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G1 versus G2 cell cycle arrest after adriamycin-induced damage in mouse Swiss3T3 cells

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Abstract Cell cycle arrest after different types of DNA damage can occur in either G1 phase or G2 phase of the cell cycle, involving the distinct mechanisms of p53/p21$^{Cip1/Waf1}$ induction, and phosphorylation of Cdc2, respectively. Treatment of asynchronously growing Swiss3T3 cells with the chemotherapeutic drug adriamycin induced a predominantly G2 cell cycle arrest. Here we investigate why Swiss3T3 cells were arrested in G2 phase and not in G1 phase after adriamycin-induced damage. We show that adriamycin was capable of inducing a G1 cell cycle arrest, both during the G0-G1 transition and during the G1 phase of the normal cell cycle. In G0 cells, adriamycin induced a prolonged cell cycle arrest. However, adriamycin caused only a transient cell cycle delay when added to cells at later time points during G0-G1 transition or at the G1 phase of normal cell cycle. The G1 arrest correlated with the induction of p53 and p21$^{Cip1/Waf1}$, and the exit from the arrest correlated with the decline of their expression. In contrast to the G1 arrest, adriamycin-induced G2 arrest was relatively tight and correlated with the Thr-14/Tyr-15 phosphorylation of cyclin B-Cdc2 complexes. The relative stringency of the G1 versus G2 cell cycle arrest may explain the predominance of G2 arrest after adriamycin treatment in mammalian cells.

Key words: CDK; Cell cycle control; Cyclin; DNA damage; p53

1. Introduction

Cyclins and cyclin-dependent kinases (CDKs) are key regulators of the eukaryotic cell cycle. Cyclin B is associated with Cdc2 and the cyclin B-Cdc2 complexes regulate entry into M phase [1]. The cyclin A-Cdk2 and cyclin E-Cdk2 complexes control progression through S phase and the G1-S transition respectively [2,3]. D-type cyclins are associated with Cdk4 and Cdk6 and the complexes are required for G1 progression [2].

The kinase activity of CDK is tightly regulated by an intricate system of phosphorylation and protein-protein interaction [4]. By definition, the activation of CDKs is dependent on the association with a cyclin subunit. CDKs are also regulated by phosphorylation: the activity of CDK holoenzyme is increased by phosphorylation of Thr-161 and inhibited by phosphorylation of Thr-14/Tyr-15. Thr-161 can be phosphorylated by CDK-activating kinase (CAK) [5], and dephosphorylated by the phosphatase KAP [6]. Thr-14/Tyr-15 can be phosphorylated by the Wee1 and Myt1, and dephosphorylated by members of the Cdc25 phosphatase family [5]. The activity of CDKs can be inhibited by binding to CDK inhibitors, which include the p21 family (p21$^{Cip1/Waf1}$, p27^{Kip1}, and p57^{Kip2}) and the p16^{INK4A} family (p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, p19^{INK4D}) [7].

Proper control of cell cycle progression requires several checkpoint controls, which regulate the activities of different CDKs to ensure that each stage of the cell cycle is completed before the next stage is initiated [8]. Deregulation of these checkpoint controls may allow cell cycle progression to become insensitive to external signals and DNA damage [9].

Following DNA damage, two strategies are generally adopted by cells to ensure that mutations are not passed on to the daughter cells. On the one hand, the cell cycle can be arrested transiently to allow time for DNA repair or be permanently arrested and enter a senescent state [10]. On the other hand, the cells with damaged DNA can be eliminated by apoptosis [11]. After DNA damage, the cell cycle can be arrested in either G1 phase or G2 phase [12]. Both cell cycle arrest and apoptosis after DNA damage involve p53 [13]. The level of p53 is negatively regulated by binding to MDM2, which targets p53 for proteasome-dependent degradation [14]. Phosphorylation of Ser-15 and Ser-37 on p53 by ATM or similar kinases after DNA damage inhibits the binding of MDM2 to p53, hence increase the level and activity of p53 [15].

Once activated, p53 induces the expression of the CDK inhibitor p21$^{Cip1/Waf1}$, which in turn may be responsible for the cell cycle arrest. Embryonic fibroblasts derived from p21$^{-/-}$ mice are impaired in their ability to arrest in G1 following DNA damage by ionizing irradiation [16,17]. In normal human fibroblasts, p21$^{Cip1/Waf1}$ is sufficient for the inhibition of cyclin A/E-Cdk2, and partly responsible for the inhibition of cyclin D-Cdk4 after DNA damage, but is not responsible for the inhibition of cyclin A/B-Cdc2 [18,19]. Instead of binding to p21$^{Cip1/Waf1}$, the mitotic cyclin A/B-Cdc2 complexes are likely to be inhibited by phosphorylation on Thr-14/Tyr-15 after DNA damage [20]. Upon DNA damage, the protein kinase Chk1 is activated and in turn phosphorylates the Ser-216 residue of Cdc25C. Phosphorylation of Cdc25C Ser-216 allows the protein 14-3-3 to bind to Cdc25C and inactivates its phosphatase activity [21–23].

We have been interested in the mechanisms of how different cyclin-CDK complexes that drive different parts of the cell cycle are turned off after DNA damage [19,20,24,25]. Given that cyclin-CDK complexes that control both G1 phase and G2 phase are turned off after DNA damage, one important question is why some DNA damaging agents arrest cells predominantly in G1 phase, while other agents arrested cells predominantly in G2 phase. When adriamycin was added to

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Abbreviations: CDK, cyclin-dependent kinase; GST, glutathione-S-transferase; Rb, retinoblastoma gene product; UV, ultraviolet light

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asynchronously growing cells, most of the cells became arrested in G2 phase. Here we investigate the basis of G1 versus G2 cell cycle arrest after adriamycin-induced damage in mouse fibroblasts.

2. Materials and methods

2.1. Cell culture

Murine Swiss3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS) in a humidified incubator with 10% CO_2 at 37°C. Adriamycin (also called doxorubicin) (Calbiochem, La Jolla, USA) was added at 0.2 μg/ml final to the medium. Cell-free extracts were prepared as described [24]. For immunoblotting analysis, the cell extracts were adjusted to 1 mg/ml in SDS-sample buffer and 10 μg was loaded onto SDS-PAGE. Flow cytometry analysis was as described [25].

2.2. Cell synchronization

For serum starvation synchronization, Swiss3T3 cells were grown in DMEM supplemented with 10% v/v FBS until the cultures reached about 50% confluence. The medium was then changed to DMEM with 0.2% v/v FBS for 48 h. Cells were released from the G0 arrest by supplying the cells with DMEM containing 10% v/v FBS. For double thymidine synchronization, cells were incubated in medium containing 2 mM thymidine for 16 h. The cells were released from the first block by removing the thymidine-containing medium, washed twice with phosphate-buffered saline (PBS) (170 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), and incubated in medium containing 24 μM deoxycytidine. After 9 h, 2 mM of thymidine was added to the medium to block the cells a second time. After 16 h, the cells were released from the second block as above. For serum starvation followed by a single thymidine block, cells were first arrested at G0 by serum starvation as described above, and were blocked in S phase by incubating in DMEM containing 10% v/v FBS and 2 mM thymidine for 24 h. The cells were then released by removing the thymidine-containing medium, washed twice with PBS, and incubated in medium containing 24 μM deoxycytidine. For mitotic block synchronization, cells were arrested at G2/M by incubating in medium containing 0.1 μg/ml nocodazole for 16 h. To harvest the M phase-arrested cells, the medium was removed and 10 ml of PBS was added to the plate followed by vortexing for 2 min. The cells in the buffer and in the medium were collected by centrifugation, washed with PBS, and replated in fresh medium. Experiments described in this study have been repeated at least twice.

2.3. Protein kinase assays

Protein kinase assays using 1 μg of histone H1 or 2 μg of GST-Rb (containing the Rb pocket only) as substrates were performed as described [19].

2.4. Antibodies and immunological methods

Anti-cyclin A monoclonal antibody E23 [24], anti-cyclin D1 polyclonal antibodies [19], anti-Cdc2 monoclonal antibody A17 [26], and rat monoclonal antibodies YL1/2 against mammalian tubulin [27] were as previously described. Rabbit anti-cyclin E antibodies were gifts from M. Ohtsubo (Kurume University, Japan). Rabbit anti-cyclin B1 polyclonal antibodies were gifts from K. Yamashita (Kanazawa University, Japan). Anti-p53 monoclonal antibody 421 was from T. Hunt (Imperial Cancer Research Fund, UK). Anti-cyclin B1 monoclonal antibodies GNS1 (sc-245) and polyclonal antibodies raised against the C-terminal peptide of $p21^{Cip1/Waf1}$ (sc-397) were from Santa Cruz Biotechnology. Immunoprecipitation and immunoblotting were performed as previously described [19].

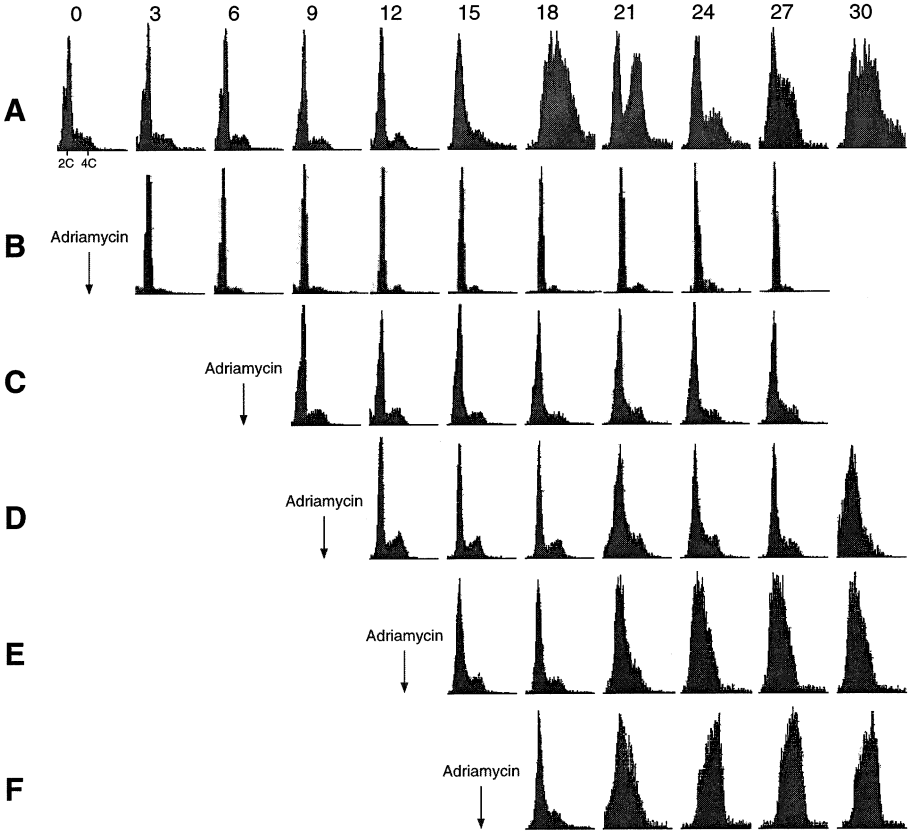

Fig. 1. Induction of cell cycle arrest by adriamycin during G0-G1 transition. Swiss3T3 cells were arrested at G0 by serum starvation as described in Section 2. The cells were released into the cell cycle by growing in medium containing 10% fetal bovine serum. Different sets of cells were either (A) untreated, or treated with adriamycin at (B) 0 h; (C) 6 h; (D) 9 h; (E) 12 h; (F) 15 h after released from serum starvation. The cells were harvested for flow cytometry analysis at the indicated time points. The arrows indicate the time of adriamycin addition.

3. Results

3.1. Adriamycin induces a G2/M arrest in Swiss3T3 cells, but can cause a G1 arrest during G0-G1

We used the mouse Swiss3T3 cells to study the DNA damage checkpoints because these cells display a p53/p21$^{Cip1/Waf1}$ response following DNA damage, and can be arrested in G0 by serum starvation or contact inhibition [20,24,25]. Treatment of asynchronously growing Swiss3T3 cells with the chemotherapeutic drug adriamycin (also called doxorubicin) induced a G2/M cell cycle arrest [25]. The majority of the Swiss3T3 cells were arrested at G2/M phase 48 h after treatment with adriamycin. The main issue that we sought to address in this study was why Swiss3T3 cells arrested in G2/M phase and not in G1 phase after adriamycin-induced damage.

We first investigated whether adriamycin is capable of causing a G1 cell cycle arrest in Swiss3T3 cells by treating cells with adriamycin during the G0-G1 progression. Cells were arrested in the quiescent G0 state by serum starvation, and the cells were released synchronously into the cell cycle by incubating in medium containing serum. Adriamycin was added to the medium at different times after release from serum starvation, and the position of the cell cycle was estimated by flow cytometry. Fig. 1A shows that after addition of serum, the cells moved progressively through S phase (between 15–18 h), G2/M phase (around 21 h), G1 phase, and again into the next S phase (around 30 h). In contrast, when adriamycin was added at the time of G0 release (Fig. 1B), the cells were arrested with a G0/G1 DNA content and did not enter the cell cycle. The G0/G1 cell cycle arrest was observed when cells were treated with adriamycin 6 h into the cell cycle from G0 release (Fig. 1C). However, when the cells were treated with adriamycin at later times (9 h and 12 h), the cells did enter S phase, albeit much delayed in comparison to untreated cells (Fig. 1D and E). When adriamycin was added at 15 h after release from serum starvation (about 1–3 h before the normal S phase), the cells did not arrest in G1 phase, but

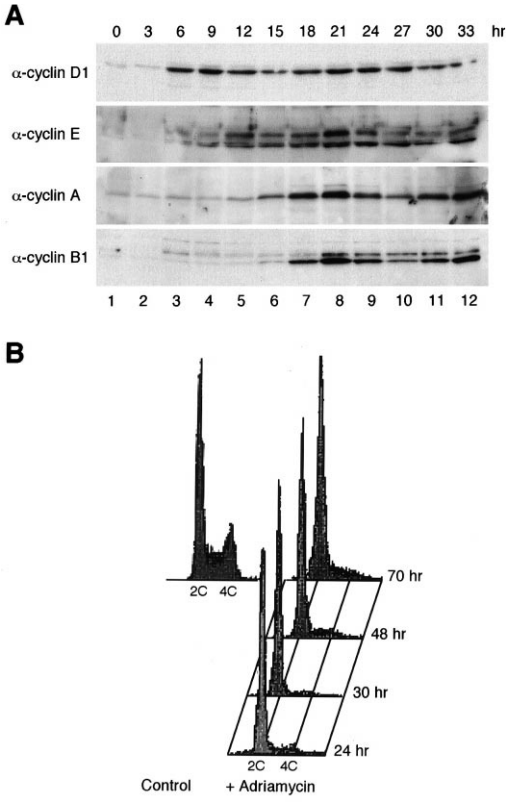

Fig. 2. Cyclin expression and cell cycle arrest in serum starved cells. (A) Swiss3T3 cells were arrested at G0 by serum starvation, and were released into the cell cycle by growing in medium containing 10% fetal bovine serum. The cells were harvested at the indicated time points and cell-free extracts were prepared. Cyclin D1, cyclin E, cyclin A, and cyclin B1 in the cell extracts were detected by immunoblotting as indicated. (B) DNA damage induced a prolong cell cycle arrest in G0 cells. Swiss3T3 cells were arrested at G0 by serum starvation. The cells were released synchronously into the cell cycle by incubating in medium containing 10% fetal bovine serum. At the time of release, the cells were treated with adriamycin and harvested after 24 h, 30 h, 48 h, and 70 h for flow cytometry analysis. Cells that were released into the cell cycle but without adriamycin were harvested at 70 h as controls. The positions of the 2C and 4C DNA contents are indicated.

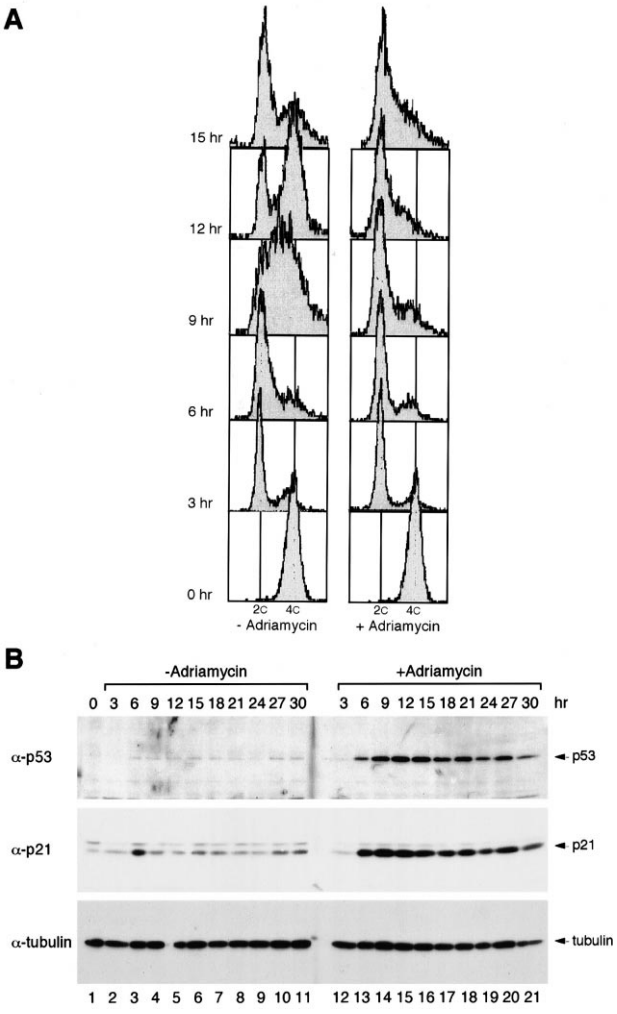

Fig. 3. Cell cycle arrest induced by adriamycin treatment in G1 phase. Swiss3T3 cells were released synchronously from G2/M phase by mitotic shake off as described in Section 2. Adriamycin was added 1 h after the cells were released. At the indicated time points after mitotic shake off, cells were harvested for (A) flow cytometry analysis and (B) cell extracts preparation. The cell extracts were dissolved in SDS-sample buffer, applied onto SDS-PAGE (lanes 1–11: untreated; lanes 12–21: adriamycin-treated), and subjected to immunoblotting with antibodies against p53 (top panel), p21$^{Cip1/Waf1}$ (middle panel), or tubulin (bottom panel).

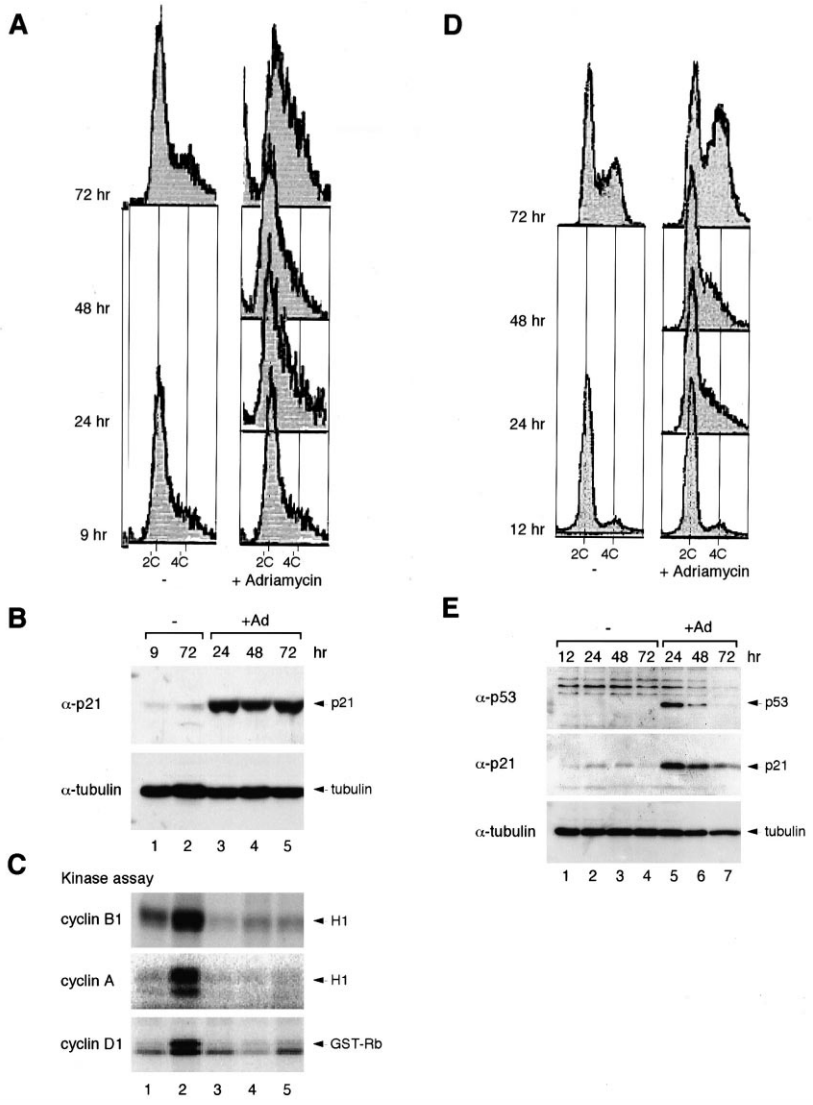

Fig. 4. Adriamycin induces a transient cell cycle arrest in G1 phase. Swiss3T3 cells were released from serum starvation and treated with adriamycin at 9 h after addition of serum. At the indicated time points after release, cells were harvested for (A) flow cytometry analysis and (B) cell extract preparation. Cell extracts were applied onto SDS-PAGE (lanes 1–2: untreated; lanes 3–5: adriamycin-treated), and subjected to immunoblotting with antibodies against $p21^{Cip1/Waf1}$ (upper panel) or tubulin (lower panel). (C) Cell extracts (200 μg each) were immunoprecipitated with antibodies against cyclin B1 (top panel), cyclin A (middle panel), or cyclin D1 (bottom panel). The kinase activities against histone H1 (for cyclin B1 and cyclin A) and GST-Rb (for cyclin D1) were assayed. (D) Swiss3T3 cells were released synchronously into the cell cycle from serum starvation, and treated with adriamycin exactly as in panel (A), except that the cells were treated at 12 h after addition of serum. Cell cycle distribution was analyzed by flow cytometry. (E) Cell extracts were prepared from cells described in panel (D), applied onto SDS-PAGE (lanes 1–4: untreated; lanes 5–7: adriamycin-treated), and subjected to immunoblotting with antibodies against p53 (top panel), $p21^{Cip1/Waf1}$ (middle panel), or tubulin (bottom panel).

continued the cell cycle to arrest in G2/M phase (Fig. 1F). The time when adriamycin could induce a G0/G1 cell cycle arrest was between the time when cyclin D1 and cyclin A were expressed, and similar to that of cyclin E (Fig. 2A).

Taken together, these data indicate that unlike in growing cells, cells in the G0-G1 transition can be arrested in G0/G1 with adriamycin. However, the G0/G1 arrest was only effective when adriamycin was added within 9 h from serum starvation release. Adriamycin treatment later in the cell cycle caused only a delay in the entry into S phase or a normal S phase entry but a G2/M cell cycle arrest. Hence the window of time that adriamycin-induced damage can result in G0/G1 cell cycle arrest was from G0 up to about 3 h from S phase, which roughly corresponded to the time of cyclin E expression and the restriction point (see Section 4).

3.2. Adriamycin induces a prolonged cell cycle arrest in G0

We next investigated whether the cell cycle arrest caused by adriamycin during G0-G1 described above was a transient cell cycle delay, or represented a longer term arrest. Serum starved cells were treated with serum and adriamycin, and harvested at time points for up to 3 days for analysis. Fig. 2B shows that the adriamycin-treated cells remained in a G0/G1 cell cycle arrest state for up to 70 h. Hence the arrest caused by adriamycin in quiescent cells probably represented a long-term cell cycle arrest.

3.3. Adriamycin induces a transient cell cycle arrest in G1 phase, which correlates with the induction of p53 and p21$^{Cip1/Waf1}$

Given that adriamycin can cause a G1 cell cycle arrest or delay when added to cells released from serum starvation, we next asked whether adriamycin could also cause a G1 phase arrest or delay during the normal cell cycle. Swiss3T3 cells were synchronously released into G1 phase by mitotic shake off. After the cells were attached to the plate, either buffer or adriamycin was added to the medium. Fig. 3A shows that in the absence of adriamycin, the majority of the cells entered G1 phase promptly after release from mitotic shake off and moved progressively through the cell cycle and into the next G1 phase. In contrast, the cells were arrested in G1 phase when adriamycin was added. Significantly, however, the cells slowly entered S phase at the later time points (12 and 15 h). To confirm that p53 and p21$^{Cip1/Waf1}$ were induced following adriamycin treatment, cell extracts were prepared from cells harvested at different time points, and were subjected to immunoblotting with the respective antibodies. Fig. 3B shows that both p53 and p21$^{Cip1/Waf1}$ were induced shortly after cells in G1 phase were treated with adriamycin, but not in the untreated cells. As a control, similar levels of tubulin were detected irrespective of the presence or absence of adriamycin. These results indicate that like the G0-G1 transition, the normal cell cycle can also be delayed by adriamycin treatment.

We next investigated the adriamycin-induced G1 arrest over longer period of time (up to 72 h). Swiss3T3 cells released from serum starvation were treated with adriamycin at either 9 h or 12 h (Fig. 4). Similar to above, adriamycin caused a G1 cell cycle arrest. But at later time points, a significant proportion of cells re-entered S phase and G2 phase. This was more marked in cells treated with adriamycin at the later time (12 h) than the earlier time (9 h). Fig. 4 shows that p21$^{Cip1/Waf1}$ was induced in adriamycin-treated cells. The level of p21$^{Cip1/Waf1}$ remained elevated during the course of the experiment when adriamycin was added at 9 h after release from starvation. Interestingly, p21$^{Cip1/Waf1}$ was also induced when adriamycin was added at 12 h, but then decreased at later time points. Hence the activation of p53/p21$^{Cip1/Waf1}$ after adriamycin treatment correlated well with the G1 cell cycle arrest, and the decrease in p53/p21$^{Cip1/Waf1}$ at later time points may correlate with the re-entry into S phase and G2 phase. To look at the kinase activities associated with the cyclin-CDK complexes, cyclins were immunoprecipitated from cell extracts and the kinase activities towards histone H1 (for cyclin B and cyclin A) or GST-Rb (for cyclin D1) were measured (Fig. 4C). In untreated cells, the kinase activities associated with the cyclins were relatively low at 9 h (early G1 phase), and increased at 72 h (loss of synchrony). In contrast, the kinase activities associated with all the cyclins remained low after adriamycin treatment. Collectively, these results show that adriamycin can induce a G1 arrest when added to Swiss3T3 cells in G1 phase, but the G1 arrest is transient, and the cells start to enter S phase and G2 phase from about 48 h.

3.4. Adriamycin induces a relatively tight cell cycle arrest in G2 phase, which correlates with the phosphorylation of Cdc2

To obtain Swiss3T3 cells in G2 phase, cells were released synchronously from early S phase by double thymidine block. At 4 h after release, adriamycin was added and the cells were harvested for flow cytometry analysis and cell extracts preparation. Fig. 5 shows that the cells entered G2 phase promptly after release from the block (3–6 h). However, after addition of adriamycin, the cells were arrested in G2 phase and did not re-enter G1 phase. Fig. 5B shows that p53 and p21$^{Cip1/Waf1}$ were detected 2 h after addition of adriamycin. Intriguingly, the level of p53 was elevated in cells treated with adriamycin for up to 15 h, but then abruptly returned to close to background level. On the other hand, p21$^{Cip1/Waf1}$ was induced throughout the experiment.

We next investigated whether adriamycin caused a transient

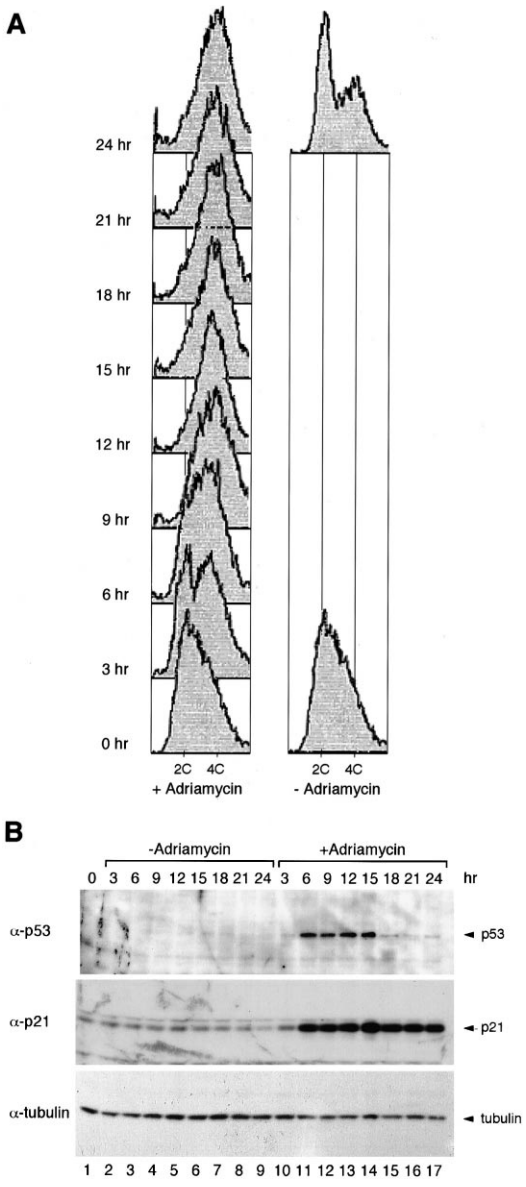

Fig. 5. G2/M cell cycle arrest induced by adriamycin in G2 phase. Swiss3T3 cells were synchronized from early S phase by double thymidine block as described in Section 2. The cells were incubated in medium in the absence (lanes 1–9) or presence (lanes 10–17) of adriamycin at 4 h after release from the second thymidine block. At the indicated time points after the cells were released, cells were harvested for (A) flow cytometry analysis and (B) cell extract preparation. The cell extracts were applied onto SDS-PAGE (lanes 1–9: untreated; lanes 10–17: adriamycin-treated), and subjected to immunoblotting with antibodies against p53 (top panel), p21$^{Cip1/Waf1}$ (middle panel), or tubulin (bottom panel).

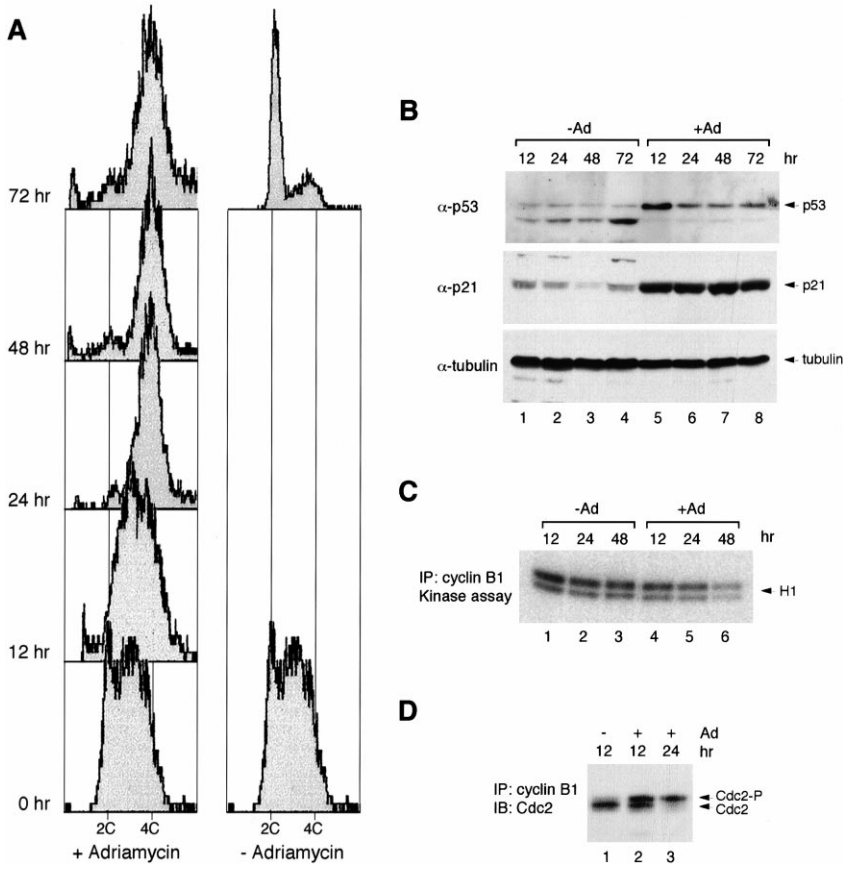

Fig. 6. Prolong G2/M cell cycle arrest induced by DNA damage in G2 cells. Swiss3T3 cells were synchronized from early S phase by serum starvation followed by thymidine block as described in Section 2. The cells were incubated in medium in the absence or presence of adriamycin at 4 h after release from the thymidine block. At the indicated time points, cells were harvested for (A) flow cytometry analysis and (B) cell extract preparation. The cell extracts were applied onto SDS-PAGE (lanes 1–4: untreated; lanes 5–8: adriamycin-treated), and subjected to immunoblotting with antibodies against p53 (top panel), $p21^{Cip1/Waf1}$ (middle panel), or tubulin (bottom panel). (C) The cell extracts (200 μg) were immunoprecipitated with antibodies against cyclin B1 and the histone H1 kinase activities were assayed. (D) Cell extracts (200 μg) derived from untreated cells harvested at 12 h (lane 1), or from cells treated with adriamycin harvested at 12 h (lane 2) and 24 h (lane 3) were immunoprecipitated with anti-cyclin B1 antibodies. The immunoprecipitates were applied onto 17.5% SDS-PAGE, and subjected to immunoblotting with anti-Cdc2 antibodies. The positions of the Thr-14/Tyr-15-phosphorylated and underphosphorylated forms of Cdc2 are indicated.

or prolonged G2/M cell cycle arrest. Fig. 6A shows that when Swiss3T3 cells in G2 phase were treated with adriamycin, the cells were arrested with a G2/M DNA content. Significantly, the cells continued to arrest in G2/M and did not go into G1 phase even after 72 h. Similar to Fig. 5, both p53 and $p21^{Cip1/Waf1}$ were induced initially after adriamycin treatment, but the level of p53 reduced at later time points (Fig. 6B). The kinase activity associated with cyclin B1-Cdc2 (the G2/M cyclin-CDK) was reduced after adriamycin treatment (Fig. 6C). Fig. 6D shows that in the absence of adriamycin, the Cdc2 that associated with cyclin B1 were in the faster migrating, active form. However, after treatment with adriamycin, the Cdc2 that associated with cyclin B1 were shifted to the slower migrating, Thr-14/Tyr-15-phosphorylated inactive form. These data suggest that in comparison to the G1 arrest, the G2/M arrest induced by adriamycin was relatively tight. Furthermore, the G2/M arrest is correlated with the inactivation of cyclin B1-Cdc2 complex and phosphorylation of Cdc2 on Thr-14/Tyr-15.

4. Discussion

Adriamycin is a potent anthracycline, and is one of the most widely used clinical cancer chemotherapeutic drugs. The cellular responses to agents like adriamycin are of key importance to tumorigenesis and cancer chemotherapy. Actions of adriamycin including DNA damage by intercalation and complex formation with topoisomerase II [28], or oxidative effects [29] have been suggested. Most of the cells become arrested in G2 phase when adriamycin is added to asynchronously growing cells. However, it is clear that $p21^{Cip1/Waf1}$ is activated and the G1 and S phase cyclin-CDK complexes are inhibited after adriamycin treatment. Since most of the cells in an asynchronously growing population are in G1 phase, one would expect a predominant G1 cell cycle arrest after adriamycin treatment. Can the predominant G2 cell cycle arrest caused by adriamycin be explained?

We show here that Swiss3T3 cells can be arrested in G1 phase by adriamycin, either during G0-G1 transition or during G1 of the normal cell cycle. However, the G1 arrest was relatively transient, and cells migrated into S phase and G2 phase after 48–72 h. In contrast, the G2 DNA damage checkpoint was more permanent. Hence over a longer period, cells that initially arrested in G1 phase may eventually accumulate in G2 phase.

Given that $p21^{Cip1/Waf1}$ was induced in G1 cells after adria-

mycin treatment, why is it that the cells moved into S phase and G2 phase after an initial delay? We show that both p53 and $p21^{Cip1/Waf1}$ could be detected within several hours following adriamycin treatment. However, the levels of p53 and $p21^{Cip1/Waf1}$ declined over further incubation, which correlated with the exit of G1 cell cycle arrest. This decline of p53 and $p21^{Cip1/Waf1}$ may explain the delayed exit from G1 arrest.

An interesting observation is that after G2 cells were treated with adriamycin, p53 was induced but disappeared after 15 h while the level of $p21^{Cip1/Waf1}$ remained high. Previously we have found that $p21^{Cip1/Waf1}$ has a half-life of about 30 min [19], hence it is unlikely that the level of $p21^{Cip1/Waf1}$ can remain elevated in the complete absence of p53. In previous work, we have also observed that after UV-induced damage, the induction of $p21^{Cip1/Waf1}$ does not correlate with the induction of p53 [19]. One possibility is that although the level of p53 decreased after 15 h, the remaining p53 may have been activated by other post-translational modification. Furthermore, it is possible that other p53-related proteins, like p73, may keep the level of $p21^{Cip1/Waf1}$ elevated.

When adriamycin was added to serum starved cells, the cells entered a prolonged G0/G1 arrest; but when adriamycin was added later during G0-G1 transition or in G1 phase, the entry into S phase and G2 phase was merely delayed. The window of time when the cells are prone to prolonged G0/G1 arrest was from G0 to about the time of cyclin E induction, which is likely to correspond to the restriction point [30]. Further investigations are needed to elucidate the exact relationship between the restriction point and the ability of adriamycin to cause a G0/G1 cell cycle arrest. Since it takes the cells about 18 h to get from G0 into S phase, but just 9 h from normal G2/M into S phase, it is possible that the ability of adriamycin to cause a G0/G1 arrest is because the cells are exposed to adriamycin for a longer period.

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References

[1] King, R.W., Jackson, P.K. and Kirschner, M.W. (1994) Cell 79, 563–571.
[2] Sherr, C.J. (1994) Cell 79, 551–555.
[3] Heichman, K.A. and Roberts, J.M. (1994) Cell 79, 557–562.
[4] Poon, R.Y.C. (1996) in: Encyclopedia of Cancer (Bertino, J.R., Ed.), pp. 246–255, Academic Press, San Diego.
[5] Morgan, D.O. (1997) Annu. Rev. Cell Dev. Biol. 13, 261–291.
[6] Poon, R.Y.C. and Hunter, T. (1995) Science 270, 90–93.
[7] Harper, J.W. (1997) Cancer Surv. 29, 91–107.
[8] Hartwell, L.H. and Weinert, T.A. (1989) Science 246, 629–634.
[9] Elledge, S.J. (1996) Science 274, 1664–1671.
[10] Di Leonardo, A., Linke, S.P., Clarkin, K. and Wahl, G.M. (1994) Genes Dev. 8, 2540–2541.
[11] Liebermann, D.A., Hoffman, B. and Steinman, R.A. (1995) Oncogene 11, 199–210.
[12] O'Connor, P.M. (1997) Cancer Surv. 29, 151–182.
[13] Levin, A.J. (1997) Cell 88, 323–331.
[14] Kastan, M.B. (1996) Bioessays 18, 617–619.
[15] Prives, C. (1998) Cell 95, 5–8.
[16] Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T. and Hannon, G.J. (1995) Nature 377, 552–557.
[17] Deng, C., Zhang, P., Harper, J.W., Elledge, S.J. and Leder, P. (1995) Cell 82, 675–684.
[18] Levedakou, E.N., Kaufmann, W.K., Alcorta, D.A., Galloway, D.A. and Paules, R.S. (1995) Cancer Res. 55, 2500–2502.
[19] Poon, R.Y.C., Jiang, W., Toyoshima, H. and Hunter, T. (1996) J. Biol. Chem. 271, 13283–13291.
[20] Poon, R.Y.C., Chau, M.S., Yamashita, K. and Hunter, T. (1997) Cancer Res. 57, 5168–5178.
[21] Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S. and Piwnica-Worms, H. (1997) Science 277, 1501–1505.
[22] Furnari, B., Rhind, N. and Russell, P. (1997) Science 277, 1495–1497.
[23] Sanchez, Y., Wong, C., Thoma, R.S., Richman, R., Wu, Z., Piwnica-Worms, H. and Elledge, S.J. (1997) Science 277, 1497–1501.
[24] Poon, R.Y.C., Toyoshima, H. and Hunter, T. (1995) Mol. Biol. Cell 6, 1197–1213.
[25] Siu, W.Y., Arooz, T. and Poon, R.Y.C. (1999) Exp. Cell Res. 250, 131–141.
[26] Kobayashi, H., Golsteyn, R., Poon, R., Stewart, E., Gannon, J., Minshull, J., Smith, R. and Hunt, T. (1991) Cold Spring Harb. Symp. Quant. Biol. 56, 437–447.
[27] Yam, C.H., Ng, R.W.M., Siu, W.Y., Lau, A.W.S. and Poon, R.Y.C. (1999) Mol. Cell. Biol. 19, 635–645.
[28] Osheroff, N., Corbett, A.H. and Robinson, M.J. (1994) Adv. Pharmacol. 29, 105–126.
[29] Keizer, H.G., Pinedo, H.M., Schuurhuis, G.J. and Joenje, H. (1990) Pharmacol. Ther. 47, 219–231.
[30] Dou, Q.P., Levin, A.H., Zhao, S. and Pardee, A.B. (1993) Cancer Res. 53, 1493–1497.