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p21 functions in a post-mitotic block checkpoint in the apoptotic response to vinblastine

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Abstract

We have shown previously that in KB-3 (HeLa) cells vinblastine causes downregulation of the CDK inhibitor p21 through a c-Jun regulated pathway. To test the hypothesis that p21 downregulation is necessary to alleviate a protective function, we transfected p21 in KB-3 cells and examined the apoptotic response to vinblastine. The results showed that cells overexpressing p21 were apoptosis-resistant, not through an ability of p21 to cause cell cycle arrest prior to mitotic arrest, but through altering the fate of mitotically arrested cells after drug treatment. Moreover, p21 null HCT116 cells were more prone to vinblastine-induced apoptosis relative to wild-type cells. The results provide support for a model whereby p21 downregulation promotes vinblastine-induced apoptosis by alleviating its protective function following mitotic arrest.

Keywords

vinblastine; p21^{CIP1/WAF1}; apoptosis; microtubule inhibitor; caspase-3

Introduction

p21^{WAF1/Cip1}, a member of Cip/Kip family and a transcriptional target of p53, functions mainly as a regulator of cell cycle progression and differentiation by inhibiting cyclin-dependent kinase (CDK) activity [1]. More recently, emerging evidence suggests additional functions for p21 including a role in regulation of apoptosis [2,3]. Protection against apoptosis has been proposed to be due to the ability of p21 to mediate growth arrest, or alternately through more direct mechanisms involving interaction with pro-apoptotic proteins [4]. In addition, several lines of evidence have suggested that p21 can exhibit pro-apoptotic properties. Thus, studies using p21 overexpression or knockout strategies have indicated that p21 can induce apoptosis [2–4]. Whether p21 plays an anti-apoptotic or pro-apoptotic role depends on the specific cellular context. Interestingly, the pro-apoptotic activity of p21 is most often displayed in cells with mutant p53 [3].

Previous studies in our laboratory have shown that a key response to treatment with microtubule inhibitors such as vinblastine is strong upregulation of c-Jun through an auto-regulatory amplification loop [5–7]. A search for transcriptional targets of c-Jun in KB-3 human cervical carcinoma cells, which have compromised p53 function, identified p21 as a leading candidate

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[7,8]. Furthermore, vinblastine caused downregulation of p21 expression concomitant with c-Jun upregulation, and it was demonstrated by chromatin immunoprecipitation that c-Jun physically associated with the p21 promoter after vinblastine treatment [7]. These results suggested a key role for c-Jun in negative regulation of p21 expression. We hypothesized that c-Jun-mediated downregulation of p21 may be a mechanism to promote efficient apoptotic cell death after mitotic arrest.

To further investigate the role of p21 in this system, we sought to express p21 in KB-3 cells and to examine the apoptotic response to vinblastine. As a complementary approach, we also used p21 null and wild-type HCT116 human colon carcinoma cells. The results show that p21 plays a protective role in vinblastine-induced apoptosis through a mechanism operating post-mitotic block. These data contribute to a developing model which provides insight into the molecular mechanisms of apoptosis induction by the microtubule inhibitor class of antitumor agents.

Materials and methods

Cell culture

The KB-3 human cervical carcinoma cell line (kindly provided by Dr. Michael Gottesman, National Cancer Institute) was maintained in monolayer culture in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% bovine growth serum (BGS), 4.5 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. Wild-type and p21 null HCT116 human colon carcinoma cell lines (kindly provided by Dr. Bert Vogelstein, Johns Hopkins University) were grown in McCoy's 5A medium supplemented with 10% BGS and antibiotics. All cell lines were maintained in a humidified atmosphere with 5% CO₂ at 37°C.

p21 overexpression

For all experiments, KB-3 cells were plated in 6-well plates to achieve about 40% confluency before being transfected the next day. Transfection conditions were optimized by varying the amount of DNA and the amount of Lipofectamine or Plus Reagent, in order to achieve 50–60% transfection efficiency and minimal cell toxicity. The following protocol was adopted for all experiments: 1 µg p21 expression plasmid (pCEP4/WAF1) or empty vector (EV) DNA plasmid/6 µl Lipofectamine/8 µl Plus Reagent in 1 ml reduced serum media (OPTI-MEM I, Invitrogen).

Fluorescence microscopy

For GFP cotransfection experiments, KB-3 cells were plated on UV sterilized coverslips. After transfection, cells were fixed with 2% paraformaldehyde for 20 min, and washed two times with phosphate buffered saline (PBS). Coverslips were mounted on the slides using vectashield (Vector Laboratories). Images were taken using a Zeiss Axio Imager Z1 fluorescent microscope with the 40x/1.3 oil objective.

Western blot analysis

After treatment, detached cells were combined with the adherent cells and washed in PBS. Cell pellets were resuspended in 100 µL lysis buffer containing protease and phosphatase inhibitors (40 mM HEPES, pH 7.5, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 20 mM beta-glycerophosphate, 1% aprotinin, 50 µg/µl leupeptin, 10 µM pepstatin, 1 µM okadaic acid, 1 mM PMSF) and incubated on ice for 45 min. Insoluble material was removed by centrifugation at 13,000 × g for 20 min. Protein concentration was determined using the Bio Rad assay. 50 or 75 µg protein per lane was resolved on 12 % polyacrylamide SDS-PAGE and then transferred to a PVDF membrane. Membranes were probed overnight at 4° C with p21

antibody (OP-64, Calbiochem) or poly(ADP ribose) polymerase (PARP) antibody (Cat # 556362, BD Pharmingen) using 1:1000 dilution and for 1 h with GAPDH antibody (Ambion) using 1:5000 dilution. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibody and the Western Lightning *Plus*-ECL Enhanced Chemoluminescence Reagent (Perkin Elmer).

Flow cytometry

p21 intracellular staining—KB-3 cells were transfected with EV or p21 plasmid as described above and 24 h post-transfection cells were washed twice with PBS and fixed using Cytofix/Cytoperm solution containing paraformaldehyde and saponin detergent (BD Pharmingen) for 20 min on ice. Cells were pelleted and washed twice with Perm/Wash Buffer (BD Pharmingen) containing saponin, fetal bovine serum and sodium azide. Cells were then stained with FITC-labeled p21 monoclonal antibody (Calbiochem, OP64F) by using 20 µg/10⁶ cells for 30 min at room temperature (RT).

Propidium iodide staining—KB-3 cells were transfected with EV or p21 expressing plasmids using the established transfection conditions. 24 h post-transfection, cells were untreated or treated with 30 nM vinblastine for defined periods, washed, fixed in 70% ethanol (overnight at -20 °C) and stained with propidium iodide (PI)/RNase staining buffer for 20 min at RT. Samples were analyzed using BD FACS Calibur (Beckton-Dickinson). Data were obtained for at least 10,000 events for each sample and analyzed by CellQuest software.

Active caspase-3 staining—KB-3 cells were transfected with p21 or EV plasmids and then untreated or treated with 30 nM vinblastine for different times; detached and adherent cells were combined; cells were washed twice with PBS and fixed using Cytofix/Cytoperm solution for 20 min on ice. Cells were pelleted and washed twice with Perm/Wash Buffer and stained using Phycoerythrin (PE) conjugated active caspase-3 antibody (PE-conjugated Monoclonal Active Caspase-3 Antibody Apoptosis Kit, BD Pharmingen) at RT. Following 30 min incubation with the antibody, cells were washed with Perm/Wash buffer, pelleted, resuspended in Perm/Wash Buffer and analysed by flow cytometry using FACS Aria (Beckton Dickinson). Data were obtained by counting at least 10,000 events for each sample and analysed using BD FACSDiva software (Beckton Dickinson).

Cell death ELISA assay

Wild-type and p21 null HCT116 human carcinoma cells were plated in 60 mm dishes one day before treatment. Cells were treated with vehicle (1% DMSO) or 100 nM vinblastine for different times, trypsinized following drug treatment, and diluted to a concentration of 5×10^4 cells/ml for measurement of apoptosis using the Cell Death Detection ELISA kit (Roche Applied Science). The assay is a quantitative photometric immunoassay for the determination of cytoplasmic histone-associated mono and-oligonucleosomes as markers for apoptosis. After dilution, cells were centrifuged at $200 \times g$ for 5 min and the cell pellet was resuspended in 0.5 ml of incubation buffer and incubated at room temperature for 30 min. After centrifugation at $16,000 \times g$ for 10 min, 0.4 ml of the supernatant was removed and diluted 1:10 in incubation buffer for analysis. The ELISA plate was prepared according to the manufacturer's instructions, and 0.1 mL of sample was added to appropriate wells in triplicate and incubated at room temperature for 90 min. After conjugation and incubation with substrate solution, the plate was incubated for approximately 10 min. The samples were subjected to photometric analysis at 405 nm using a Bio-Tek ELx800 microplate reader Quant (Biotek Instruments) and KC4 software version 3.4 (Biotek Instruments).

Results

Overexpression of p21 in KB-3 cells

In order to optimize the transfection parameters, KB-3 cells were cotransfected with pCEP4-WAF1 plasmid encoding full-length human p21 protein and a GFP expression plasmid in a 1:4 ratio using Lipofectamine and Plus Reagent (Invitrogen). Conditions, including the presence or absence of serum and the concentrations of Lipofectamine and Plus Reagent, were varied, and transfection efficiency estimated by fluorescence microscopy. Fig. 1A presents a representative image taken 24 h post-transfection. Ten microscopic fields were examined and the green fluorescent cells were counted versus total number of cells for each field. Transfection efficiency was consistently 50–60%, and no autofluorescence was observed when GFP plasmid was omitted (data not shown). In order to establish the minimum required concentration of p21 plasmid to induce overexpression of p21 protein, KB-3 cells were transfected with either pCEP4-WAF1 (1, 2 or 2.5 µg DNA in 1 mL medium) or EV for 24, 48 or 72 h. Cell extracts were prepared and assessed by immunoblotting using p21 specific antibody. Fig. 1B shows a representative immunoblot and indicates that p21 protein expression is sustained over 72 h with maximum expression 24 h post-transfection. To further confirm that p21 is overexpressed in KB-3 cells, cells were analyzed by flow cytometry using FITC-labeled p21 antibody after transfection with the p21 or EV plasmids. A shift to the right was observed indicating an increase in p21 positive cells from basal, endogenous levels of 6% to 47% in p21 overexpressing KB-3 cells (Fig. 1C). This result is consistent with the transfection efficiency obtained by using GFP cotransfection.

Cell cycle distribution of p21 expressing KB-3 cells

If transient expression of p21 affected cell cycling in untreated cells, this would complicate interpretation of data obtained after vinblastine treatment, which is effective in cells traversing G2/M phase. In order to determine whether p21 expression affected cell cycle distribution, EV or p21 plasmids were transfected into cells using established conditions. Twenty hours post-transfection, cells were washed, fixed in ethanol, and stained with PI. Cells were analyzed for cell cycle distribution using flow cytometry. Representative results are presented in Fig. 2 (panels A, B) and average values obtained after analysis of three independent experiments are shown in Fig. 2C. The data demonstrate that the distribution among the major cell cycle phases was similar for EV and p21 transfected KB-3 cells.

p21 expressing KB-3 cells are protected from vinblastine-induced apoptosis

We have previously shown that caspase-3 activation is a good marker for apoptosis in response to vinblastine in KB-3 cells [8]. KB-3 cells were transfected with either p21 or EV expression plasmids and 20 h after transfection were treated with either vehicle (1% DMSO) or 30 nM vinblastine. Apoptosis was assessed by using PE-labeled active caspase-3 antibody and flow cytometry analysis. The results in Fig. 3A represent percentages of active caspase-3 positive cells (P3) out of total number of counted cells (P2 + P3) and show that p21 expressing cells have a reduced apoptotic response (21% active caspase-3 positive cells) when compared to empty vector transfected cells (52% active caspase-3 positive). As an independent marker of apoptosis, extracts were subjected to immunoblotting for PARP. As shown in Fig. 3B, PARP was less susceptible to cleavage at early time-points in p21 expressing versus EV expressing cells. The results of Fig. 3 results suggest a protective role of p21 in vinblastine-induced apoptosis in KB-3 cells.

p21 expression promotes DNA endoreduplication

To determine if p21 exerted its effect by altering the fate of vinblastine treated cells, cell cycle distribution was examined after vinblastine treatment. One of the complicating factors is that

vinblastine typically induces G2/M arrest prior to cell death. Therefore cells with apoptotic DNA in this case have *sub-G2/M DNA* and cannot necessarily be distinguished in the flow cytometry profiles from cells still in G1 or S phase. For this reason, for vinblastine treated cells, we chose to analyze the proportion of cells distributed among three gates: <G2M, these would be largely cells with apoptotic DNA, but could also contain residual unaffected cells in G1 or S phases; G2/M cells, the limits of this gate were established from the untreated cells (top panels, Fig. 4A); >G2/M, these are cells that are undergoing DNA endoreduplication. The results are shown graphically in Fig. 4A and in tabulated form in Fig. 4B. It is evident that there was a distinct difference in the behavior of the cells following vinblastine treatment. In EV-transfected cells, at 16 h, the DNA content was mostly G2/M or <G2/M with only a small proportion >G2/M. In p21-transfected cells, in contrast, a much larger proportion of cells exhibited >G2/M DNA, indicative of DNA endoreduplication. This trend was more pronounced at 24 h of treatment, with 40% of the p21-transfected cells showing DNA endoreduplication compared to 18% of EV-transfected cells. Given that transfection efficiency was 50–60%, these results suggest that the majority of p21 transfected cells have an altered response and following mitotic block re-enter an abortive cell cycle.

p21 null cells are more sensitive to vinblastine-induced apoptosis

Wild-type and p21 null HCT116 colon carcinoma cells were treated for different times with vinblastine and the extent of apoptosis assessed using a colorimetric assay, as described in Materials and methods. Fig. 5A shows that vinblastine induced a much greater apoptotic response in p21 null cells versus p21 positive cells that was highly statistically significant (p value < 0.0001). These results are consistent with an anti-apoptotic or protective role of p21 in vinblastine-induced apoptosis in HCT116 human colon carcinoma cells. To confirm p21 status in the HCT116 cell lines, p21 immunoblots were performed. Wild-type HCT116 cells expressed a low level of p21 which was strongly induced after treatment with DNA damaging agent, VP-16, whereas p21 was not detectable in p21 null cells under either condition (Fig. 5B).

Discussion

Previously we have presented data showing that the cyclin-dependent kinase inhibitor p21^{WAF1/Cip1} is an authentic target of c-Jun in vinblastine treated KB-3 cells [7,8]. We have hypothesized that p21 downregulation is an important mechanism facilitating apoptosis in this system. In this study we have sought to further explore this hypothesis by overexpressing p21 in KB-3 cells. The results demonstrate that p21-overexpressing KB-3 cells have a reduced and delayed apoptotic response to vinblastine. This conclusion was supported by data using p21 null cells, which were more sensitive to vinblastine-induced apoptosis than wild-type cells. The results strengthen our hypothesis and suggest that vinblastine-induced p21 downregulation [7,8] facilitates apoptosis by removing a strong protective function.

Importantly, p21 overexpression did not alter the cell cycle distribution profile of cells before vinblastine addition (Fig. 2). The results are consistent with other reports where pRb-deficient cells were not arrested at the G1 phase when p21 was overexpressed [9]. This is likely because KB-3 cells, a HeLa subline, are p53 and pRb functionally deficient since they express E6 and E7 human papilloma virus proteins [10,11]. Thus KB-3 cells represent a good model system to study the effects of p21 on the fate of cells following drug-induced mitotic arrest without the complication of p21-induced effects prior to drug treatment.

Given the importance of p21 in regulation of such fundamental processes as cell growth and cell death, it is perhaps not surprising that p21 has been reported to strongly influence the response of tumor cells to chemotherapeutic drugs [3]. In response to DNA-damaging agents, p21 appears to play pro- and anti-apoptotic functions depending on the context. The apoptotic

response to cisplatin was increased when p21 was overexpressed in glioma [12] and ovarian carcinoma [13] cell lines. In contrast, p21 overexpression protected lung cancer cells from doxorubicin [14]. In a study using camptothecin, low concentrations induced growth arrest in wild-type HCT116 cells but apoptosis in p53/p21 null cells, and high concentrations induced apoptosis regardless of p53/p21 status [15]. In response to microtubule inhibitors, other reports have suggested a protective role for p21, but through mechanisms that operate either pre- or post-mitotic arrest. For example, p21 overexpression has been shown to protect cells from apoptosis induced by paclitaxel and vincristine in human sarcoma cells lacking both p53 and functional Rb protein through a mechanism involving suppression of the S-G2 checkpoint [16]. In another report using HCT116 cells, p21-deficient cells displayed a dose-dependent, enhanced chemosensitivity to paclitaxel and vincristine, and sensitivity was reduced by induction of ectopic p21 protein [17].

Analysis of cell cycle distribution after vinblastine treatment was revealing with regard to the mechanism of resistance in p21-expressing cells. It was clear that DNA endoreduplication was much more pronounced in p21-expressing cells than in EV-expressing cells (Fig. 4). Thus, while control cells proceed to die more directly from the arrested state, p21 expression promoted cell cycle re-entry and DNA endoreduplication prior to apoptotic induction. The time spent in a futile new cycle explains the pronounced delay and reduction in apoptosis at comparable time points (Fig. 3). Thus, p21 expression in KB-3 cells appears to provide a different route of cell death. This may seem counter-intuitive, as p21 expression might be considered to block cell cycle re-entry after mitotic slippage. Thus, in certain cell types such as fibroblasts, p21 functions in a checkpoint that normally prevents post-mitotic DNA endoreduplication [18]. However, a study of the effects of p21 in Rb-proficient or Rb-deficient cell lines showed that Rb plays a key role in preventing DNA endoreduplication in G2-arrested cells [9]. Indeed, p21 expression promoted DNA endoreduplication in Rb-deficient cell lines. KB-3 cells, as a Rb-deficient HeLa subline [9], would be predicted to undergo DNA endoreduplication after G2/M arrest, and this is entirely consistent with the results reported here. The requirement for c-Jun-mediated p21 downregulation in vinblastine-treated KB-3 cells [8] become more apparent when it is considered that apoptosis is more direct and efficient in the absence of p21 (Fig. 3).

Finally, the results emphasize that the response to agents that cause cell cycle arrest depends on the complex interplay of many regulatory pathways. Overall, while the utility of p21 targeted therapies as an adjunct to chemotherapy may be difficult to predict in the case of DNA damaging agents, disabling of p21 would be predicted to facilitate the therapeutic action of drugs that target microtubules.

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Abbreviations

CDK	cyclin-dependent kinase
EV	empty vector
PBS	

phosphate buffered saline

BGS

bovine growth serum

DMSO

dimethyl sulphoxide

GAPDH

glyceraldehyde-3-phosphate dehydrogenase

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Fig. 1.

Overexpression of p21 in KB-3 cells. (A) KB-3 cells were cotransfected with plasmids encoding GFP and p21/WAF1 as described in the text. Images were taken 24 h post-transfection and are representative of several fields and several independent experiments. (a) phase contrast; (b) green fluorescence. (B) KB-3 cells were untransfected or transfected with empty vector (EV) or p21/WAF1 expression plasmid. At 24, 48 and 72 h post-transfection, cell extracts were prepared and subjected to immunoblotting for p21 and GAPDH as a loading control. (C) 24 h after transfection, cells were washed, fixed and incubated with FITC-conjugated p21 monoclonal antibody. Samples were analyzed by flow cytometry using FACS Aria. (a) no p21 antibody as negative control; (b) untransfected KB-3 cells; (c) p21 transfected cells. Percentages of p21 negative cells (P2 fraction) and p21 positive cells (P3 fraction) are given and are representative of data from two independent experiments.

Fig. 2.

Cell cycle distribution is unchanged in p21 expressing cells. KB-3 cells were transfected with empty vector (EV) (A) or p21/WAF1 plasmid (B) using established transfection conditions. 24 hours after transfection, cells were washed, fixed and incubated with propidium iodide/RNase for 20 min. DNA content was analyzed by flow cytometry. 10,000 events were analyzed for each sample. Data presented in the table (panel C) represent averages from three independent experiments (mean + S.D.). p-values > 0.2, no significant differences.

Fig. 3.

p21-expressing cells are more resistant to vinblastine-induced apoptosis. (A) KB-3 cells were transfected with empty vector (EV) or p21 expression plasmid and after 20 h were treated with vehicle (1% DMSO) or 30 nM vinblastine for 36 h. Cells were washed, fixed and incubated with PE-conjugated anti-active caspase-3 specific antibody using the manufacturer's instructions (BD Biosciences) and analyzed by flow cytometry using FACS Aria. Percentages of cells negative for active caspase-3 (P2) or positive for active caspase-3 (P3) are indicated and are representative of two independent experiments. (B) KB-3 cells were transfected as in (A) and treated with 30 nM vinblastine for the times indicated. Whole cell extracts were immunoblotted for PARP, with uncleaved (110 kDa) and cleaved (85 kDa) species indicated. GAPDH was used as a loading control.

Fig. 4. p21 expression promotes DNA endoreduplication. KB-3 cells were transfected with empty vector (EV) or p21/WAF1 plasmid using established transfection conditions. Twenty-four post-transfection, cells were left untreated or treated with vinblastine for 16, 24 or 36 h, then stained with PI and analyzed by flow cytometry. At least 10,000 events were counted for each sample. A. DNA content profiles determined by flow cytometry. B. Tabulation of data of panel A.

Fig. 5.

p21 null cells are more sensitive to vinblastine-induced apoptosis. (A) Wild-type and p21 null HCT116 human colon carcinoma cells were treated with vehicle (1% DMSO) or 100 nM vinblastine for 24 or 48 h. After cell lysis and centrifugation, nucleosomes were detected in the soluble fraction using the Cell Death ELISA kit (Roche) following manufacturer's instructions. Data represent mean $\pm$ S.D. (n = 3). (B) Wild-type and p21 null HCT116 cells were untreated or treated with the DNA damaging agent VP-16 (15 μ M, 16 h) and cell extracts were subjected to immunoblotting for p21 and GAPDH as a loading control.