Abstract. Roscovitine, a cyclin-dependent kinases (CDKs) inhibitor, has been reported to have anti-tumor effects in some cancer cell lines by inducing apoptosis. However, the exact underlying mechanisms are not fully understood. Here, we report that roscovitine induces expression and cleavage of the universal CDK inhibitor p21 Waf1/Cip1 in non-small cell lung cancer (NSCLC) A549 cells in a dose-dependent manner. Western blots of roscovitine-treated cells undergoing apoptosis consistently demonstrated a 15 kDa band that was not detected in control cultures. CDK2 activity and PCNA expression were repressed with increasing dose of roscovitine. Accompanying these molecular changes was a progressive arrest of G2 phase and decreasing of 5-bromo-2-deoxyuridine (Brdu) incorporation of S phase cells. Caspase-3 inhibitor z-DEVD-fmk almost completely abolished roscovitine-induced apoptosis, as well as the appearance of 15 kDa band, indicating that p21 Waf1/Cip1 cleavage was mediated by caspase-3 activity. Furthermore, this band was predominant in the floating apoptotic cells, while weakened in the adherent cells which were vital and pre-apoptotic. We also showed that roscovitine induced an enhanced expression of γ-H2AX, which was blocked by caspase-3 inhibition, suggesting that p21 Waf1/Cip1 cleavage may interfere with DNA repair, leading to increased frequency of double strand breaks (DSBs) and enhanced apoptosis. Here we show, for the first time, that p21 Waf1/Cip1 cleavage, which is mediated by caspase-3 activity, is involved in roscovitine-induced apoptosis.

Introduction

Deregulation of cyclin-dependent kinase (CDK) activity occurs universally in human cancers and CDK2 inhibition has become recently an attractive therapeutic approach for anti-cancer drug development (1). Several chemical inhibitors of CDKs have been found for their ability to inhibit cell cycle progression and/or to induce apoptosis in cancer cells and some are in the development for clinical cancer treatment (2-4). Roscovitine is a purine analogue that inhibits the activity of cyclin-dependent kinases (CDKs), including the progression of cells into S phase (CDK2) and mitosis (cdc2) and the facilitation of transcription (CDK7) (5). In addition to CDK inactivation, it has been found to act as an anti-tumor agent, which induces apoptosis in some human cancer cells, including Ewing's sarcoma family tumor cells (6), prostate cancer cells (7), breast cancer cells (8), leukemia cells (9), HeLa cervix carcinoma cells (10), head and neck squamous cell carcinoma (11) and non-small cell lung cancer A549 cells (12). However, the mechanism by which roscovitine triggers apoptosis is not fully understood, although it has been reported that the accumulation of p53 in the nucleus via MDM2 suppression (13,14), caspase-3 activation and X-linked inhibitor of apoptosis protein (XIAP) reduction are involved (6).

The cyclin-dependent kinase inhibitor p21 Waf1/Cip1, which is transcriptionally activated in a P53-dependent way, plays an important role in DNA-damage-induced cell cycle arrest by binding to CDK2 and inhibiting the kinase activity. However, the role of p21 Waf1/Cip1 in apoptosis still remains to be elucidated. Several observations suggest that high level of p21 Waf1/Cip1 expression can inhibit apoptosis, thus dramatically limiting the effectiveness of some anti-cancer drugs (15,16). On the other hand, p21 Waf1/Cip1 has been demonstrated to be an effector molecule for caspases in apoptosis induction in colorectal cancer cells by butyrate, an inhibitor of histone deacetylase (HDAC) (17) and in leukemia ML-1 cells by γ-irradiation (18). Tyagi et al (19) showed that, a natural flavonolignan silibinin-induced apoptosis was mediated in part via p21 Waf1/Cip1 cleavage by caspase, which was reversed by p21 Waf1/Cip1 siRNA. Recently, we have also observed p21 Waf1/Cip1 cleavage HDAC inhibitor trichostatin A-induced apoptosis in A549 cells (20). Whether p21 Waf1/Cip1 is targeted by caspase activity in roscovitine-induced apoptosis in A549 cells has not been reported.
To investigate the role of p21Waf1/Cip1 in roscovitine-induced cell cycle arrest and apoptosis, we analyzed the molecular events in A549 cells after roscovitine treatment. We report here that roscovitine induces expression and cleavage of p21Waf1/Cip1 in A549 cells in a dose-dependent manner and that p21Waf1/Cip1 cleavage, which is mediated by caspase-3 activity, is involved in roscovitine-induced apoptosis.

Materials and methods

Cell culture and treatments. NSCLC cell line A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum, 10 mg/ml antibiotics (penicillin and streptomycin) and 2 mmol/l L-glutamine at 37°C under 5% CO₂ and saturated moisture. Roscovitine (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma), final concentrations of 5, 10, 20 and 40 μM were used to treat the cells and proper amount of DMSO was used as vehicle control.

Cell cycle and BrdU incorporation analysis. Cells were collected by trypsinization at indicated time points. 5-bromo-2-deoxyuridine (BrdU, Sigma, 100 μM) was pulsed 1 h before harvesting. Samples were fixed in 70% ethanol at 4°C overnight. FITC labeled anti-BrdU antibody (BD Pharmingen, USA) and propidium iodide (PI, Sigma) staining were performed. BrdU incorporation and DNA content were analyzed by flow cytometry with FACScan (Becton-Dickinson, Mountain View, CA, USA) using the CELLQuest program (Becton-Dickinson). Data were analyzed by WinMDI software.

Reverse transcription and polymerase chain reaction (RT-PCR). Total RNA was isolated with TRIZol reagent (Invitrogen) according to the manual. First-strand cDNA was synthesized using the Reverse Transcription System (Promega) following the instructions. PCR was performed with initial denaturation for 2 min at 94°C, followed by 30 cycles of PCR amplification (94°C for 30 sec, 52°C for 30 sec and 72°C for 60 sec). The primers used for RT-PCR were: 5'-ATGATCTTGAGGCTGTTGAGAG-3' for GAPDH mRNA. PCR products were analyzed by running samples on 1.5% agarose gel.

Annexin V-FITC and PI staining. Cells were digested with trypsin and washed twice with PBS. The experiment was performed using the Annexin V-FITC apoptosis detection kit (BD Pharmingen) according to the manual. Cells were treated with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) staining solution in the dark at room temperature (RT) for 15 min. The cell samples were analyzed by flow cytometry on a FACScan station with Cell Quest software using the FL1 and FL2 range for Annexin V-FITC and PI, respectively.

TUNEL assay. Apoptosis was also detected by the terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end-labeling (TUNEL) technique. Briefly, cells were treated as described above, at indicated time points, both adherent and non-adherent cells were harvested and fixed overnight (15 h) with 1% paraformaldehyde (1 h) and then 70% ethanol. The next day, the cells were processed using APO-BRDU kit (BD Pharmingen), according to the manufacturer's instructions. Cells were incubated with terminal deoxynucleotidyl transferase (TdT) and BrdUTP. BrdU was detected using the FITC-labeled anti-BrdU monoclonal antibody. Samples were analyzed on a FACSscan station with Cell Quest software using the FL1 range for FITC-BrdU labeling.

Analysis of active caspase-3 and γ-H2AX. Cells were collected by trypsin and the experiments were performed following the manual. Briefly, cells were fixed and permeabilized using the Cytofix/Cytoperm™ kit (BD Pharmingen) for 20 min at RT, pelleted and washed with Perm/Wash™ buffer (BD Pharmingen). Cells were then stained with FITC labeled anti-caspase-3 active form (BD Pharmingen) or anti-γ-H2AX (Upstate) (60 min) followed by FITC-labeled goat anti-mouse IgG for 60 min at RT in the dark. Samples were analyzed by flow cytometry on a FACSscan station with Cell Quest software using the FL1 for FITC labeled caspase-3 active form or γ-H2AX.

Western blotting. Cells were rinsed in PBS and then lysed in lysis buffer containing 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH 8.0), and 1:25 protease inhibitor cocktail. The lysates were kept on ice for 30 min before centrifuging at 14,000 rpm to remove any cellular debris. Protein concentrations of the lysates were determined by the Bradford protein assay system (Bio-Rad, Hercules, CA). Equal amounts of protein (20 μg protein each lane) were separated by SDS-PAGE, transferred to nitrocellulose membranes (Hybond C, Amersham, UK). Immunoblots were blocked with 5% skim milk in TBS/Tween-20 (0.05%, v/v) for 1 h at RT. The membrane was incubated with primary antibody overnight at 4°C. Antibody against polyadenylribosyl polymerase (PARP) were from BD Pharmingen, antibodies against p21Waf1/Cip1, CDK2, p-CDK2 (Thr 160) and PCNA were purchased from Santa Cruz Biotechnology Inc, and ß-Actin antibody was from Upstate Biotechnology Inc, and ß-Actin antibody was purchased from Sigma. Following several washes with PBS containing 0.1% Tween-20, the membrane was incubated with corresponding secondary antibody conjugated with horseradish peroxidase (Sigma), diluted in 5% skim milk (1:5000) at RT for 1 h. The blots were developed using an enhanced chemiluminescence Western blotting detection system (Amersham Bioscience, UK).

Statistical analysis. All data represent at least three independent experiments. Statistical comparisons were made using Student's t-test. P<0.05 was considered to represent a statistically significant difference.

Results

Induction of growth arrest and apoptosis in roscovitine-treated A549 cells. To investigate the effects of roscovitine on cell cycle changes and DNA synthesis, cells were treated with 5, 10, 20 and 40 μM roscovitine for 24 h. One hour before harvesting, Brdu was pulsed by adding to cell culture medium.
Cells were collected for analysis of DNA content and BrdU incorporation by flow cytometry. The results (Fig. 1A and B) showed that roscovitine induced G2 phase arrest in a dose-dependent way. S phase cells were also decreased with increasing drug concentration, except for roscovitine 20 μM. The reason why roscovitine caused an unexpectedly increase of S phase cells is not known. However, BrdU incorporation analysis revealed that roscovitine caused a decrease of BrdU-positive cells in S phase dose-dependently, with the initial effective concentration as low as 5 μM (Fig. 1C). Interestingly,
Western blotting. Analyzed at both the mRNA level by RT-PCR and (C) protein level by Western blotting with anti-p21 Waf1/Cip1 and anti-Actin antibodies were extracted and Western blotting with anti-p21 Waf1/Cip1 and anti-Actin antibodies was performed. For p21 Waf1/Cip1, both intact form (21 kDa) and cleaved fragment (15 kDa) were detected. (B) A549 cells were incubated with roscovitine (Ros) 40 μM for 0, 6, 12, 18 and 24 h. Induction of p21 Waf1/Cip1 was observed at both the mRNA level by RT-PCR and (C) protein level by Western blotting.

Figure 2. Roscovitine inhibits expression of CDK2 and PCNA. A549 cells were treated as described in Fig. 1. Western blotting with antibodies against CDK2, p-CDK2 (Thr 160), PCNA and Actin was performed.

Since it has been well established that roscovitine inhibits CDK2 activity, we also detected CDK expression by Western blotting using antibodies against CDK2 and p-CDK2 (Thr160). PCNA and Actin was performed. Proteins were extracted for p21 Waf1/Cip1 expression by Western blotting. The results (Fig. 3A) showed that roscovitine caused p21 Waf1/Cip1 expression in a dose-dependent manner. The initial concentration for an obvious increase of p21 Waf1/Cip1 expression was 20 μM. Meanwhile, a 15 kDa band was detected in cells treated with roscovitine 20 and 40 μM, with the latter having a much stronger signal. We then chose roscovitine 40 μM to treat A549 cells and collected cells at 0, 6, 12, 18 and 24 h after drug exposure. The kinetic induction of p21 Waf1/Cip1 was analyzed at both the mRNA and protein levels by RT-PCR and Western blotting. As shown in Fig. 3B, p21 Waf1/Cip1 mRNA level increased with time of roscovitine incubation and reached the peak at 12 h. The protein expression level increased in a time-dependent manner with the starting time point at 12 h after roscovitine exposure (Fig. 3C). p21 Waf1/Cip1 cleavage as indicated by the 15 kDa band showed up at 18 h after roscovitine treatment and became stronger at 24 h. Taken together, these results indicate that roscovitine induces p21 Waf1/Cip1 expression and cleavage in a time- and dose-dependent manner.

there was a sharp decrease in Brdu-positive rates in S phase cells when the concentration of roscovitine was switched from 20 to 40 μM, suggesting that the DNA synthesis ability of S phase cells was almost completely abolished by 40 μM roscovitine treatment. Apoptosis was confirmed by TUNEL staining (Fig. 1D). The results showed that roscovitine induced apoptosis in a dose-dependent manner, with initial effective dose at 20 μM.

Since it has been well established that roscovitine inhibits CDK activity, we also detected CDK expression by Western blotting using antibodies against CDK2 and p-CDK2 (Thr160) which is an active form of CDK2. As shown in Fig. 2, both total CDK2 and active form of CDK2 expression decreased dose-dependently as expected, with the initial effective dose as low as 10 μM. However, roscovitine 40 μM did not cause significantly decrease of CDK2 activity compared to 20 μM, which was not consistent with the inhibitory effect of roscovitine on DNA synthesis. PCNA expression also showed the same pattern with CDK2.

Taken together, these results hint that increasing the concentration of roscovitine to 40 μM, which could effectively induce cell death, may trigger other cellular pathways in which inhibition of CDK2 activity is not indispensable.

Roscovitine induces p21 Waf1/Cip1 expression and cleavage. Since p21 Waf1/Cip1 is a key cyclin-dependent kinase inhibitor (CDKI) which is involved in cell cycle arrest after DNA damage, we analyzed p21 Waf1/Cip1 expression by Western blotting after roscovitine treatment. A549 cells were incubated with varying concentrations of roscovitine (5, 10, 20 and 40 μM) for 24 h. Proteins were extracted for p21 Waf1/Cip1 expression by Western blotting. The results (Fig. 3A) showed that roscovitine caused p21 Waf1/Cip1 expression in a dose-dependent manner. The initial concentration for an obvious increase of p21 Waf1/Cip1 expression was 20 μM. Meanwhile, a 15 kDa band was detected in cells treated with roscovitine 20 and 40 μM, with the latter having a much stronger signal. We then chose roscovitine 40 μM to treat A549 cells and collected cells at 0, 6, 12, 18 and 24 h after drug exposure. The kinetic induction of p21 Waf1/Cip1 was analyzed at both the mRNA and protein levels by RT-PCR and Western blotting. As shown in Fig. 3B, p21 Waf1/Cip1 mRNA level increased with time of roscovitine incubation and reached the peak at 12 h. The protein expression level increased in a time-dependent manner with the starting time point at 12 h after roscovitine exposure (Fig. 3C). p21 Waf1/Cip1 cleavage as indicated by the 15 kDa band showed up at 18 h after roscovitine treatment and became stronger at 24 h. Taken together, these results indicate that roscovitine induces p21 Waf1/Cip1 expression and cleavage in a time- and dose-dependent manner.

p21 Waf1/Cip1 cleavage is caspase-dependent and is associated with apoptosis. It has been reported that caspase-3 is responsible for p21 Waf1/Cip1 cleavage in γ-irradiation-induced apoptosis in human myeloblastic leukemia ML-1 cells (18). Therefore, we wondered whether caspase-3 activation was involved in p21 Waf1/Cip1 cleavage in roscovitine-induced apoptosis in A549 cells. A549 cells were treated with roscovitine (40 μM), caspase-3 inhibitor z-DEVD-fmk (100 μM) or both. The active form of caspase-3, apoptosis and p21 Waf1/Cip1 cleavage were analyzed by flow cytometry and Western blotting. The results showed that roscovitine 40 μM effectively caused apoptosis in A549 cells, as indicated by Annexin V-positive cells (Fig. 4C and D) and cleaved PARP 85 kDa band (Fig. 4E), which was accompanied by an increased caspase-3 activation (Fig. 4A and B) and p21 Waf1/Cip1 cleavage (Fig. 4E). Caspase-3 inhibitor z-DEVD-fmk almost completely abolished roscovitine-induced apoptosis, as well as caspase-3 activation and p21 Waf1/Cip1 cleavage. These results suggest that roscovitine-induced p21 Waf1/Cip1 cleavage is caspase-3-dependent and associated with apoptosis.

To further confirm the involvement of p21 Waf1/Cip1 cleavage in roscovitine-induced apoptosis, cells were treated with 40 μM roscovitine for 24 h. Floating cells and adherent cells were collected for analysis of apoptosis and p21 Waf1/Cip1 cleavage by
Western blotting. As shown in Fig. 5, after 40 μM roscovitine treatment for 24 h, apoptotic cells were enriched in floating cells as indicated by the cleaved form of PARP. p21 Waf1/Cip1 was completely cleaved as only a 15 kDa band was detected. However, in adherent cells, which were enriched with non-apoptotic cells, much less fractions of p21 Waf1/Cip1 and PARP were cleaved, suggesting that p21 Waf1/Cip1 cleavage is prior to cell detachment, which is consistent with previous study (17). Taken together, p21 Waf1/Cip1 cleavage is an early event during apoptosis, which is caspase-3-dependent.

p21Waf1/Cip1 cleavage influences DNA repair capacity. p21Waf1/Cip1 has been reported to co-localize with PCNA in the nucleus at the repair foci during the DNA damage response (17), suggesting a role for p21Waf1/Cip1 in DNA repair process. Since roscovitine could effectively induce p21Waf1/Cip1 cleavage which may interfere with the interaction between p21Waf1/Cip1 and PCNA. We then tempted to study whether roscovitine-induced p21Waf1/Cip1 cleavage could impair DNA repair capacity. Cells were treated with varying concentrations of roscovitine. γ-H2AX level was detected by flow cytometry and Western blotting. The results shown in bar graph (Fig. 6A, B and C) showed that roscovitine caused increasing level of γ-H2AX dose-dependently. Pretreatment with caspase-3 inhibitor z-DEVD-fmk almost completely abolished the effect of roscovitine (Fig. 6D and E). These results suggest that roscovitine impairs DNA repair capacity and causes prolonged presence of DNA lesions, which is correlated with caspase-3-dependent p21Waf1/Cip1 cleavage.
Discussion

Increased activity of cyclin-dependent kinases (CDKs) are involved in human cancer development. Because of their critical role in cell cycle progression and the association of their activities with apoptotic pathways, CDK inhibition has become an attractive therapeutic approach (1). A growing number of CDK inhibitors have been shown with promising anti-cancer properties and are under evaluation in clinical trials. These drugs exert their growth inhibitory effects not only by inhibiting CDKs and inducing cell cycle arrest, but also by directly causing apoptosis in cancer cells. Among them, the purine analogue roscovitine has been tested in a variety of solid tumors for its growth inhibitory and anti-tumor effects.

In the current study, we have shown that the growth inhibitory effects of roscovitine in A549 cancer cells are associated with a G2 phase arrest and a concomitant decrease of CDK2 activity and DNA synthesis ability. Consistently, p21Waf1/Cip1 expression was induced with increasing amounts of roscovitine. High level of p21 Waf1/Cip1 expression has been thought to inhibit apoptosis (15,16). In TRAIL death receptor DR4-mediated apoptosis of human solid tumor cell lines, overexpression of p21Waf1/Cip1, or its N-terminal 91 amino acids, may suppress apoptosis and enhance cell survival by blockage of initiator caspase activation (21). However, in the current study, we showed that in A549 cells treated with...
roscovitine, the growth arrest was accompanied with apoptosis. Interestingly, using a p21\textsuperscript{Waf1/Cip1} antibody which could recognize the C-terminus of p21\textsuperscript{Waf1/Cip1}, a 15 kDa band was constantly detected with roscovitine treatments (20 and 40 μM) which could effectively induce apoptosis. Furthermore, the cleaved p21\textsuperscript{Waf1/Cip1} fragment was enriched in apoptotic cells and the caspase-3 inhibitor almost completely abolished roscovitine-induced p21\textsuperscript{Waf1/Cip1} cleavage and apoptosis, suggesting that caspase-3-mediated p21\textsuperscript{Waf1/Cip1} cleavage is involved in roscovitine-induced apoptosis in A549 cells. Indeed, some DNA damage agents have already been shown to induce caspase-dependent p21\textsuperscript{Waf1/Cip1} cleavage, which is associated with apoptosis. These studies strongly indicate that the cleaved form of p21\textsuperscript{Waf1/Cip1} is closely related to apoptosis.

Gervais et al (18) showed that p21\textsuperscript{Waf1/Cip1} was cleaved by caspase-3 at aspartic acid 112 site which is located at the carboxyl region of p21\textsuperscript{Waf1/Cip1}. Dong et al (22) reported that a synthetic peptide corresponding to residues 139-160 of the carboxyl terminus of p21\textsuperscript{Waf1/Cip1} induced rapid apoptosis, which is associated with apoptosis. These studies strongly indicate that the cleaved form of p21\textsuperscript{Waf1/Cip1} is closely related to apoptosis.

In addition to binding and inhibiting CDKs activity, p21\textsuperscript{Waf1/Cip1} also associates and co-localizes with the proliferation cell nuclear antigen (PCNA) by its C-terminus at the DNA repair foci (18,23), suggesting that p21\textsuperscript{Waf1/Cip1} may play a role during the DNA damage repair. Gervais et al (18) have reported that a direct effect of p21\textsuperscript{Waf1/Cip1} cleavage is to abolish its interaction with PCNA, which may interfere with DNA repair, leading to prolonged presence of DNA lesions that may trigger apoptosis. In the current study, we also showed that roscovitine caused enhanced expression of γ-H2AX, a marker of DSBs, dose-dependently, in the same pattern by which roscovitine induces p21\textsuperscript{Waf1/Cip1} cleavage. Pretreatment with caspase-3 inhibitor not only abolished p21\textsuperscript{Waf1/Cip1} cleavage, but also suppressed the formation of γ-H2AX, leading to inhibition of apoptosis.

Taken together, caspase-3-mediated p21\textsuperscript{Waf1/Cip1} cleavage plays an important role in roscovitine-induced apoptosis (Fig. 7). The current data provide a novel insight into how roscovitine may induce apoptosis, strongly suggesting that roscovitine may be a valuable therapeutic agent for clinical treatment of NSCLC.