G_1 arrest induction represents a critical determinant for cisplatin cytotoxicity in G_1 checkpoint-retaining human cancers

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Cisplatin has been used effectively to treat various human cancer types; yet, the precise mechanism underlying its cytotoxicity remains unknown. In eukaryotes, progression through G_1 is monitored by a checkpoint, which executes G_1 arrest in the event of DNA damage to allow time for repair before initiating DNA replication. The retinoblastoma tumor suppressor gene is an integral component of the mammalian G_1 checkpoint. The utility of the retinoblastoma gene as a therapeutic for human cancers has been investigated. Intriguingly, the cytotoxicity profile of the retinoblastoma gene therapy closely parallels the clinical targets of cisplatin. It prompted an investigation into the potential role of the checkpoint-induced G_1 arrest in cisplatin cytotoxicity. Here, the evidence that G_1 arrest induction represents a critical step in cisplatin-induced lytic path is presented. First, cisplatin-treated human cancer cells undergo a prolonged G_1 arrest before dying. Second, triggering G_1 arrest via infection with a recombinant adenovirus expressing the human retinoblastoma gene is sufficient to potentiate lethality in the absence of cisplatin. Third, the extent of the lethality induced correlates with the G_1-arresting potential of the ectopically expressed human retinoblastoma polypeptide. Fourth, human cancer cells resistant to cisplatin do not undergo G_1 arrest despite cisplatin treatment. The above mechanism may be exploited to develop therapeutics that preserve the efficacy of cisplatin yet bypass its mutagenicity associated with the formation of secondary tumors. *Anti-Cancer Drugs* 18:411–417 © 2007 Lippincott Williams & Wilkins.

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Introduction

Cisplatin has been used effectively to treat a variety of human cancers including the cancers of the head and neck, bladder and others [1]. Yet, its mutagenicity resulting in the formation of secondary tumors in cancer patients dampens cisplatin's therapeutic efficacy [2]. Inside the cells, cisplatin reacts with multiple molecular entities, but its cytotoxicity is primarily attributed to the crosslinking of DNA. The DNA–DNA crosslinks occur via an exchange reaction involving cisplatin's chloride ions with nucleophile groups [3]. With DNA, cisplatin forms a bidentate lesion mostly with two adjacent guanine bases (pGpG), although it may form additional adducts with pApG sequences. Cisplatin also generates interstrand crosslinks, which may interfere with DNA replication. Understanding of the lytic path induced by cisplatin is critical as it may facilitate the development of a therapeutic with greater efficacy.

In eukaryotes, DNA modification by cisplatin activates the G_1 checkpoint. The checkpoint exists to halt cell cycle progression in the event of DNA damage to allow time for repair before proceeding with DNA replication [4]. A key component of this checkpoint is the prototypic tumor suppressor protein retinoblastoma (Rb) [5], whose inactivation predisposes to Rb in humans [6]. The function is consistent with a 'houskeeping' role inferred from its genomic structure [7,8]. Rb is a nuclear phosphoprotein with an intrinsic ability to interact with DNA [9], as evidenced by the ability of purified human recombinant Rb to form a complex with double-stranded DNA [9,10]. Rb has an additional intrinsic property to associate with itself [11]. Phosphorylation serves to regulate negatively self-interaction necessary for Rb oligomerization [10,12,13]. This has led to the proposal that Rb may form a higher-ordered structure to execute its function *in vivo* [13,14]. That Rb closely interacts with chromatin remodeling proteins to regulate gene expression is in keeping with this view [15]. In Rb-deficient cells, DNA damage by cisplatin fails to induce G_1 arrest, confirming Rb's role in the G_1 checkpoint [5].

Following the cloning of the RB gene, the feasibility of extending Rb's tumor suppressing property to clinics was investigated. This has led to the development of RB gene therapy, which successfully suppressed tumorigenesis for several human cancer types in the laboratory. Intriguingly, the cytotoxicity profile of the RB gene therapy closely

parallels that of cisplatin, i.e. ectopically expressed Rb selectively induces lethality in human bladder or head and neck cancers [16,17]. As Rb represents a component of the G_1 checkpoint, it prompted an investigation into the potential role of the checkpoint-induced G_1 arrest in cisplatin cytotoxicity. In this report, the evidence that G_1 arrest induction represents a critical step in cisplatin-induced lytic path is presented. The above mechanism may be exploited to develop therapeutics that preserve the efficacy of cisplatin yet bypass its mutagenicity.

Materials and methods

Cells

Cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12) containing 10% fetal bovine serum (FBS) supplemented with antibiotics in 5% CO_2. MDA138Tu-CDDP^R cells were maintained in DMEM/F12 containing cisplatin (4 μmol/l).

Adenovirus infection

Ad-RB56 or Ad-RB110 recombinant adenovirus was constructed as described in [18]. Briefly, a cassette containing the cytomegalovirus promoter, human RB cDNA and the polyadenylation signal from the bovine growth hormone gene was inserted into the E1 gene-deleted region in Ad-5 adenovirus. Viral stocks were propagated using human 293 cells. The cells were harvested 36–40 h after the infection, pelleted, resuspended in phosphate-buffered saline (PBS) and lysed. Cell debris was removed by centrifugation through a CsCl density gradient. Concentrated virus stock was dialyzed to remove CsCl and stored at −80°C with glycerol. Viral infection was performed in 2 ml of DMEM/F12 containing 2% FBS in 60-mm tissue culture plates. After incubating at 37°C for 1 h, 3 ml of complete medium (DMEM/F12 with 10% FBS) was added and incubation was continued at 37°C in 5% CO_2 environment.

Western blot analysis

Western blot analysis was performed as described previously [19]. Briefly, cells were suspended in sample buffer (2% sodium dodecyl sulfate, 20% glycerol, 0.12 mol/l Tris, pH 6.8), sonicated and boiled for 2 min. After centrifuging at 12 000 r.p.m. to remove debris, the lysate was separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. To detect p110^{RB}, monoclonal antibody 14001A (BD PharMingen, San Diego, California, USA), which recognizes Rb amino acids 332–344, was used. To detect p56^{RB}, monoclonal antibody 14061A (BD PharMingen), which recognizes Rb amino acids 662–665, was used. To detect tubulin, monoclonal antihuman tubulin antibody 1111876 (Boehringer Mannheim, Indianapolis, Indiana, USA) was used. Alkaline phosphatase-conjugated antimouse immunoglobulin G antibody was used as the secondary antibody, and the blot was developed using NBT/BCIP (Promega, Madison, Wisconsin, USA).

Viability assay

To determine the number of viable cells, cells were dissociated with trypsin and incubated with trypan blue dye in PBS for 5 min at 37°C. Viable cells impermeable to the dye were counted with a hemocytometer.

Flow cytometry

Flow cytometry was performed as described in [19]. Briefly, cells were harvested with trypsin and permeabilized in 70% ethanol for 16 h at 4°C. Propidium iodide was added (100 Kunitz U/ml) with RNase A (50 μg/ml) and the DNA content per cell was measured with an EPICS Profile II flow cytometer (Coulter Corp, Hialeah, Florida, USA) in its standard configuration. At least 10 000 events were counted for each sample.

Reverse transcriptase-polymerase chain reaction analysis

RNA was isolated from cell lysates by using a Triazol kit (BRL, Rockville, Maryland, USA), converted into cDNA using avian myeloma virus reverse transcriptase, and amplified via polymerase chain reaction (PCR) using Taq polymerase (Promega) and primers spanning the Rb nucleotides 2220–2530, a region present in both the p56^{RB} and p110^{RB} cDNAs.

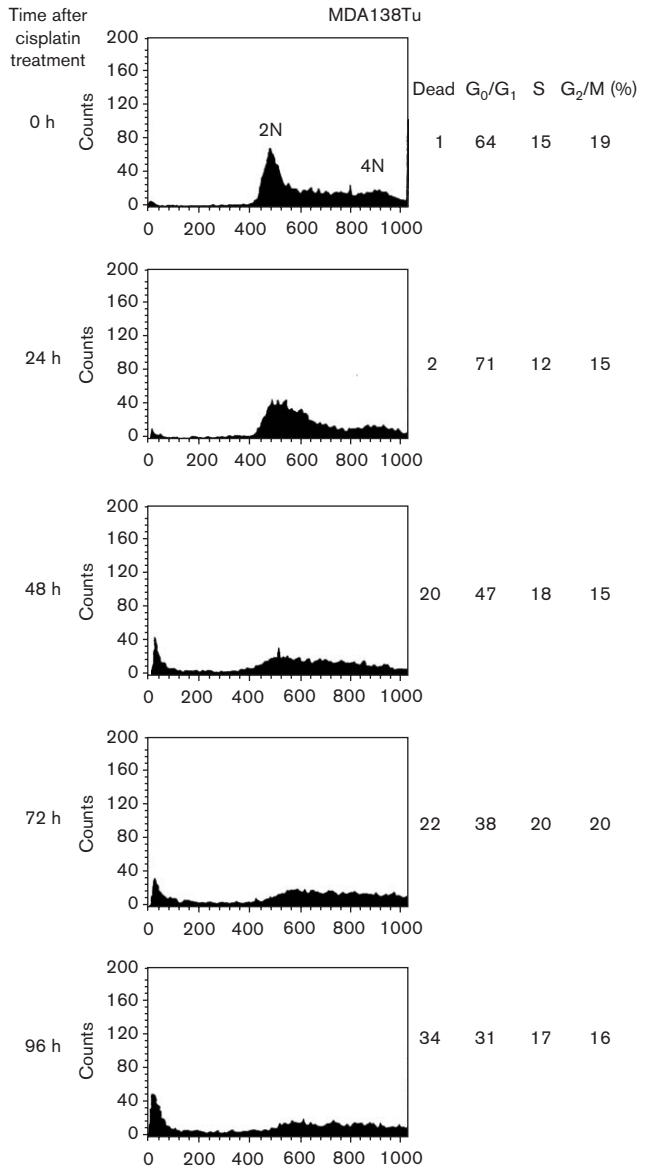
Results

G_1 arrest induction precedes cell death following cisplatin treatment

In G_1 checkpoint-retaining human cancer cells, the presence of damaged DNA activates the checkpoint to induce G_1 arrest. The arrest occurs to allow time for repair before proceeding with DNA replication. The checkpoint thus serves a critical function of keeping the genome intact to maintain cell viability. As such, the onset of G_1 arrest is frequently observed following a *transient* treatment with cisplatin. In clinics, however, cisplatin is usually administered intravenously in a continuous manner over a finite period, which represents a *persistent* treatment. In the laboratory, when exposed to a persistent cisplatin treatment, even the cells that retain the G_1 checkpoint die eventually. What is unclear is whether these cells undergo G_1 arrest before dying.

To determine whether cisplatin-treated cells undergo G_1 arrest before dying, MDA138Tu human head and neck cancer cells were treated with cisplatin and monitored by flow cytometry at various times thereafter. To mimic the clinical situation, cells were treated with cisplatin persistently. The percentage of cells in G_0/G_1 arose to 71% after 24 h of cisplatin treatment, indicating the onset of G_1 arrest (Fig. 1). Intriguingly, by 96 h of treatment, the percentage of G_0/G_1 cells declined sharply from 71 to 31% with the concomitant rise in dead cells from 2 to

Fig. 1



Cisplatin-treated cells undergo G_1 arrest before dying. A DNA content flow cytometric histogram is shown. MDA138Tu cells (5×10^5) were treated with cisplatin (3 μmol/l) for the specified time, and analyzed as described in Methods. 2N, G_0/G_1 phase cells; 4N G_2/M phase cells. The results shown reflect the outcome of three independent experiments.

34%. The percentage of S and G_2/M phase cells remained relatively constant during this period, indicating that the dead cells were mainly the G_0/G_1 cells. The results thus suggest that cisplatin-treated MDA138Tu cells undergo G_1 arrest before dying.

Construction of a recombinant adenovirus expressing human $p56^{RB}$

The above findings suggest that G_1 arrest induction may represent a critical step the in the cisplatin-induced lytic path. If this is the case, then triggering G_1 arrest alone should be sufficient to induce lethality in the absence of cisplatin. To test this, a recombinant adenovirus capable of expressing the RB gene was constructed. As mentioned in the Introduction, Rb is an integral component of the mammalian G_1 checkpoint, which responds to DNA damage by executing G_1 arrest. Previously, it was shown that $p56^{RB}$, an N-terminally truncated human RB polypeptide retaining the E2F-interacting domains, exhibits a potent G_1-arresting potential [20].

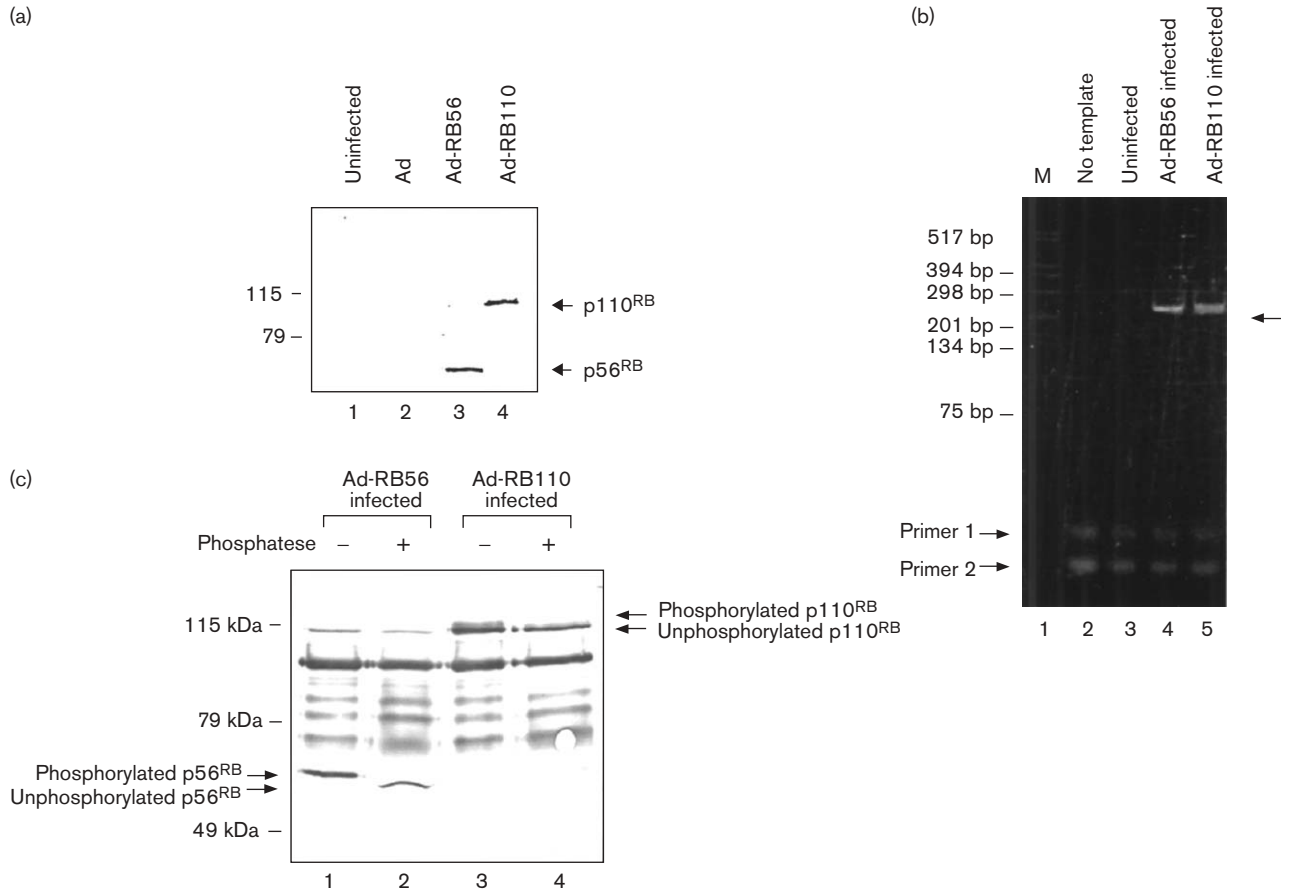
The construction of Ad-RB56 recombinant adenovirus expressing the human $p56^{RB}$ transgene was performed using Ad-5 (lacking the E1 gene) as the parental virus. To express, the gene encoding $p56^{RB}$ was placed under the control of a cytomegalovirus promoter. To stabilize the transcript, a polyadenylation signal from the bovine growth hormone gene was added. For transfection, MDA182Tu human head and neck cancer cells (rather than MDA138-Tu cells) were used as MDA182Tu cells contain a lower level of endogenously expressed Rb, making it easier to detect the transgene expression (this is critical for the comparison experiments involving the $p110^{RB}$ transgene expression described below). MDA182Tu cells were infected with Ad-RB56 at the multiplicity of infection (MOI) of 30 (as little transgene expression was detected after infecting at lower MOIs, indicating that MDA182Tu cells may express a lower level of the adenovirus receptor). Virus titer was determined as plaque-forming units, rather than as viral particles, to quantify the number of viruses that are functional, i.e. infectious. The Ad-RB56 infection led to $p56^{RB}$ expression in MDA182Tu cells as shown by Western blot analysis using antihuman Rb antibody (Fig. 2a). Western blot analysis performed on identical samples with irrelevant antibodies did not show the Rb bands (not shown).

G_1 arrest induction is sufficient to induce lethality in the absence of cisplatin

To determine if ectopic expression of $p56^{RB}$ leads to G_1 arrest, the cell cycle distribution of Ad-RB56-infected MDA182Tu cells was analyzed at various times after the infection. Flow cytometry performed on 2 days post-infection (d.p.i.) showed an increase in G_0/G_1 cells from 68 to 83%, confirming the onset of G_1 arrest (Fig. 3). That no such significant increase was observed with Ad-5-infected cells indicated that the G_1 arrest was specifically owing to ectopically expressed $p56^{RB}$.

Intriguingly, by 5 d.p.i., there was a dramatic increase from 6 to 74% in dead Ad-RB56-infected MDA182Tu cells that was accompanied by a concomitant decrease in G_0/G_1 cells (from 83 to 19%) (Fig. 3). The percentages of S and G_2/M cells stayed relatively constant during this period, which indicated that most of the dead cells were G_0/G_1 cells.

Fig. 2



The retinoblastoma (Rb) transgene expression following Ad-RB56 or Ad-RB110 infection. (a) MDA182Tu cells infected with Ad-RB110 or Ad-RB56 express similar transgene levels. Cells were infected with the indicated virus at a multiplicity of infection of 30 for 48 h and lysed. Equivalent amounts of lysates were loaded and separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Western blot analysis was performed using anti-Rb antibodies as described in Methods. The $p110^{RB}$ and $p56^{RB}$ bands are indicated. Lane 1, uninfected; lane 2, Ad-5-infected; lane 3, Ad-RB56-infected; lane 4, Ad-RB110 infected. (b) Reverse transcriptase-polymerase chain reaction (RT-PCR) detects similar levels of the transgene transcript in Ad-RB56- or Ad-RB110-infected cells. RT-PCR was performed as described in Methods. The amplified products were electrophoresed through a 6% polyacrylamide gel. The arrow indicates the expected 230-bp amplification product. Lane 1, DNA size markers. Lane 2, no template; lane 3, uninfected MDA182Tu cells; lane 4, Ad-RB56-infected MDA182Tu cells; lane 5, Ad-RB110-infected MDA182Tu cells. (c) Both the adenovirally expressed $p56^{RB}$ and $p110^{RB}$ are phosphorylated. The Rb polypeptides in Ad-RB56- or Ad-RB110-infected MDA182Tu cell lysate were immunoprecipitated using the anti-Rb antibodies 14001A and 14061A, treated with calf intestinal alkaline phosphatase (25 units) at 37°C for 15 min, separated by 10% SDS–PAGE, and subjected to Western-blot analysis as described in Methods. Lanes 1 and 2, Ad-RB56-infected; lanes 3 and 4, Ad-RB110-infected; lanes 2 and 4, phosphatase-treated. Molecular size markers are in kilodaltons.

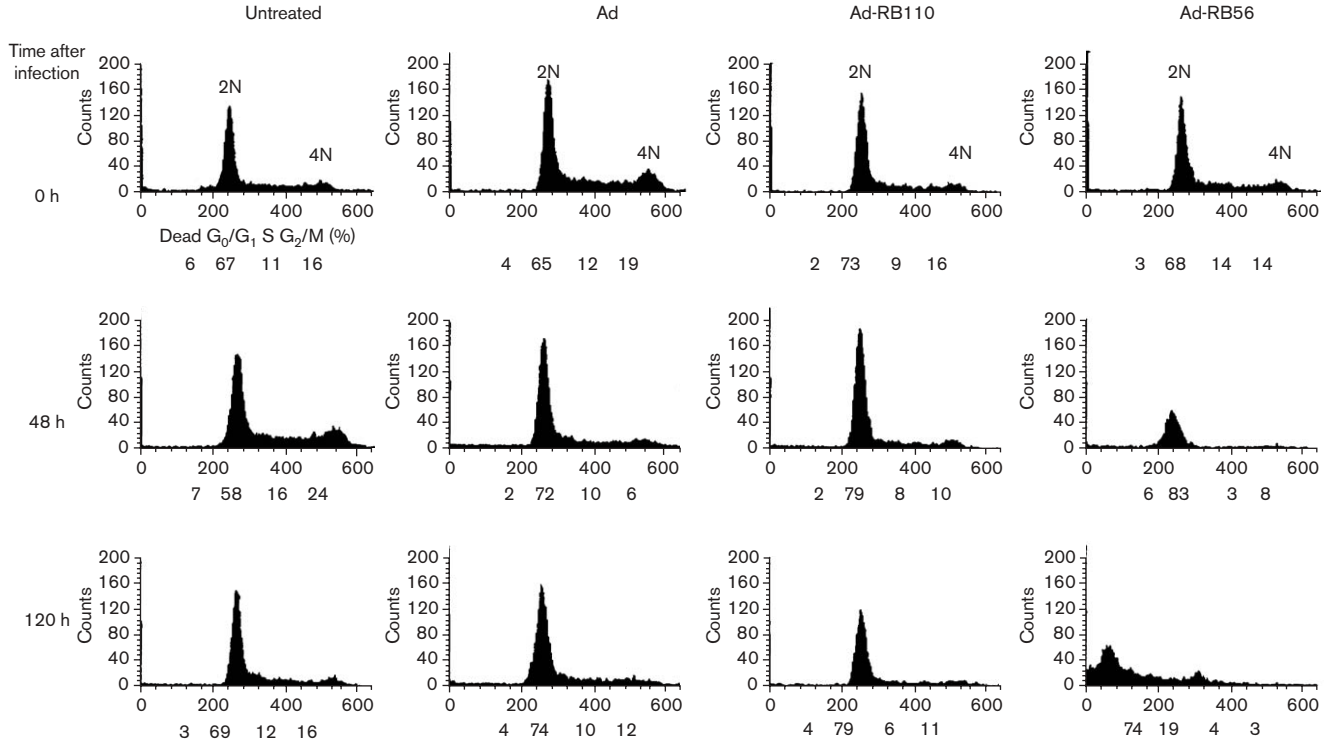
To confirm the death of the Ad-RB56-infected MDA182-Tu cells, an independent experiment was performed in which cell death was monitored by trypan blue dye exclusion. After the Ad-RB56 infection, cells grew for 2 days, albeit more slowly than did the control virus Ad-5-infected cells. This initial increase in cell number probably resulted from the division of cells that were in phases beyond the 'restriction point' (the window during which cells can be G_1 arrested) at the time of Ad-RB56 infection and so were immune to $p56^{RB}$'s G_1-arresting capacity. But, by 5 d.p.i., around 32% of cells present at 2 d.p.i. had died. By 7 d.p.i., the number of dead cells had further increased to around 67% of the cells present at 2 d.p.i. (Fig. 4).

Microscopic examination of the Ad-RB56-infected cells showed enlarged vacuoles and disrupted cell membranes, consistent with cell death (not shown). Death owing to adenovirus-associated cytotoxicity was excluded as MDA182Tu cells infected with the control virus Ad-5 at the same MOI continued to grow throughout this period (Fig. 4). The results thus suggestd that artificially triggering and sustaining G_1 arrest is sufficient to kill MDA182Tu cells in the absence of cisplatin.

The extent of lethality induced correlates with the G_1-arresting potential of retinoblastoma polypeptide

If G_1 arrest induction is required to induce lethality in G_1 checkpoint-retaining cells, then decreasing its G_1-arresting

Fig. 3



G_1 arrest alone is sufficient to induce lethality. A DNA content flow cytometric histogram is shown. MDA182Tu cells (5×10^5) were infected with the indicated virus at a multiplicity of infection of 30 for the specified time, treated and analyzed as described in Methods. 2N, G_0/G_1 phase cells; 4N, G_2/M phase cells. The results shown reflect the outcome of three independent experiments. Ad, Ad-5 parental virus-infected cells; Ad-RB56, Ad-RB56-infected cells; Ad-RB110, Ad-RB110-infected cells.

potential should, in turn, decrease the lytic potential of $p56^{RB}$. This can be tested by ectopically expressing a modified $p56^{RB}$ with an attenuated G_1-arresting potential. The approach, however, may not be entirely valid if $p56^{RB}$ contains two distinct yet overlapping domains for inducing G_1 arrest versus lethality (as then a mutation that affect its G_1-arresting potential may also affect its lytic potential). To exclude this possiblity, the full-length human Rb protein $p110^{RB}$, which exhibits a lesser G_1-arresting potential than $p56^{RB}$, was used [20].

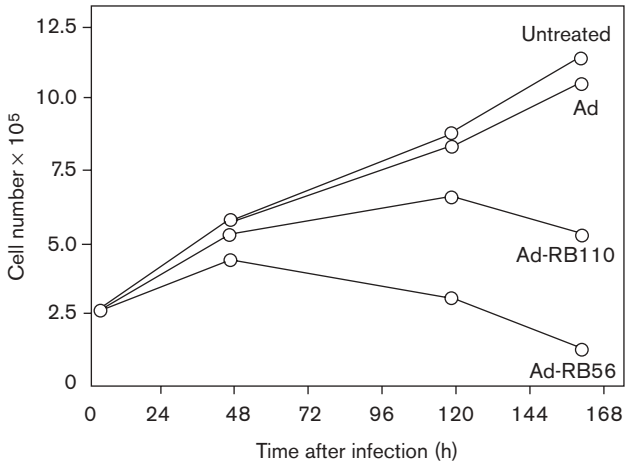
MDA182Tu cells were infected with Ad-RB110 at the same MOI as Ad-RB56 infection. The expression level of $p110^{RB}$ after the Ad-RB110 infection was comparable with that of $p56^{RB}$ after Ad-RB56 infection (Fig. 2a). RT-PCR revealed similar levels of transgene transcript in Ad-RB56- versus Ad-RB110-infected MDA182Tu cells (Fig. 2b).

Phosphatase treatment abolished the slower migrating species of $p110^{RB}$ as well as $p56^{RB}$, which indicated that both proteins were capable of undergoing phosphorylation *in vivo* and so were biochemically functional (Fig. 2c). The phosphorylated and unphosphorylated Rb species are indicated with arrows in Fig. 2c. Other cross-reacting bands represent antibodies used for immunoprecipitation.

Flow cytometry showed that unlike Ad-RB56 infection, Ad-RB110 infection did not significantly increase the percentage of G_0/G_1 cells, confirming that $p110^{RB}$ has lesser ability to induce G_1 arrest than does $p56^{RB}$ (Fig. 3).

Ad-RB110-infected MDA182Tu cells were also examined for lethality. The infected cells continued to grow for at least 5 days, unlike cells infected with Ad-RB56, a significant fraction of which had died by 5 d.p.i. (Fig. 4). By 7 d.p.i., the growth had stopped, and 18% of the cells viable at 5 d.p.i. were dead (Fig. 4). Thus, ectopic expression of $p110^{RB}$ triggered significantly less lethality than did $p56^{RB}$ in MDA182Tu cells. The alternate possibility that the lytic phenotype was not yet manifested during the 7-day study period is highly unlikely as the number of viable cells had increased by 10 d.p.i. (not shown). A similar result was obtained with MDA138Tu and MDA167Tu cells (not shown). The

Fig. 4



The death of Ad-RB56- or Ad-RB110-infected cells. MDA182Tu cells were infected with the indicated virus for the specified time and analyzed for viability using the trypan blue dye exclusion method. Cells (5×10^5) were infected with the indicated adenovirus at a multiplicity of infection of 30. To determine the number of viable cells, infected cells were dissociated using trypsin and incubated with trypan blue dye in phosphate-buffered saline for 5 min at 37°C. Both the attached and floating cells were pooled for the analysis: Ad, Ad-5 parental virus-infected cells; Ad-RB56, Ad-RB56-infected cells; Ad-RB110, Ad-RB110-infected cells. Hour 0 refers to the time point when virus infection started. The data shown are the average of three independent experiments.

results thus suggest that attenuating the G_1-arresting potential decreases the lytic potential of the ectopically expressed Rb protein.

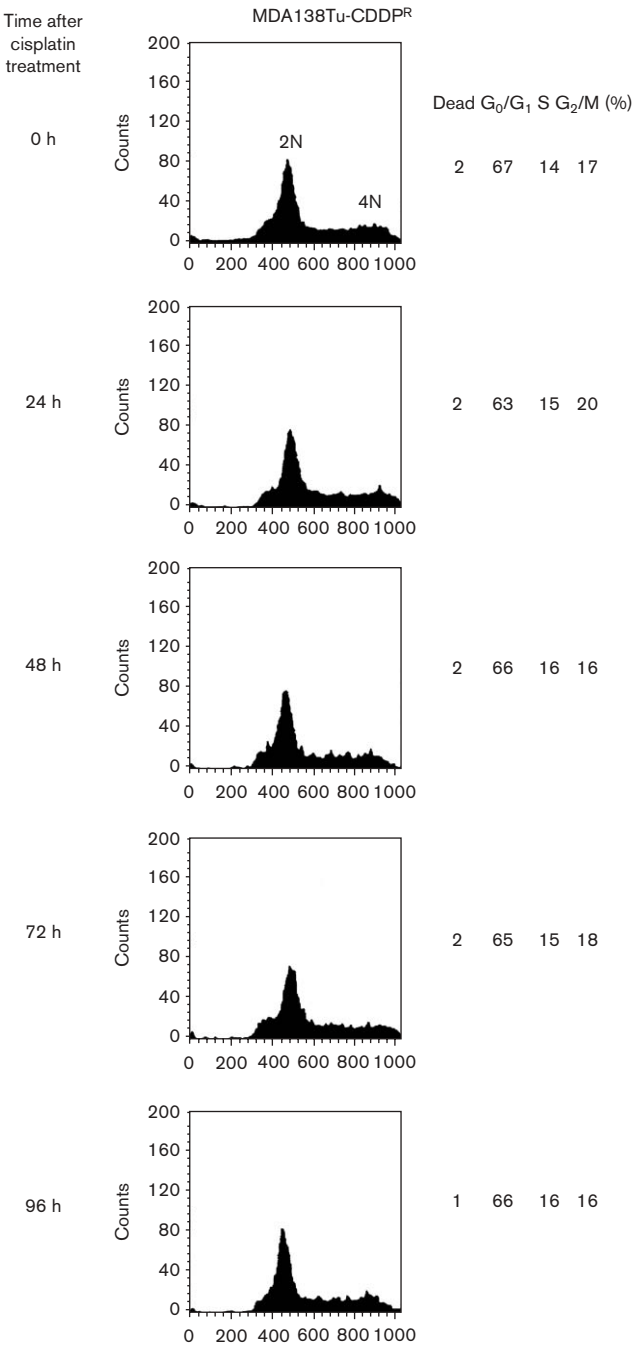
Cisplatin-resistant cells lack the ability to undergo G_1 arrest induction

Finally, genetic evidence for the requirement of G_1 arrest induction in cisplatin cytotoxicity was sought. MDA138-Tu-CDDP^R is a cisplatin-resistant cell line derived from MDA138Tu through a stepwise selection. The ability of MDA138Tu-CDDP^R to undergo G_1 arrest following cisplatin treatment was examined. Flow cytometry detected no significant increase in G_0/G_1 cells even after 4 days of cisplatin treatment (Fig. 5), suggesting that MDA138Tu-CDDP^R cells do not undergo G_1 arrest in the presence of cisplatin. Taken together, the above results suggest that G_1 arrest induction represents a critical determinant for cisplatin cytotoxicity in G_1 checkpoint-retaining human head and neck cancer cells.

Discussion

The mechanism through which cisplatin induces lethality in human cancer cells was investigated. Our results indicate that G_1 arrest induction represents a critical step in cisplatin-induced lytic path for G_1 checkpoint-retaining human head and neck cancers. First, in multiple human head and neck cancer cell lines, persistent cisplatin treatment (mimicking continuous infusion) causes a prolonged G_1 arrest before death. Second,

Fig. 5



Cisplatin-resistant cells do not undergo G_1 arrest after cisplatin treatment. A DNA content flow cytometric histogram is shown. MDA138Tu-CDDP^R cells (5×10^5) were incubated with cisplatin (3 μmol/l) for the specified time, treated, and analyzed as described in Methods. 2N, G_0/G_1 cells; 4N, G_2/M cells. The results shown reflect the outcome of three independent experiments.

triggering G_1 arrest through infection with a recombinant adenovirus expressing the RB gene is sufficient to potentiate lethality in the absence of cisplatin. Third, the extent of lethality induced correlates with the G_1

arresting potential of the ectopically expressed Rb polypeptide. Fourth, cisplatin-resistant cells fail to undergo G_1 arrest despite the presence of cisplatin, providing evidence at the genetic level.

Previously, it was shown that cisplatin also induces G_2 arrest. The reason why most of the cisplatin-treated cells described in this report underwent G_1 arrest is largely because the majority of cells were in G_1 phase (around 64%; Fig. 1) at the time of cisplatin treatment. Hence, the majority of cisplatin-treated cells suffered intra-G_1 DNA damage and became arrested at G_1 owing to the activation of the G_1 checkpoint. Nevertheless, there were some cells that became G_2 arrested as indicated by the lingering presence of cells as G_2/M peak (around 15%) even after 96 h of cisplatin treatment (Fig. 1).

Analysis of the genomic DNA of Ad-RB56-infected human head and neck cancer cells showed a DNA degradation that appears random than at distinct inter-nucleosomal sites (not shown). Morphologically, the Ad-RB56-infected cells showed vacuolization of cytoplasm, degradation of cell organelles and the disintegration of membrane systems. Thus, it seems to induce cell death via necrosis rather than apoptosis. The extent of necrosis induced following the infection with Ad-RB110 was less pronounced, consistent with its mitigated lytic potential.

The above finding has several implications for therapy. Exploiting the mechanism delineated here, therapeutic agents capable of inducing a prolonged G_1 arrest (via interacting with the components of the checkpoint machinery) without requiring a persistent cisplatin treatment may be developed. Through such approaches, the occurrence of secondary tumors in cancer patients resulting from the mutagenicity of the cisplatin may be avoided. Alternatively, a combination therapy employing such therapeutics in conjunction with cisplatin could be envisioned. Already, several works have been reported along this line. These include ectopic expression of $p21^{WAF1/Cip1}$ that led to an enhanced cisplatin cytotoxicity in human ovarian or hepatoma cells [21,22]. Thus, therapeutic innovations based on the enhanced understanding of lytic mechanism may lead to the further refining of cisplatin therapy.

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