Selective Sensitization of Retinoblastoma Protein-deficient Sarcoma Cells to Doxorubicin by Flavopiridol-mediated Inhibition of Cyclin-dependent Kinase 2 Kinase Activity

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INTRODUCTION

Sensitivity of human tumor cells to various DNA-damaging agents, including anticancer drugs, depends greatly on cell cycle checkpoint function (1). The G1-S checkpoint is regulated by the cyclin D1/cdk4,6 and cyclin E/cdk2 complexes, which target the pRb and inactivate this protein by phosphorylation (2). pRb is a positive regulator that binds to members of the E2F transcriptional factor family (E2F-1–3) in its hypophosphorylated form and inhibits E2F-1-mediated G1-S progression (3). An absence of pRb or phosphorylation of this protein by cyclin D1/cdk4,6 and cyclin E/cdk2 leads to release of functional E2F-1 and increases transition from G1 to S phase. However, in S phase, E2F-1 is phosphorylated by cyclin A/cdk2 complex, resulting in loss of its DNA-binding activity and transactivation function (4). Thus, in the presence of pRb, inhibition of cyclin D1/cdk4,6 or cyclin E/cdk2 by cyclin/cdk inhibitors such as p21 will reduce release of E2F-1 and dominantly arrest cells at G1. In the absence of pRb, inhibition of cyclin A/cdk2 by cyclin/cdk inhibitors would increase unphosphorylated E2F-1, which may lengthen the S phase or block cells in the S-G2 stage and enhance sensitivity of cells to S-phase-specific drugs such as DOX (5).

FP, a flavone derivative with potent antitumor activity, has been demonstrated to be a potent cdk inhibitor (6, 7). FP inhibits activity of cdk2 and cdk4 by binding to the ATP-binding site of these cdkks and causes a G1 block (8). FP may also directly inhibit cyclin B1/cdc2 kinase activity and result in induction of G2 arrest (9). Several studies (10, 11) indicate that at higher concentrations, FP could inhibit other tyrosine protein kinases and decrease expression of cyclin D1 and bcl-2. Therefore, FP may play an important role in mediating cellular response to anticancer drugs by regulating cell cycle checkpoints.

In this study, we tested the hypothesis that in pRb-positive cells, FP may mainly arrest cells in G1 and decrease the cellular response to S-phase-specific drugs. In contrast, in cells that lack functional pRb, FP may dominantly inhibit cyclin A/cdk2 activity, thus increasing functional E2F-1 activity and resulting in S-G2 arrest. As a consequence, cells lacking pRb will be more sensitive to S-phase-specific drugs. We found that FP selectively sensitizes pRb-deficient SaOs-2 cells but not pRb-reconstituted SaOs-2 cells to DOX and show that this may be attributed to a FP-mediated S-G2 block because of inhibition of cdk2 kinase activity.
The blots were probed with various primary antibodies using standard techniques (18). Protein was detected using enhanced chemiluminescence detection.

Immunoprecipitation and Histone-H1 Kinase Assay. Cells were exposed to DOX and DOX plus FP for 24 h and were lysed with a solution containing: 100 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2% NP-40, 0.5% sodium deoxycholate, 0.2% SDS, and protease inhibitors. After centrifugation at 4°C for 15 min, the supernatant was centrifuged and precleared with protein A/G agarose for 30 min. Precleared protein extract (200 μg) was combined with lysis buffer and 10 μg of anti-cdk2/agarose conjugate (Santa Cruz Biotechnology) and incubated for 1 h at 4°C with shaking. Agarose beads were collected by centrifugation and washed four times in lysis buffer and once in kinase buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 2 mM EGTA, and 1 mM DTT). The resuspended beads were reconstituted in 20 μl of kinase buffer containing 20 μM ATP, 100 mg/ml histone H1 (Boehringer Mannheim), and 200 μCi/ml [γ-32P]ATP. After incubation for 20 min at 30°C, the reaction mixture was then subjected to 7.5% SDS-PAGE followed by autoradiography.

RESULTS

FP Increases DOX-induced Cell Killing and DOX-induced Activation of Caspase-3 in pRb-deficient Cells. The sensitivity of parental SaOs-2 cells and two Rb-restored sublines, SaOs-2/9B and SaOs-2/10B, to FP was first determined. Cells were treated with FP at different concentrations for 5 days. As shown in Fig. 1A, sensitivity of SaOs-2/9B and 10B cell lines to FP was similar to that of SaOs-2neo cells. IC50 values for these cell lines were approximately 500 nM. The sensitivity to DOX in these cell lines was measured next, and no obvious difference in sensitivity was observed between SaOs-2neo cells and pRb-restored cells (Fig. 1B). To determine whether FP would affect sensitivity of pRb-deficient or -restored cells to DOX, the effect of DOX combined with FP (100 nM), a dose that caused less than 10% inhibition of cell growth, was examined on these cell lines. As shown in Fig. 1B, the effect of DOX on SaOs-2 cells was greatly enhanced by the addition of this concentration of FP. IC50 values for DOX combined with FP were 7-fold lower compared with that for DOX alone (2.6 nM versus 18.5 nM) in Rb-deficient cells. In contrast to this result, IC50 values for DOX combined with FP were similar to that for DOX alone in Rb-restored cells. To understand whether FP enhancement of DOX-induced cell killing links to increased apoptotic response to DOX in pRb-deficient cells, we further determined caspase-3 activation, which correlates with apoptosis (16). As shown in Fig. 2, FP alone did not increase caspase-3 activity in either pRb-deficient cells or pRb-restored cells. DOX caused a moderate increase in caspase-3 activity in both pRb-deficient and -restored cells. However, when FP was combined with DOX, substantially enhanced DOX-induced activation of caspase-3 (about 2-fold higher than that for DOX alone) was observed after either 4-h or 24-h exposure to drugs in pRb-deficient cells but not in pRb-restored cells.

FP Decreases DOX-induced Cell Accumulation in S Phase in Rb-reconstituted Cells. Because FP is a cdk inhibitor, we examined the effect of FP on cell cycle distribution before and after treatment of cells with DOX to understand whether FP-modulated sensitivity of these cells to DOX is related to alteration of cell cycle distribution. As shown in Fig. 3, treatment with DOX decreased the proportion of cells in G1 and increased S-phase accumulation in both SaOs-2neo and SaOs-2/9B cells. Treatment with 100 nm FP had little effect on cell cycle distribution in Rb-deficient cells and decreased S-phase accumulation and arrested cells in the G1 phase in Rb-restored cells. When treated with a combination of FP and DOX, whereas pRb-deficient cells were accumulated in S phase (48.9%), pRb-restored cells were arrested in the G1 phase (80.2%).

FP Induces Expression of p21 in pRb-deficient Cells. To further understand the mechanisms of FP-modulated different sensitivity to DOX in pRb-deficient and -restored cells, we examined the levels of proteins involved in G1-S regulation after treatment of cells with FP or DOX combined with FP. As shown in Fig. 4, analysis of cyclin/cdk complexes revealed that expression of cyclin D1, cyclin A, and cdk2 proteins were not significantly changed by exposure to FP in either pRb-deficient or -restored cells. However, the increased level of cyclin A by DOX was reduced by addition of FP in Rb-deficient cells. The elevated level of E2F-1 by DOX was slightly decreased by the addition of FP. Interestingly, expression of p21, barely detectable in SaOs-2 cells, was induced by FP in Rb-deficient cells but not in Rb-restored cells. Because the bcl-2 family is involved in the regulation of drug sensitivity or resistance, we also determined whether FP affected the expression of bcl-2 family proteins. Bcl-2 was barely detectable in both Rb-deficient and -restored cells (data not shown). Expression of bcl-X/L (Fig. 4) and bax (data not shown) was not altered by FP or FP combined with DOX in these cells.

FP Decreases cdk2 Kinase Activity in pRb-deficient Cells. As the level of p21 was elevated by FP in pRb-deficient cells, we examined cdk2 kinase activity after treatment with FP and FP combined with DOX in both Rb-deficient and -restored cells. Increased expression of p21, as shown in our previous studies (5), would result in decreased cyclin A/cdk2 kinase activity and, as a consequence,
reduction of E2F-1 phosphorylation, which would increase S-G2 stay of cells and increase S-phase-specific drug sensitivity. As shown in Fig. 5, cdk2 kinase activity was reduced to 62% compared with untreated control by FP in Rb-deficient cells. DOX-induced increase (169%) in cdk2 kinase activity in these cells was also attenuated (98%) by addition of FP. In contrast, cdk2 kinase activity was slightly increased by FP (138%) in Rb-restored cells, and the DOX-induced increase (135%) in pRb-restored cells was not attenuated (140%) by addition of FP.

DISCUSSION

The effect of FP combined with various anticancer agents on human tumor cell lines was reported recently (19). These experiments showed that cytotoxic synergy between FP and the anticancer drugs may depend on the sequence of administration, and the mechanisms may involve inhibition of FP on p21 expression, down-regulation of bcl-2, increased S-phase fraction, and FP-mediated apoptosis (10, 20–21). However, the role of pRb status on the effect of the combination of FP with anticancer drugs has not been explored previously.

In this study, we demonstrated that FP selectively enhances the cytotoxicity of DOX in Rb-deficient sarcoma cells. Although FP was reported to have both functions of inducing apoptosis and arresting cell cycle, enhancement of DOX effect by FP in this study is more likely through its cell cycle arrest rather than apoptosis because the concentration of FP used here is subtoxic and different cell cycle distributions generated by FP were observed between Rb-deficient and -restored cells. By measuring the levels of cell cycle-regulatory proteins and associated kinase activity, additional results indicated that FP-modulated DOX sensitivity in Rb-deficient SaOs-2 cells may involve preferable inhibition of cdk2 activity. Inhibition of cyclin A/cdk2 activity would reduce phosphorylation of E2F-1, which results in an increase of cells in late S phase and, as a consequence, increases susceptibility of cells to DOX-induced apoptosis as shown previously (5) and in this study. Therefore, a pathway that links FP enhancement of DOX sensitivity is inhibition of S-phase cyclin/cdkks by FP and pRb status of the cell.

Several studies have demonstrated that, in the absence of pRb, no or little cdk4 activity is detected, and cyclin D1 is dispensable (6, 22). As a consequence, the regulatory interplay between cdk4, t-type cyclins, p16, and pRb may be lost (23). Therefore, in the absence of pRb, interaction of FP with cdk4 may be attenuated, and cdk2 would become a major target of FP. Thus, these studies also seem to support the possibility that FP preferably targets S-phase cdks in pRb-deficient cells.

In the presence of pRb, FP does not induce p21 expression or even inhibit DNA damaging agents-induced p21 expression (20). We also observed a lack of induced p21 expression by FP in pRb-positive cells. However, we did observe that FP increased p21 expression in pRb-deficient cells. Increased expression of p21 in the absence of pRb...
has been demonstrated to increase the S-phase fraction of human sarcoma cells and enhance sensitivity of these cells to anticancer drugs (5, 24). Therefore, increased p21 expression by FP may partially contribute to enhancement of DOX sensitivity in pRb-deficient cells. It is not clear how FP increases p21 expression in the absence of pRb. However, several studies showed that E2F-1 is able to induce p53-independent p21 expression by directly transactivating the p21 promoter (25). Because “free” E2F-1 is much higher in pRb-deficient cells than in pRb-positive cells (14) and FP may further increase activity of E2F-1 by inhibition of cdk2, the FP-induced increase in p21 expression in the absence of pRb may be through the E2F-1-p21 pathway.

Although DOX is one of the most active drugs to treat human sarcomas, its effectiveness against this disease is limited. Because loss of functional pRb occurs frequently in sarcomas (26), administration of FP combined with DOX to selectively enhance the DOX sensitivity in pRb-deficient cells may improve the antitumor effects of DOX in patients with sarcomas. This combination is worthy of further exploration in this disease and in other pRb-deficient tumors.

REFERENCES

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