Inhibition of X-ray and Doxorubicin-induced Apoptosis by Butyrolactone I, a CDK-specific Inhibitor, in Human Tumor Cells

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(Received, July 26, 2000)
(Revision received, November 6, 2000)
(Accepted, September 8, 2000)

apoptosis/CDK/doxorubicin/p21/X-ray

Cell-cycle progression is coordinately regulated by cyclin-dependent kinases (CDKs). The inhibition of CDKs by p21Waf1/Cip1/Sdi1 prevents the apoptosis of cells treated with DNA-damaging agents. In this study, we found that butyrolactone I, a specific inhibitor of CDC2 family kinases, blocks the X-ray- or doxorubicin-induced apoptosis of DLD1 (p21+/+) human colorectal carcinoma cells in a dose-dependent manner. We also found that butyrolactone I inhibits the CDK2 activity and enhances cell survival after an X-ray irradiation or doxorubicin treatment in both DLD1 (p21–/–) and DLD1 (p21+/+) cells. These findings suggest that butyrolactone I prevents apoptosis by the direct inhibition of CDK and also, possibly, by CDK-inhibition through p53-independent p21-induction. Our findings indicate that CDK activity is required for DNA-damaging agent-induced apoptosis.

INTRODUCTION

Apoptosis is a physiological process of cell death that functions to control cell populations in the developing process of higher organisms. Apoptosis also occurs in certain types of cells when they suffer DNA damage. The signaling pathway that regulates apoptosis is not fully

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Abbreviations: CDK; cyclin dependent kinase, DOX; doxorubicin, PBS; phosphate buffered saline
understood. Evidence exists to suggest that the cell cycle and apoptosis may be interconnected. For example, in response to DNA damage, cells undergo cell-cycle arrest at both the G1/S and G2/M transitions before entry into subsequent stages to allow time for repair. However, some damaged cells still proceed with their cell cycles and thus escape from arrest to undergo apoptosis. Progression through G1 and entry into the S phase are regulated by the activation of cell cycle-dependent kinase 2 (CDK2), which makes a complex with cyclin A or cyclin E. The complexes phosphorylate pRb, thereby reversing Rb-mediated repression of those genes whose functions are required for DNA synthesis and entry into the S phase of the cycle.

Apoptosis has been shown to be accompanied by changes in the CDK activity. In a previous study, we demonstrated that DNA-damaging agents, doxorubicin (DOX) and X-rays, induce apoptosis, which is prevented by the ectopic expression of p21 (Waf1/Cip1/Sdi1) due to its CDK-binding or CDK-inhibitory activity in human colorectal carcinoma cells (DLD1). We also demonstrated that DOX- or X-ray-induced apoptosis was not prevented by mutated p21 lacking its CDK-binding or CDK-inhibitory activity, suggesting that the inhibition of CDK activity is required to prevent apoptosis.

Butyrolactone I, \(\alpha\)-oxo-\(\beta\)-(p-hydroxyphenyl)-\(\gamma\)-(p-hydroxy-m-3, 3-dimethylallyl benzyl)-\(\gamma\)-methoxycarbonyl-\(\gamma\)-butyrolactone, is a specific inhibitor of CDC2 family kinases which regulate the cell cycle by phosphorylation of the RB protein and H1 histone. If the prevention of apoptosis by p21 is mediated by the inhibition of CDK activity, apoptosis should also be inhibited by butyrolactone I. To verify this postulation in the present study, we examined whether butyrolactone I inhibits CDK activity and prevents the DOX- or X-ray-induced apoptosis of p21 (+/+ ) and p21 (−/−) DLD1 cells.

**MATERIALS AND METHODS**

**Cell culture**

DLD1 (p21−/− ) and its parental DLD1 (p21+/+) cells were kindly supplied by Dr. B. Vogelstein. Both cells have a point mutation in the p53 gene. The cells were cultured and maintained at 37°C in Dulbecco’s modified Eagle’s minimum essential medium (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

**CDK2 kinase assay**

Cell extracts (100 µg) obtained after the treatment of cells with butyrolactone I (0, 30, 40, 50, 60 µM) for 24 h were incubated with an anti-CDK2 antibody (SC-163), and immune complexes were collected with 80 µl protein G-plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA.) The cyclin-dependent kinase activity was measured using a CDK assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, the immune complexes (40 µg) were mixed with \([γ^{32}P]\) ATP (3000 Ci/mmol), histone H1 (2 µg) as a substrate and a reaction buffer containing inhibitors of other kinases. After incubation for 10 min at 30°C, phosphorylated histone H1 was resolved by 16% polyacrylamide-SDS gel electrophoresis and detected by autoradiography.
Western blotting

The cells at 90% confluence were washed with phosphate-buffered saline (PBS) and lysed at 4°C for 10 min in a buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, 0.05% SDS, 1% NP40, 1% trasylol and 0.2 mg/ml phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). Cellular debris was removed by centrifugation, and the protein content of the cell lysate was determined by the Bradford calorimetric assay method (Biorad, Richmond, CA). A 40 µg aliquot of the cell lysate was resolved by 16% polyacrylamide-SDS gel electrophoresis and transferred to a Hybond-C Super membrane (Amersham, UK). The p21 protein was detected with an anti-p21 antibody (SC-187, Santa Cruz Biotechnology, Santa Cruz, CA.) and horseradish peroxidase-conjugated protein A using a Renaissance Chemiluminescence kit (Dupont, Boston, MA).

Cell survival after X-ray irradiation or a doxorubicin treatment

Details of the method were reported by Chen et al. In brief, exponentially growing cells were trypsinized, serially diluted and plated in 60 mm dishes, and then incubated in a medium supplemented with or without 60 µM butyrolactone I for 24 h. After the cells were irradiated with X-rays or treated with DOX for 3 h, they were cultured in a medium with or without 60 µM butyrolactone I for 24 h and then washed with PBS, followed by further incubation in a normal medium for 2 weeks. Colonies were stained with crystal violet and scored.

Observation of apoptotic cells after X-ray irradiation or a doxorubicin treatment

Cells were cultured in a medium containing butyrolactone I (0, 30, 40, 50, 60 µM) after X-ray irradiation or a DOX (3 µg/ml) treatment under the same conditions as mentioned above. They were then washed with PBS, stained with the DNA-binding dye Hoechst 33258 (0.5 µg/ml in PBS), and observed under a fluorescence microscope. Cells showing condensed nuclear morphology and nuclear lobulations were regarded as being apoptotic.

DNA fragmentation in cells after X-ray irradiation or doxorubicin treatment

Cells were cultured in a medium with or without 60 µM butyrolactone I after an X-ray irradiation (14 Gy) or DOX (3 µg/ml) treatment, as mentioned above. DNA extracted with the Apoptosis Ladder Detection Kit (Wako, Osaka, Japan) was separated by agarose gel electrophoresis and stained with SYBR Green I (Molecular Probes, Eugene, OR).

RESULTS

In a previous preliminary study, we demonstrated that butyrolactone I (50 µM) markedly reduces a fraction of apoptotic cells that were produced by an X-ray or DOX treatment. In the present study, we first treated DLD1 (p21+/+) cells with different concentrations of butyrolactone I (0, 30, 40, 50, 60 µM) after exposing them to X-rays or DOX, and examined their nuclear morphology by staining with the DNA-binding dye Hoechst 33258. A fraction of apoptotic cells having a condensed nuclear morphology and nuclear lobulations was reduced by a butyrolactone I treatment in a dose-dependent manner (Fig.1A, B). X-ray- or DOX-induced apoptosis was
Fig. 1. Dose-dependent suppression of doxorubicin- or X-ray-induced apoptosis by butyrolactone I. DLD1 (p21+/+) cells were cultured in a medium with butyrolactone I (0, 30, 40, 50, 60 µM) after a doxorubicin (3 µg/ml) treatment for 3 h (A) or X-ray irradiation (14 Gy) (B). The cells were fixed and stained with Hoechst 33258, and apoptotic cells were counted under a microscope. An error bar of the graphs shows the standard error of the mean. Agarose gel electrophoresis patterns of DNA obtained from DLD1 (p21+/+) cells grown for 24 h in a medium with or without butyrolactone I (60 µM) after a doxorubicin (3 µg/ml) treatment for 3 h or X-ray irradiation (14 Gy) are shown (C).

completely inhibited by 60 mM butyrolactone I.

To confirm that the abnormal nuclear morphology which can be blocked by butyrolactone I is caused by apoptosis, DNA fragmentation of the DLD1 (p21+/+) cells after an X-ray or DOX treatment was examined by agarose gel electrophoresis (Fig. 1C). The apoptotic DNA ladder formation due to its fragmentation was greatly suppressed in cells treated with butyrolactone I (60 µM). These findings indicate that the inhibition of CDK is required to prevent the X-ray- or DOX-induced apoptosis of the cells.

In a previous study, we reported that although the ectopic expression of the CDK inhibitor
Fig. 2. Inhibition of CDK activity by butyrolactone I in DLD1 (p21−/−) and (p21+/+) cells. The phosphorylation of histone H1 by CDK2 in extracts of DLD1 (p21−/−) (top) and DLD1 (p21+/+) (bottom) cells treated with butyrolactone I for 24 h was measured.

Fig. 3. Effects of butyrolactone I on cell survival after a doxorubicin or X-ray treatment in DLD1 (p21−/−) (A, B) and (p21+/+) (C, D) cells. Cells were cultured in a medium supplemented with butyrolactone I (60 µM) from 16 h before through 24 h after a doxorubicin (3 µg/ml) treatment (A, C) or 14 Gy X-ray irradiation (B, D), then cultured in a regular medium until colonies formed (†). Cells were cultured without butyrolactone I (□). The error bar shows the standard error of the mean from three independent experiments.
p21 protects DLD1 cells from X-ray- or DOX-induced apoptosis, that of mutated p21 lacking CDK-inhibitory activity does not. To clarify whether butyrolactone I prevents X-ray- or DOX-induced apoptosis in DLD1 cells through the induction of p21 or by the direct inhibition of CDK, we treated p21-deficient and its parental DLD1 cells with butyrolactone I (0, 30, 40, 50, 60 \( \mu M \)) for 24 hours, and then measured the CDK2 kinase activity of cell extracts (Fig. 2). Butyrolactone I substantially inhibited CDK2 activity not only in parental DLD1 (p21+/+), but also in DLD1 (p21−/−) cells in a dose-dependent manner. The inhibition of CDK2 by butyrolactone I occurred at lower concentrations in DLD1 (p21+/+) cells than in DLD1 (p21−/−) cells, which may be due to the induction of p21 in DLD1 (p21+/+) cells, in addition to the direct inactivation of CDK2.

The cell survival after an X-ray irradiation or DOX treatment in the presence or absence of butyrolactone I is shown in Figure 3. In both DLD1 (p21+/+) and (p21−/−) cells, survival after an X-ray (Fig. 3B, D) or DOX treatment (Fig. 3A, C) was enhanced by butyrolactone I (60 \( \mu M \)). The enhancement of the cell survival was greater in DLD1 (p21+/+) cells (Fig. 3C, D) than in DLD1 (p21−/−) cells (Fig. 3A, B). These findings indicate that butyrolactone I protects DLD1 cells from X-ray- or DOX-induced death, which may be due to both its induction of p21 and direct CDK-inhibition.

To confirm that the butyrolactone I treatment induces endogenous p21, we examined the p21 protein level by Western blotting in the DLD1 (p21+/+) and (p21−/−) cells. Figure 4 clearly shows that butyrolactone I enhanced the p21 protein level only in DLD1 (p21+/+) cells.

Fig. 4. Endogenous p21 expression levels after a 24 h butyrolactone I treatment (60 \( \mu M \)) detected by Western blotting with an anti-p21 antibody in the DLD1 (p21+/+) and DLD1 (p21−/−) cells.

DISCUSSION

In the present study we demonstrated that X-ray or doxorubicin-induced apoptosis is suppressed by butyrolactone I. This suppression is due to the inhibition of CDK directly by butyrolactone I as well as by mediation of the endogenous CDK-inhibitory protein p21 induced by butyrolactone I. It is therefore reasonable that the apoptosis was suppressed more markedly in p21-positive cells than in p21-defective cells. The p21 induction by butyrolactone I is not mediated by p53 because DLD1 cells lack wild-type p53.

Changes in the activity of CDK and cyclins in apoptosis have been observed in some cell
For example, an increase in cyclin E-associated CDK activity is associated with cell death caused by the DNA-damaging agent camptothecin in HL60 cells\(^9\). Cyclin A-associated CDK activity is upregulated by other apoptosis-inducing pharmacological agents\(^10,11\). The expression of the dominant negative mutants of CDK2 suppresses apoptosis in Hela cells\(^12\). Aberrant activation of CDC2 is associated with the Fas-induced apoptosis of hematopoietic cells\(^13\). In a previous study, we showed that p21 arrests the cell cycle at the G1 phase and prevents X-ray- and DOX-induced apoptosis\(^3\). These studies suggested that the inhibition of apoptosis was due to the inhibition of CDK by p21. This was supported by our direct findings that p21 with a mutation at codon 46 (Arg to Cys) or 140 (Arg to Gly), which neither binds to CDK2 nor affects CDK2 activity \textit{in vivo}, fails to prevent apoptosis\(^3,14\). Since many CDKs regulate in coordination at each step of the cell-cycle progression, it is reasonable to suggest that inappropriate CDK activation triggers the apoptosis of the cells suffering DNA damage. Using butyrolactone I in this study, we further confirmed that the inhibition of CDK prevents apoptosis, i.e. CDK activity is necessary for apoptosis.

Three mechanisms can be suggested for suppressing apoptosis by p21. 1) The p21 protein binds to CDKs and inhibits their activities. It is known that G1 CDKs phosphorylate pRb and subsequently activate E2F1. Recent findings have suggested that the E2F1 promotes cell apoptosis\(^15\). 2) p21 directly inactivates caspase 3 by binding to procaspase 3\(^16\). Caspase 3 is an essential factor for apoptosis induction. 3) p21 functions as a cell-cycle checkpoint protein during the G1 and G2 phases. During cell-cycle arrest after an X-ray- or DOX-treatment, DNA repair may be carried out and cell apoptosis is prevented.

A function of many cancer therapeutic agents, including X-ray and doxorubicin, is to cause the apoptosis of tumor cells\(^17,18\). Our findings suggest that keeping high CDK activity in tumor cells and avoiding a concurrent treatment with other agents causing p21 induction or CDK inhibition are important to enhance the curative effect of the chemotherapeutic agents.

ACKNOWLEDGEMENTS

We thank Dr. B. Vogelstein for kindly supplying DLD1 (p21-/-) and DLD1 (p21+/+) cell lines. This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Culture and Sports, Japan.

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