase due to the transcriptional activation (Pines and Hunter, 1989) and inactivation of the polyubiquitination-derived degradation pathway (Peters, 2002). The degradation of cyclin B1 begins at the metaphase–anaphase transition in mitosis and lasts until G1 phase (Peters, 2002). In response to DNA damage, transcriptional suppression (Maity et al., 1996; Crawford and Piwnica-Worms, 2001) and mRNA destabilization (Maity et al., 1995, 1996) are involved in the decrease in cyclin B1 levels for long-term G2 arrest. We, however, found no significant change in the transcriptional level (Figure 3B), and we recently reported that cyclin B1 is degraded during G2 arrest in response to DNA damage (Nakayama et al., 2009). Indeed, we showed that cyclin B1 levels are decreased after accumulation of cyclin B1 in 4 N cells (Figures 3A and 4A), suggesting that degradation is involved in the decrease in cyclin B1 levels. We found a decrease in cyclin B1 levels but not in CDK1 levels (Figure 3A), suggesting the involvement of the polyubiquitination-derived degradation pathway. These results suggest that degradation of cyclin B1 may be involved in polyploidization of DNA-damage-induced senescent cells.

Polyploidization was less induced by treatment with a cytotoxic concentration (200 ng/ml) of Adriamycin than a subcytotoxic concentration (20 ng/ml) of Adriamycin (Figure 4B). Western blotting analysis showed that the level of cyclin B1 was decreased in cells treated with a cytotoxic concentration of Adriamycin (Figure 3A). Importantly, the kinetics of change in cyclin B1 levels was delayed (Figure 3A), and cell death was induced in cells treated with a cytotoxic dose of Adriamycin (Figures 1A and 4B). Therefore, it is likely that cell death is induced instead of induction of polyploidization upon treatment with a cytotoxic concentration of Adriamycin.

The activation of p53 is responsible for the induction of growth arrest in senescent cells through the transcriptional regulation of

target genes (Roninson, 2003). One of the most relevant targets of p53 for inducing senescence is p21^{Waf1}, an inhibitor of cyclin/CDK complexes. p21 expression causes growth arrest in senescent cells (Roninson, 2003). In HeLa cells, wild-type p53 protein is sequestered by HPV/E6 viral proteins, resulting in degradation of p53 protein (Scheffner et al., 1990). However, increases in protein levels of p21 in senescent HeLa cells have been shown (Michishita et al., 2002; Suzuki et al., 2002; Huang et al., 2003). These findings suggest that senescence may be induced through a p53-independent pathway in HeLa cells.

Several reports show that cyclin B1 levels are elevated in cancer cells (Dutta et al., 1995; Mashal et al., 1996; Kushner et al., 1999; Yasuda et al., 2002; Grabsch et al., 2004). Overexpression of cyclin B1 is associated with a poor prognosis for patients with squamous cell carcinomas of the esophagus (Murakami et al., 1999; Takeno et al., 2002), larynx (Dong et al., 2002), lung (Soria et al., 2000) and tongue (Hassan et al., 2001). In addition, overexpression of cyclin B1 contributes to metastasis, probably by promoting an epithelial–mesenchymal transition of esophageal squamous cell carcinoma cells (Song et al., 2008) and colorectal tumours (Li et al., 2003). Our results show that cyclin B1 levels are decreased in cells undergoing senescence upon treatment with Adriamycin. Since senescence-associated growth arrest of cancer cells is generally considered to be irreversible (Chang et al., 1999), it is possible to evaluate clinical outcome by measuring cyclin B1 levels after treatment with anticancer drugs. However, escape from chemotherapy-induced senescence was also reported, where the cells resumed proliferation and showed aneuploidy (Roberson et al., 2005; Sliwiska et al., 2009). Aneuploid cells may contribute to cancer development and aggressiveness and are thought to be generated from polyploid cells via aberrant cell division (Kops et al., 2005). If polyploid cells in senescence would resume proliferation, induction of polyploidization in senescence is critical for production of aneuploid cells and relates to prognosis. Although in DNA-damage-induced senescence, polyploid cells are generated via a decrease in cyclin B1 levels, it is plausible that cyclin B1 is restored when polyploid cells resume cell division. Therefore, cyclin B1 levels alert us to the risk of escape from senescence and generation of aneuploid cells. It is thus of considerable interest to analyse correlations between prognosis and cyclin B1 levels following treatment with chemotherapeutic agents, including Adriamycin.

In conclusion, a subcytotoxic concentration of Adriamycin induces senescence in HeLa cells, in which the decrease in cyclin B1 levels is involved in the polyploidization of DNA-damage-induced senescence. Our results support the hypothesis that cyclin B1 is a useful prognostic marker for cancer patients.

Author contribution

Ikue Kikuchi performed the flow cytometry experiments and immunostaining experiments. Yuji Nakayama took part in the whole study, wrote the manuscript and was responsible for the study. Takao Morinaga took part in the Western blotting experiments. Yasunori Fukumoto and Naoto Yamaguchi supervised the experiments, and Naoto Yamaguchi was responsible for the study.

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