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Implications of caspase-dependent proteolytic cleavage of cyclin A1 in DNA damage-induced cell death

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ABSTRACT

Cyclin A1 is an A-type cyclin that directly binds to CDK2 to regulate cell-cycle progression. In the present study, we found that doxorubicin decreased the expression of cyclin A1 at the protein level in A549 lung cancer cells, while markedly downregulating its mRNA levels. Interestingly, doxorubicin upregulated caspase-1 in a concentration-dependent manner, and z-YAVD-fmk, a specific inhibitor of caspase-1, reversed the doxorubicin-induced decrease in cyclin A1 in A549 lung cancer and MCF7 breast cancer cells. Active caspase-1 effectively cleaved cyclin A1 at D165 into two fragments, which *in vitro* cleavage assays showed were further cleaved by caspase-3. Finally, we found that overexpression of cyclin A1 significantly reduced the cytotoxicity of doxorubicin, and knockdown of cyclin A1 by RNA interference enhanced the sensitivity of cells to ionizing radiation. Our data suggest a new mechanism for the downregulation of cyclin A1 by DNA-damaging stimuli that could be intimately involved in the cell death induced by DNA damage-inducing stimuli, including doxorubicin and ionizing radiation.

1. Introduction

There are two A-type cyclins in mammals: cyclin A1 (CCNA1) and A2 (CCNA2). Whereas cyclin A2 is ubiquitously expressed and serves to regulate the cell cycle at the G1/S to G2/M transition, the expression and function of cyclin A1 is limited to the testis and hematopoietic stem cells [1,2]. Human cyclin A1 is also highly expressed in several myeloid leukemia cell lines independently of cyclin A2 expression. It has recently been reported that cyclin A1 also contributes to G1-to-S progression of the cell cycle in somatic cells [3], suggesting the functional importance of cyclin A1 in diseases caused by dysfunction of cellular proliferation, such as cancer. Indeed, downregulation of cyclin A1 expression inhibits proliferation of human leukemia cells and induces apoptosis through activation of the DNA-damage response [4,5].

The expression of cyclins is tightly regulated by the ubiquitin-dependent proteasome pathway during cell-cycle progression. Interestingly, the cyclin A2 of *Xenopus* is also cleaved by caspases during ionizing radiation-induced apoptosis to generate a truncated form whose Cdk2 binding activity is normal [6]. The truncated cyclin A2–Cdk2 complex possesses kinase activity that is insensitive to inhibition by Cdk inhibitors and degradation by the proteasome. Cyclin D1 is similarly targeted by caspases during radiation-induced apoptosis, suggesting that caspases may cleave cyclins to promote specific apoptotic events in *Xenopus*.

Doxorubicin is known to bind to DNA-associated enzymes, intercalate between DNA base pairs, and target multiple molecular targets to produce a range of cytotoxic effects [7]. Treatment of p53-positive cell lines with doxorubicin induces expression of Ipaf, a recently identified positive regulator of caspase-1 [8]. Subsequently, activated caspase-1 causes the release of mitochondrial proteins, such as cytochrome c and the serine peptidase Omi, through activation of the pro-apoptotic Bcl-2 family member, Bax [9].

In the present study, we found that downregulation of cyclin A1 is involved in DNA damage-induced cell death. Overexpression of cyclin A1 significantly reduced the cytotoxicity of doxorubicin, and knockdown of cyclin A1 expression by small interfering RNA (siRNA) enhanced the sensitivity of cells to ionizing radiation. Interestingly, downregulation of cyclin A1 by doxorubicin was attributable to caspase-dependent cleavage at the D165 residue. Our data strongly suggest that targeting degradation of cyclin A1

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by caspase could be a beneficial strategy in cancer therapies using DNA damage-inducing agents, including doxorubicin or ionizing radiation.

2. Materials and methods

2.1. Cell cultures and reagents

A549 human non-small cell lung cancer cells and MCF7 human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in the recommended growth medium (Life Technologies, Grand Island, NY, USA). Doxorubicin was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Immunoblotting

Cell lysis and immunoblotting were performed according to a previous report [10]. Membranes were probed with anti-cyclin A1, anti-cyclin A2, anti-p53, anti-caspase-1, anti-cleaved PARP, and anti-FLAG antibodies, from Santa Cruz Biotechnology (Dallas, TX, USA), and another anti-cyclin A1 and anti-β-actin antibody from Sigma–Aldrich. The caspase-1 inhibitor, z-YAVD-fmk, was purchased from EMD Millipore (Billerica, MA, USA).

2.3. Measurement of cell viability

Cell viability was determined by measuring the mitochondrial conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) to a colored product. Cells were treated with the specified drugs, and the medium was exchanged with serum-free medium containing 1 mM MTT. After 4 h of incubation at 37 °C, cells were solubilized with dimethylsulfoxide (DMSO). The amount of formazan, the converted form of MTT, was determined by measuring absorbance at 570 nm. IC_{50} values were determined with Prism software (GraphPad Software Inc., San Diego, CA) using non-linear regressions.

2.4. In vitro cleavage assay

A human cyclin A1 construct in pcDNA3.1(+) containing the full-length cyclin A1 coding sequence was kindly provided by Dr. Carsten Müller-Tidow (University of Münster, Münster, Germany). Truncated forms of cyclin A1 (cyclin A1-ΔC, 1–165 aa; cyclin A1-ΔN, 166–465 aa) incorporating *Eco*RI sites at the ends were generated by polymerase chain reaction (PCR) and cloned into the corresponding sites of the pcDNA4/HisMax C expression vector (Life Technologies) following amplification. Two potential cleavage site mutants (D165E and D249E) were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All constructs were verified by restriction enzyme digestion and DNA sequencing. ^{35}S-methionine labeled wild-type cyclin A1, cyclin A1-D165E, and cyclin A1-D249E were generated by *in vitro* transcription and translation using the TNT T7-coupled Reticulocyte Lysate System (Promega, Madison, WI, USA). The labeled proteins (2 μl) were incubated with different recombinant caspases (EMD Millipore) in caspase buffer (50 mM HEPES pH 7.2, 50 mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% glycerol, 10 mM dithiothreitol [DTT]) in a total volume of 10 μl for 3 h at 37 °C. Cleavage was terminated by addition of sodium dodecyl sulfate (SDS) sample buffer and boiling for 5 min. The products of cleavage reactions were separated by SDS–PAGE, and the gels were dried and autoradiographed.

2.5. Real-time PCR

Total RNA was isolated using TRI Reagent (Sigma–Aldrich). An aliquot of total RNA was transcribed into cDNA using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). Real-time PCR assays were conducted using IQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a BioRad CFX96 real-time PCR machine (Bio-Rad Laboratories). Primer sequences for real-time PCR were as follows: CCNA1, 5′-TTCCCGCAATCATGTAC CCTG-3′ (forward) and 5′-TGTAGCCAGCACAACTCCACT-3′ (reverse); CCNA2, 5′-TGGACCTTCACCAGACCTAC-3′ (forward) and 5′-GGTTGACCAGAGAAACACCA-3′ (reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as internal standard), 5′-GAACATCATCCCTGCCTCTAC-3′ (forward) and 5′-CTCCGAC GCCTGCTTCACC-3′ (reverse). The thermocycling protocol involved an initial 10 min denaturing step at 95 °C, followed by 39 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s. Amplification of a single product was confirmed by a melting curve analysis (from 65 to 95 °C). Relative quantification of CCNA1 and CCNA2 expression levels was determined by the $2^{-\Delta\Delta Ct}$ method [11]. The fold induction relative to the control groups was expressed as means ± standard deviation of three independent experiments.

2.6. Transfections

The cyclin A1 construct was subcloned into the pFLAG-CMV-5b expression vector (Sigma–Aldrich) for expression of FLAG-tagged cyclin A1 (FLAG-CCNA1). siRNA targeting cyclin A1, caspase-1, and control (scrambled) siRNA were purchased from Ambion (Life Technologies). A549 and MCF7 cells were transfected with 1 μg of plasmid or 60 nM siRNA using Lipofectamine 2000 (Life Technologies), according to the manufacturer's recommendations.

3. Results

3.1. Doxorubicin reduces cyclin A1 expression at the protein level

We initiated our studies by examining the cytotoxic effects of doxorubicin in the human non-small cell lung cancer line A549 using MTT assays. Doxorubicin alone caused a concentration-dependent decrease in the viability of A549 cells; the calculated 50% inhibitory concentration (IC_{50}) was 0.25 μg/ml (Fig. 1A). Doxorubicin is well known to be a DNA damage-inducing agent. Thus, we examined the expression level of proteins associated with the DNA damage response. p53 accumulated after treatment with doxorubicin, as previously reported [7]. Interestingly, both cyclin A1 and A2 were downregulated by doxorubicin treatment, and caspase-1 was upregulated (Fig. 1B). To further investigate the decrease in cyclin A1 and A2 in A549 cells treated with doxorubicin, we examined the corresponding mRNA levels using real-time RT-PCR. As shown in Fig. 1C, cyclin A2 mRNA levels decreased upon doxorubicin treatment, consistent with the observed reduction in cyclin A2 protein levels. In contrast, doxorubicin induced an increase in cyclin A1 mRNA levels, despite downregulation of cyclin A2 protein, suggesting that the decrease in cyclin A1 induced by doxorubicin is not due to transcriptional regulation of the *CCNA1* gene. Exogenously expressed FLAG-cyclin A1, introduced by transient transfection, was also decreased by doxorubicin treatment, supporting the post-transcriptional downregulation of cyclin A1 in these cells.

3.2. Caspase-1 mediates doxorubicin-induced downregulation of cyclin A1

Since caspase-1 was induced by doxorubicin, we used the caspase-1 specific inhibitor, z-YAVD-fmk to test whether cyclin A1

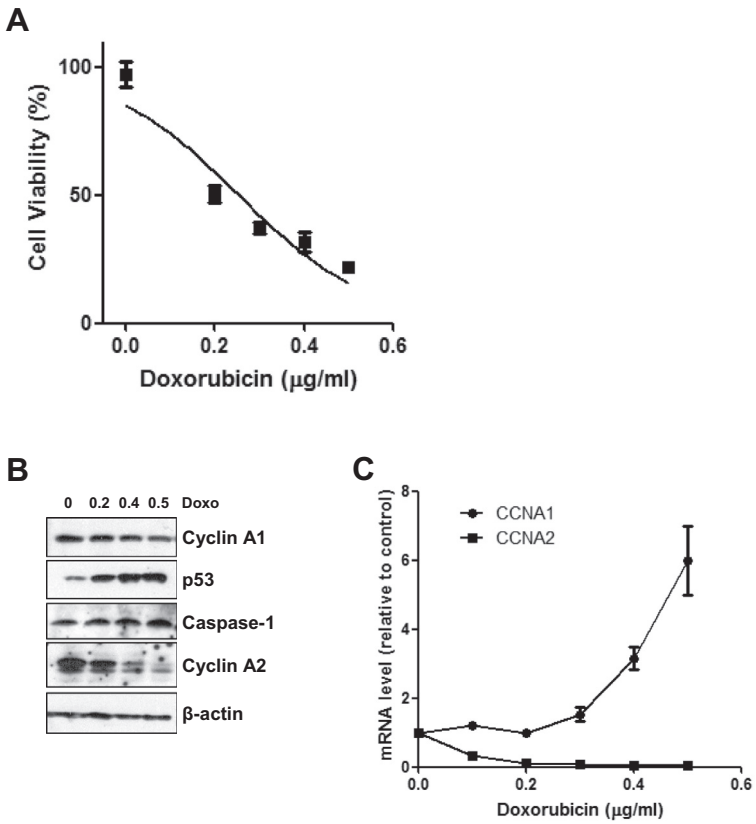

Fig. 1. Inhibition of survival and cyclin A1 expression in A549 cells by doxorubicin treatment. (A) Cells were treated with 0–0.5 μg/ml doxorubicin for 48 h, and then assayed for viability by MTT assay as described in Section 2. Survival relative to the control (defined as 100%) was determined and IC_{50} values were calculated. (B) Cells were treated with 0–0.5 μg/ml doxorubicin for 24 h and immunoblotted as described in Section 2. Immunoblots are representative of at least two independent experiments. (C) A549 cells were treated with 0–0.5 μg/ml doxorubicin for 24 h. The levels of cyclin A1 (CCNA1) and cyclin A2 (CCNA2) mRNA were measured by real-time PCR as described in Section 2. Each value represents the mean ± standard deviation of three independent experiments.

is cleaved by caspase-1 in cells treated with doxorubicin. Endogenous cyclin A1 expression levels were rescued by pretreatment of A549 cells with z-YAVD-fmk prior to exposure to doxorubicin (Fig. 2A). To determine if this effect was cell-type specific, we examined downregulation of cyclin A1 by doxorubicin in the human breast cancer cell line, MCF7. Treatment with doxorubicin (0.2 μg/ml) resulted in a considerable reduction of cyclin A1 protein expression, an effect that was reversed by pretreatment with z-YAVD-fmk, indicating that this downregulation is not cell line specific (Fig. 2B). To further confirm caspase-1-dependent cleavage of cyclin A1, we transfected MCF7 cells with caspase-1 siRNA before treatment of doxorubicin. The expression level of cyclin A1 was markedly recovered by depletion of caspase-1 in the doxorubicin-treated cells (Fig. 2C). In addition, PARP cleavage, a marker of apoptosis, was decreased in the caspase-1-knockdown cells (Fig. 2C), suggesting the cleavage of cyclin A1 might mediate apoptosis in doxorubicin-treated cells.

3.3. Cleavage of cyclin A1 by caspases in vitro

To further confirm the caspase-dependent cleavage of cyclin A1, we incubated cyclin A1 with various active caspases *in vitro*. As shown, two cleavage products were evident in reactions containing caspase-1, -3, -6, -9, or -10 (Fig. 3A). Caspase-1 and caspase-3 more efficiently cleaved cyclin A1 than did the other caspases. Starting from a consideration of the sizes of the cleavage products (~33 and ~20 kDa), we examined the sequence of cyclin A1 for putative caspase recognition and cleavage sequences. We identified two such sites, one at 162–165 and the second at 246–249 corresponding to the amino acid sequences Tyr-Glu-Val-Asp (YEVD) and Ile-Leu-Val-Asp (ILVD), respectively. Using site-directed mutagenesis, we mutated the Asp residues at positions 165 and 249 to Glu, and then tested the ability of active caspase-1 and -3 to cleave proteins translated *in vitro* from these cyclin A1 mutants. These *in vitro* cleavage assays revealed that wild-type cyclin A1 and the D249E mutant were efficiently cleaved by caspase-1 and -3 (Fig. 3B). In contrast, the D165E mutant was resistant to caspase-mediated cleavage, indicating that the caspase recognition and cleavage sequence in cyclin A1 corresponds to position 165. Caspase-3 rapidly eliminated full-length cyclin A1, suggesting that it might cleave cyclin A1 at multiple sites. Indeed, the N-terminal fragment was likely further cleaved by caspase-3 (Fig. 3A). To further examine this possibility, we generated truncated variants of cyclin A1 containing only the N-terminus (ΔC) or C-terminus (ΔN), and tested *in vitro* cleavage of these variants by caspase-3. As shown in Fig. 3C, caspase-3 cleaved both ΔN and ΔC into smaller fragments. To see whether doxorubicin induces the cleavage of cyclin A1 at 165, we overexpressed wild-type cyclin A1 or the D165E mutant in MCF7 cells, followed by exposure of doxorubicin. The D165E mutant was significantly resistant to the doxorubicin-induced cleavage compared with the wild-type (Fig. 3D). The mutation at 165 did not completely block the cleavage of cyclin A1, suggesting that cyclin A1 may have multiple cleavage sequences for caspases activated by doxorubicin. Furthermore, the overexpression of the D165E mutant suppressed the induction of PARP cleavage by doxorubicin (Fig. 3D). These results support that doxorubicin may induce apoptotic cell death through the cleavage of cyclin A1 at position 165.

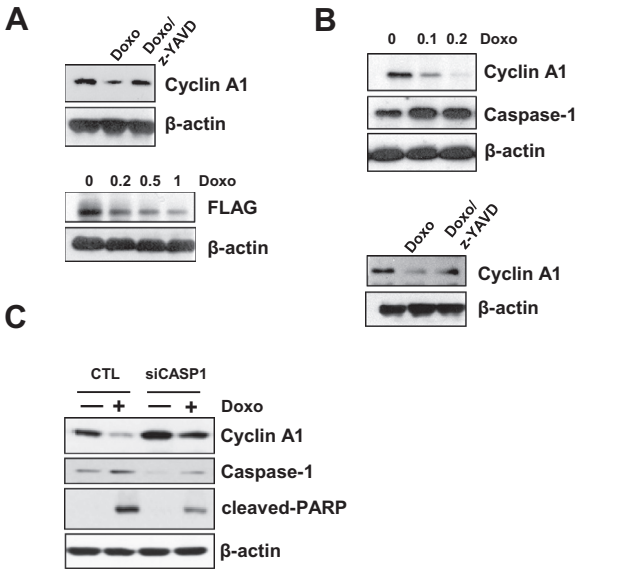

Fig. 2. Downregulation of cyclin A1 by doxorubicin is dependent on the activity of caspase-1. (A) A549 cells were pretreated with z-YAVD-fmk (100 μM) for 1 h and then exposed to 0.5 μg/ml doxorubicin for 24 h (upper panel). Cells were transfected with 1 μg of FLAG-CCNA1 expression plasmid. After 24 h, cells were treated with 0–1 μg/ml doxorubicin (lower panel). Cells were lysed and lysates were analyzed by immunoblotting. (B) MCF7 cells were treated with the indicated concentration of doxorubicin for 24 h with (lower panel) or without (upper panel) pretreatment with z-YAVD-fmk. Cell lysates were analyzed by immunoblotting. The images are representative of at least two independent experiments. (C) MCF7 cells were transfected with control siRNA or caspase-1 siRNA (siCASP1) and incubated with or without 0.5 μg/ml doxorubicin for 24 h. Cells were lysed and lysates were analyzed by immunoblotting.

3.4. Cyclin A1 expression is involved in the induction of cell death by DNA-damaging stimuli

Next, we examined the effect of cyclin A1 expression on the viability of A549 cells treated with DNA-damaging stimuli. Overexpression of FLAG-cyclin A1 by transfection of cells with the FLAG-CCNA1 vector markedly protected cells from doxorubicin-induced cytotoxicity (Fig. 4A). To further investigate the protective role of cyclin A1 in A549 cells following DNA damage, we tested the effects of cyclin A1 siRNA in cells exposed to ionizing radiation. Knockdown of cyclin A1 with siRNA significantly sensitized cells to ionizing radiation (5 Gy) compared with controls (Fig. 4B). These results support the conclusion that cyclin A1 plays a protective role in the response of cells to DNA damage.

4. Discussion

Expression of cyclin A1 in human cancer cells has been reported to increase in response to UV and γ-irradiation through direct activation of the cyclin A1 promoter by p53, although rather high doses of irradiation (>25 Gy) were used to detect the increase in cyclin A1 in these studies [4,12]. Here, we also observed an increase in cyclin A1 mRNA expression following doxorubicin treatment in A549 cells, suggesting that induction of p53 by doxorubicin activates cyclin A1 transcription. However, despite this increase at the mRNA level, cyclin A1 was decreased at the protein levels in doxorubicin-treated A549 and MCF7 cells, an effect that was concentration dependent. We further demonstrated that caspase-1 activated by p53 mediates downregulation of cyclin A1 in A549 and MCF7 cells. We speculate that the reduction in cyclin A1 protein levels is attributable to dominance of the cleavage-mediated degradation process mediated over the transcriptional activation of the corresponding gene. Thus, p53 may function as both a negative and positive regulator of cyclin A1 expression, depending on cellular context and dose of DNA-damaging stimulus.

Induction of p53 regulates the expression of a number of target genes that contribute to cellular responses. Caspase-1 is also known to be a transcriptional target of p53 [13]. We observed induction of caspase-1 in the p53-positive cell lines A549 and MCF7, but not in the p53-negative cell line H1299 (data not shown). In addition, cyclin A1 protein levels were not reduced by doxorubicin in H1299 cells, further supporting the p53-dependent downregulation of cyclin A1.

It has been suggested that proteolytic cleavage of cyclins leads to altered substrate specificity of the associated kinases [6,14]. Caspase-mediated cyclin E cleavage inhibits cyclin E interaction with Cdk2 and therefore inactivates Cdk2 kinase activity. Furthermore, the p18-cyclin E fragment generated by caspase-mediated cleav-

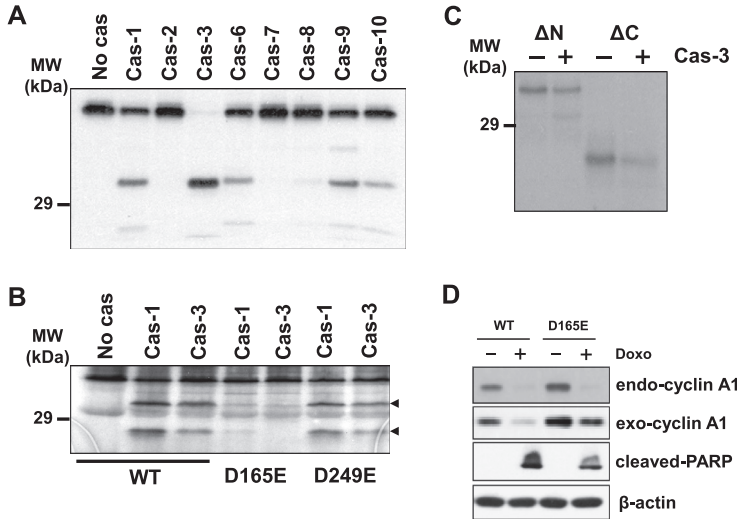

Fig. 3. Caspase-dependent cleavage of cyclin A1. (A) ^{35}S-labeled cyclin A1 was incubated with recombinant, active caspases for 3 h at 37 °C. (B) Two potential cleavage sites (Asp-165 and Asp-249) were mutated to Glu residue by *in vitro* mutagenesis. ^{35}S-labeled wild-type or mutant (D165E and D249E) cyclin A1 was incubated with recombinant, active caspase-1 or caspase-3 for 3 h at 37°. (C) ^{35}S-labeled ΔC (aa 1–165) or ΔN (aa 166–465) was incubated with caspase-3 for 3 h at 37 °C. Cleavage of cyclin A1 was examined by SDS–PAGE and autoradiography. The autoradiograph shown is representative of at least two separate experiments. (D) MCF7 cells were transfected with 1 μg pcDNA-cyclin A1 wild-type (WT) or pcDNA-cyclin A1 mutant (D165E) plasmid and incubated with or without 0.5 μg/ml doxorubicin for 24 h. Cells were lysed and lysates were analyzed by immunoblotting.

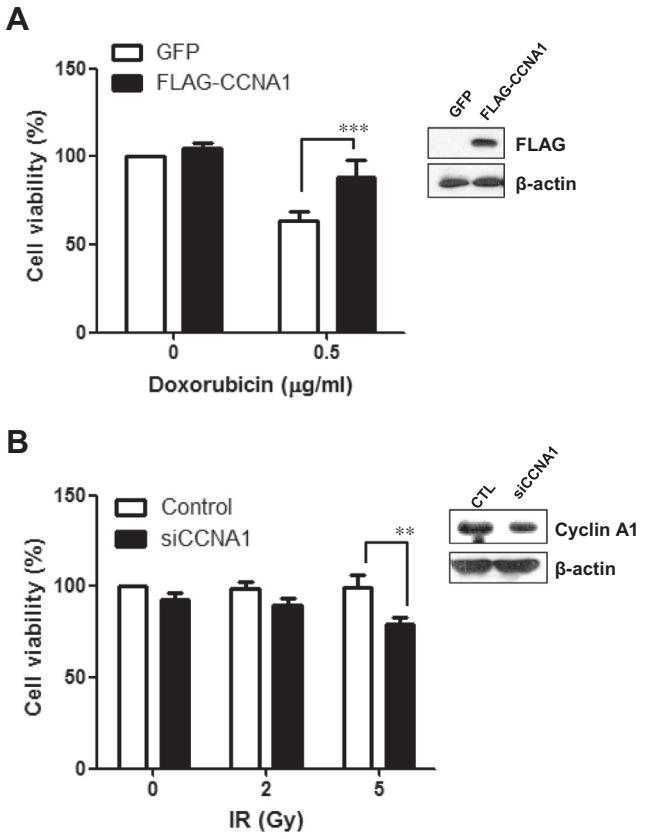

Fig. 4. Cyclin A1 expression inhibits the cytotoxicity of doxorubicin and irradiation (IR) in A549 cells. (A) Cells were transfected with 1 μg GFP (control) or FLAG-CCNA1 plasmid and incubated with or without 0.5 μg/ml doxorubicin for 24 h. (B) Cells were transfected with control siRNA or cyclin A1 siRNA (siCCNA1) and left untreated or were treated with IR (2 or 5 Gy) for 24 h. Viability was determined by MTT assay, as described in Section 2. The viability of control cells was defined as 100%, and survival relative to the control was calculated. The expression of cyclin A1 and FLAG-cyclin A1 were assessed by immunoblotting. Columns represent means ± standard deviation of three independent experiments ($^{**}p < 0.01$, $^{***}p < 0.001$).

age triggers apoptosis in hematopoietic cells. In *Xenopus,* cyclin A2 is cleaved by caspases to generate a truncated form that complexes with Cdk2. The complex has increased kinase activity, leading to apoptosis through phosphorylation of histone H2B. A truncated form of cyclin D1 generated by caspase has a higher affinity for the CDK inhibitor, p27, leading to failure to phosphorylate the retinoblastoma protein. Our results showed that two cyclin A1 fragments generated by caspase-1 were further cleaved by caspase-3 at unknown sites. In fact, we found that overexpression of ΔN or ΔC had no effect on the viability of A549 cells (data not shown). Thus, it is unlikely that the truncated forms have cellular functions different from those of intact cyclin A1. It is known that cyclin A1 has an anti-apoptotic role in the DNA-damage response. Here, we discovered a new pathway for regulation of cyclin A1 expression through which cancer cells can be sensitized to radiation therapies.

Conflicts of interest

The authors have no potential conflicts of interest to disclose.

Acknowledgments

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