Failure to Dephosphorylate Retinoblastoma Protein in Drug-resistant Cells

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Abstract

Hypophosphorylation of retinoblastoma protein (RB) accompanies the DNA damage-induced, p53-independent G1 arrest and apoptosis in two p53-null human leukemic cell lines, HL-60 and U937 (Q. P. Dou et al., Proc. Natl. Acad. Sci. USA, 92: 9019–9023, 1995). When an HL-60 cell line resistant to cytosine arabinoside was exposed to this DNA-damaging agent, neither RB hypophosphorylation nor apoptosis were observed. In contrast, treatment of these cells with another DNA-damaging agent, etoposide, dramatically induced these events, which were inhibitable by the addition of zinc chloride, a protein tyrosine phosphatase inhibitor. Induction of hypophosphorylation of RB may be an important novel strategy for treating drug-resistant cancers.

Introduction

The tumor suppressor and transcription factor p53 regulates a DNA damage-triggered G1 checkpoint (1). In some cell systems, the induction of p53 by DNA-damaging agents results in transcriptional activation of p21, an inhibitor of cyclin-dependent kinases (also called WAF1/CIP1/SDI1), which inhibits cyclin-dependent kinase-mediated phosphorylation of RB,3 leading cells to G1 arrest (1–3). In other cells, the induced p53 activates expression of Bax (a cell death inducer) and/or suppresses that of Bcl-2 (a cell death inhibitor), leading to apoptosis (4, 5). Recent experiments also suggest a p53-dependent, DNA damage-triggered G1 checkpoint (6), although the involved molecular mechanisms remain largely unknown. By using two p53-null human leukemic cell lines, HL-60 and U937, we have reported recently that the induction of an RB serine/threonine phosphatase activity rather than p21 by DNA-damage signals, and the consequent hypophosphorylation of RB, may be one mechanism responsible for p53-independent G1 arrest and apoptosis (7). Resistance to chemotherapy by neoplastic cells is a major problem in the treatment of human cancers. Understanding of the biochemical mechanisms(s) of drug resistance is, therefore, invaluable for improvements in therapy. Here we report that failure to dephosphorylate RB may be a mechanism responsible for drug resistance, at least in an HL-60 cell line resistant to cytosine arabinoside (HL-60-Ara-C-resistant).

Materials and Methods

Materials. Purified mouse monoclonal antibody to human RB protein, G3-245, was purchased from PharMingen; calyculin A was from Life Technologies, Inc.; Ara-C, etoposide (VP-16), dimethyl sulfoxide, DNA markers (0X 174 DNA HaeIII digest), zinc chloride, and all other chemicals were from Sigma Chemical Co. Ara-C (60 μM) was dissolved in PBS, VP-16 (400 mM) was dissolved in 100% dimethyl sulfoxide, calyculin A (0.1 mM) was dissolved in 10% (v/v) dimethyl sulfoxide, and zinc chloride (1 μM) was dissolved in distilled water.

Results and Discussion

Ara-C Fails to Induce the RB Hypophosphorylation and Apoptosis in HL-60-Ara-C-resistant Cells. We had reported that when HL-60 cells were treated with a variety of anticancer agents, RB became hypophosphorylated; this was accompanied by the accumulation of cells with pre-G1, DNA content (apoptotic peak) and preceded the internucleosomal fragmentation of DNA (180-bp DNA ladder; Ref. 7). We speculated that if induction of hypophosphorylation of RB is critical for the initiation of apoptosis, failure to dephosphorylate RB should be associated with drug resistance. In this study, we used a pair of HL-60 lines that were sensitive (7) or resistant (8) to the anticancer agent Ara-C. Both HL-60 cell lines were treated with Ara-C, followed by the measurement of RB protein (by Western blot assay), cell cycle distribution (by flow cytometry), and apoptosis. Apoptosis was assayed by the induction of pre-G1, apoptotic peak (by flow cytometry), formation of 180-bp DNA ladder (DNA gel electrophoresis), and loss of cell viability (trypan blue exclusion).

As reported previously (7), treatment of HL-60 cells with Ara-C for 3–5 h converted RB from the hyperphosphorylated (p120/hyper) to a hypophosphorylated form (p115/hypo) (Fig. 1A, Lanes 3–5 versus lanes 1, 2, and 6). A 5-h treatment with Ara-C caused an ~30% increase in the G1 population of these cells (Fig. 2, b versus a). This treatment also induced apoptosis, as evident by an ~25% increase in the pre-G1 apoptotic population (Fig. 2, b versus a) and production of a 180-bp DNA ladder (Fig. 1B, lower, lanes 4 and 5). Apoptotic cell death was confirmed by trypan blue exclusion assay; ~50% of these cells incorporated trypan blue dye after 24 h.

In contrast, when HL-60-Ara-C-resistant cells were exposed to Ara-C for up to 5 h, no hypophosphorylation of RB could be detected (Fig. 1A, Lanes 7–11 versus Lane 12). These cells also failed to undergo apoptosis, since neither increase in pre-G1 population (Fig. 2, e versus d) nor the 180-bp DNA ladder (Fig. 1B, Lanes 7–11 versus Lane 12) were detected. Consistent with this observation was that only ~5% of the resistant cells lost their membrane permeability after 24 h.
FAILURE TO DEPHOSPHORYLATE RB IN RESISTANT CELLS

Treatment of HL-60 cells with VP-16 for up to 5 h resulted in induction of the hypophosphorylation of RB (Fig. 3A, Lanes 3–5 versus Lanes 1, 2, and 6), G1 arrest (an ~35% increase), apoptotic peak (an ~35% increase; Fig. 2, c versus a), and a 180-bp DNA ladder (Fig. 3B, Lanes 4 and 5). Approximately 70% of HL-60 cells lost their viability after a 27-h treatment with VP-16. Similar to HL-60 cells, HL-60-Ara-C-resistant cells also converted RB from the p120/hyper to p115/hypo form when treated with VP-16 for 3–5 h (Fig. 3A, Lanes 9–11, versus Lanes 7, 8, and 12). Consistent with the RB hypophosphorylation was a 30% increase in G1 and a 55% increase in pre-G1 population of these cells after 5 h treatment with VP-16 (Fig. 2, f versus d). This treatment also induced production of the 180-bp DNA ladder (Fig. 3B, Lanes 9–11). After 27 h treatment, ~80% of HL-60-Ara-C-resistant cells lost their membrane permeability, as measured by incorporation of trypan blue dye. None of these events

treatment with Ara-C. These data strongly suggest that failure to dephosphorylate RB is tightly associated with resistance to induction of apoptosis in HL-60-Ara-C-resistant cells.

Exposure of these HL-60-Ara-C-resistant cells to Ara-C for 5 h, however, slightly increased their G1 population (Fig. 2, e versus d). This increase was much less than that detected in parental HL-60 cells (5% versus 30%; Fig. 2). This was consistent with the previous observation that the accumulation of a small portion (5–10%) of cells in G1 phase preceded the hypophosphorylation of RB (7). Failure to dephosphorylate RB, therefore, is at least associated with resistance to major G1 arrest in these cells.

VP-16 Induces G1 Arrest- and Apoptosis-specific RB Hypophosphorylation in HL-60-Ara-C-resistant Cells. It had been shown that HL-60-Ara-C-resistant cells lacked deoxycytidine kinase and were unable to efficiently incorporate Ara-C (8). Treatment of these cells with Ara-C, therefore, would not cause sufficient DNA damage, and this may be responsible for their failure to induce RB hypophosphorylation, G1 arrest, and apoptosis (Figs. 1 and 2). We, therefore, investigated whether HL-60-Ara-C-resistant cells were sensitive to another DNA-damaging agent, VP-16, which acts via different mechanisms (as a topoisomerase II inhibitor). If these cells were sensitive to VP-16, we expected that such a treatment would induce the hypophosphorylation of RB, G1 arrest, and apoptosis.

Fig. 2. DNA content assay in HL-60 and HL-60-Ara-C-resistant cells treated with either Ara-C or VP-16. HL-60 (Sensitive) and HL-60-Ara-C-resistant (Resistant) cells grown exponentially (Oh, a and d) were treated with 10 μM of Ara-C (b and e) or VP-16 (c and f) for 5 h, followed by analysis of DNA contents. The cell cycle distribution was calculated as the percentage of cells that contain DNA of G1, S, G2, and M phases (total number of cells with G1, S, G2, and M DNA content was 100%), judged by propidium iodide staining. The apoptotic population (Ap) was calculated as the percentage of cells with <G1 DNA content (total number of cells with pre-G1, G1, S, G2, and M DNA content was 100%), judged by propidium iodide staining. The apoptotic population (Ap) was calculated as the percentage of cells with <G1 DNA content (total number of cells with pre-G1, G1, S, G2, and M DNA content was 100%). Cells treated with PBS or dimethyl sulfoxide for 5 h gave similar results to those grown exponentially (Oh). The results shown here are a representative of two to five independent experiments.
Ara-C-resistant cells. Ara-C-resistant cells were pretreated with VP-16 for 1 h, washed, and incubated in drug-free medium for an extended period, and aliquots of cells were prepared. A, Western blot assay. B, DNA fragmentation assay. Similar results are obtained in at least three independent experiments.

Fig. 3. Induction of RB hypophosphorylation and 180-bp DNA fragmentation by VP-16 in HL-60 and HL-60-Ara-C-resistant cells. Both cell lines were treated with 10 μM of VP-16 or an equal percentage (0.003%) of dimethyl sulfoxide (D) for the indicated times, and aliquots of cells were prepared. A, Western blot assay. B, DNA fragmentation assay. Similar results are obtained in at least three independent experiments.

were observed in the Ara-C-resistant cells treated with dimethyl sulfoxide, the solvent of VP-16 (Figs. 2 and 3), demonstrating that the effect was drug induced. The kinetics of the VP-16-induced RB hypophosphorylation, G1 arrest, and apoptosis in HL-60-Ara-C-resistant cells was very similar to that in HL-60 cells (Figs. 2 and 3), indicating that the induction pathways for these events were intact in HL-60-Ara-C-resistant cells.

Addition of Zinc Chloride but not Calyculin A Inhibits the VP-16-induced Events in HL-60-Ara-C-resistant Cells. We had shown previously that the addition of zinc chloride into HL-60 cells pretreated with Ara-C or VP-16 effectively blocked induction of RB hypophosphorylation, G1 arrest, and apoptosis (7). We, therefore, tested whether zinc could inhibit VP-16-induced events in HL-60-Ara-C-resistant cells. Ara-C-resistant cells were pretreated with VP-16 for 1 h, washed, and incubated in drug-free medium for an additional 4 h in the absence or presence of zinc chloride. The addition of zinc effectively prevented the VP-16-induced hypophosphorylation of RB (Fig. 4a, Lanes 3 and 4 versus Lane 2), the accumulation of cells in G1 (Fig. 4, d versus c), appearance of the apoptosis-specific pre-G1 peak (Fig. 4, d versus c), and formation of the 180-bp DNA ladder (data not shown). The addition of zinc into the control cells had no effects (Fig. 4a, Lanes 7 and 8 versus Lanes 5 and 6; Fig. 4b). These data strongly suggest that a VP-16-inducible, zinc-inhibitable activity (probably a tyrosine phosphatase; see discussion below) is required for hypophosphorylation of RB, G1 arrest, and apoptosis in the Ara-C-resistant cells.

The addition of a specific protein-serine/threonine phosphatase inhibitor, calyculin A (13), into the VP-16-pretreated HL-60 cells also prevented the hypophosphorylation of RB (Ref. 7; Fig. 4e, Lanes 3–5 versus Lane 2 versus Lane 1) and formation of the 180-bp DNA ladder (7) in a dose-dependent manner. However, under the same conditions, the addition of calyculin A (up to 400 nm) into HL-60-Ara-C-resistant cells, which had been pretreated with VP-16 for 1 h, had very little inhibitory effect on either the hypophosphorylation of RB (Fig. 4e, Lanes 8–10 versus Lane 7 versus Lane 6) or formation of the apoptosis-specific 180-bp DNA ladder (data not shown). Consistent with this, the addition of calyculin A (up to 1 mm) into a protein extract, prepared from the VP-16-pretreated HL-60 cells (7) but not the Ara-C-resistant cells (data not shown), prevented the in vitro-reproduced RB hypophosphorylation. These data suggest that the mechanism of induction of RB hypophosphorylation in the Ara-C-resistant cells is different from that in HL-60 cells.

We have provided evidence that VP-16, but not Ara-C, dramatically induced the hypophosphorylation of RB, G1 arrest, and apoptosis in HL-60-Ara-C-resistant cells (Figs. 1–3). This is probably because VP-16, but not Ara-C (8), is able to be incorporated efficiently into these cells, producing sufficient DNA damage. The following evidence suggests that the signal transduction pathways responsible for these DNA damage-induced events may be very similar in both HL-60 and HL-60-Ara-C-resistant cell lines: (a) the kinetics of the VP-16-induced events in HL-60-Ara-C-resistant cells was very similar to that in HL-60 cells (Figs. 2 and 3); (b) the addition of zinc prevented all of the VP-16-induced events in both HL-16 (7) and HL-60-Ara-C-resistant cells (Fig. 4, a–d). Zinc is believed to be an inhibitor of a protein-tyrosine phosphatase activity (14), which might control activation of the RB serine/threonine phosphatase activity in HL-60 cells (7). Therefore, the major machinery for regulating the p53-independent, RB-mediated G1 checkpoint is undamaged in HL-60-Ara-C-resistant cells.

However, the involved checkpoint regulators may not be the same in both HL-60 cell lines. In parental HL-60 cells, induction of an RB protein-serine/threonine phosphatase activity (which is inhibitable by calyculin A at a very low concentration) but not p21 was responsible for the hypophosphorylation of RB and consequent G1 arrest and apoptosis (Ref. 7; Fig. 4e). In contrast, the addition of calyculin A to the VP-16-pretreated HL-60-Ara-C-resistant cells did not suppress either RB hypophosphorylation or apoptosis (Fig. 4e), suggesting an altered mechanism. If treatment of the Ara-C-resistant cells with VP-16 activates a protein-serine/threonine phosphatase activity responsible for the hypophosphorylation of RB, this phosphatase should be very different from that found in HL-60 cells (7). Alternatively, some other mechanisms (such as induction of p21) might be responsible for the VP-16-induced RB changes in the Ara-C-resistant cells. In any case, our studies suggest that the hypophosphorylation of RB is a new marker for detection of apoptosis, and induction of the RB hypophosphorylation might be an important novel strategy for developing new drugs for treating drug-resistant cancers.
Fig. 4. Zinc but not calyculin A inhibits VP-16-induced RB hypophosphorylation, G1 arrest, and apoptosis in HL-60-Ara-C-resistant cells. Both HL-60 (Sensitive; e, Lanes 1—5) and HL-60-Ara-C-resistant cells (Resistant; a—d, and e, Lanes 6—10) were treated with 20 μM of VP-16 or an equal percentage (0.005%) of dimethyl sulfoxide (DMSO) for 1 h, washed, and incubated in drug-free medium for additional 4 h in the absence (1h + 4h, −) or presence of zinc chloride (1 mM in a, Lanes 3 and 7; 1.5 mM in a, Lanes 4 and 8, and b and d) or calyculin A (e, 10 nM in Lanes 3 and 8; 50 nM in Lanes 4 and 9; and 200 nM in Lanes 5 and 10, respectively). Assays of RB protein (a and e) and DNA contents (b—d) were performed. The percentages of cells in apoptotic peak and the cell cycle were calculated as described in the legend to Fig. 2.

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References

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