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COMPARATIVE EFFECTS OF PROTEIN PHOSPHATASE INHIBITORS (OKADAIC ACID AND CALYCULIN A) ON HUMAN LEUKEMIA HL60, HL60/ADR AND K562 CELLS

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Inhibitors of protein phosphatases 1/2A (okadaic acid and calyculin A) exhibited differential cytotoxicity toward three human leukemia cell lines, in an increasing order of resistance, HL60 < HL60/ADR < K562 cells. Cytotoxicity of the toxins was associated with marked mitotic arrest of the cells, characterized by chromatid scattering/overcondensation and abnormal mitotic spindles. In all cases, calyculin A was more potent than okadaic acid. Protein phosphorylation experiments in intact cells revealed that HL60/ADR, the adriamycin-resistant variant, showed a higher overall phosphorylation of nuclear proteins than the drug-sensitive parental HL60, and that phorbol ester (protein kinase C activator) and calyculin A appeared to more specifically stimulate phosphorylation of p66 and p60, respectively. It was suggested that the toxins might be useful in delineating mechanisms underlying certain properties of cancer cells (such as multidrug resistance, mitosis and differentiation) related to protein phosphorylation/dephosphorylation reactions.

It has been shown that cell cycle is regulated by protein phosphorylation/dephosphorylation reactions. The catalytic subunit of maturation promoting factor, $p34^{cdc2}$ kinase, and expression of cdc2 family of gene are essential for eukaryotic cells to progress from G1 to M phase (1-3). Phosphorylation of a number of nuclear proteins by $p34^{cdc2}$ kinase is critical for initiation and progression of M phase, whereas dephosphorylation of these proteins to their interphase level of phosphorylation by protein phosphatases 1 and 2A (PP1/2A) is required for mitotic progression after metaphase and for cells to escape from mitosis (4-7). Role of PP1/2A in mitosis is supported by recent findings that profound mitotic arrest was demonstrated in ganglions of drosophila with a mutant gene encoding PP1 (6) and in human leukemia K562 cells incubated with a PP1/PP2A inhibitor okadaic acid (8). In the present study, we examined the effects of PP1/2A inhibitors (okadaic acid and calyculin A) on three leukemia cell lines, including HL60/ADR, an adriamycin-resistant variant of HL60 cells (9).

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EXPERIMENTAL PROCEDURES

Materials: Okadaic acid and calyculin A were purchased from Moana Bioproducts or LC Services; [^{3}H]thymidine and ^{32}Pi were from ICN Radiochemicals; 12-O-tetradecanoyl-phorbol-13-acetate (TPA), colchicine, anti-β-tubulin antibodies and fluorescein-conjugated anti-mouse antibodies were from Sigma; human leukemia cell lines HL60 and K562 were from American Type Culture Collection; HL60/ADR subline, originally developed by Bhalla et al. (9), was a gift from Dr. Robert I. Glazer; medium and supplies for cell culture were from GIBCO.

Cell studies: Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum, in a humidified incubator at 37°C in 95% air/5% CO_2. [^{3}H]Thymidine uptake, cell viability and microscopic examination of mitotic phase (8) and immunofluorescence visualization of mitotic spindles (6), were carried out as described.

Protein phosphorylation in HL60 and HL60/ADR cells: The procedures for metabolical labeling of cells with ^{32}Pi, preparation of crude nuclear fraction and two-dimensional electrophoresis and autoradiography of ^{32}P-proteins were essentially the same as described earlier for KG-1 (10) or K562 cells (8).

RESULTS AND DISCUSSION

Calyculin A was more potent (up to 10-fold) than okadaic acid in inhibiting thymidine uptake (cell proliferation) and causing cell death of three leukemia cell lines examined, with a relative resistance to the cytotoxicity of the two toxins of, in an increasing order, HL60 < HL60/ADR < K562 cells (Fig. 1). Because the toxins inhibited cell proliferation at concentrations lower than those required for cell killing, it was unlikely that the toxins, PP1/PP2A inhibitors, were directly cytocidal.

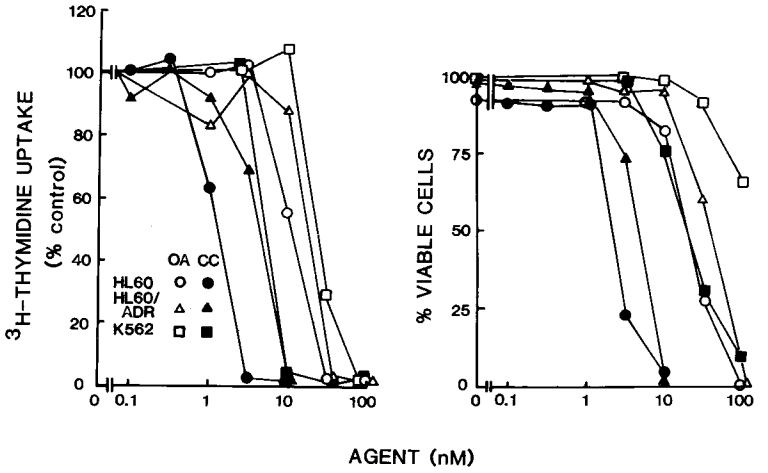

Fig. 1. Effects of okadaic acid (OA) and calyculin A (CC) on K562, HL60 and HL60/ADR cells. The cells (2 x 10^5/ml/well) were incubated for 2 days with various concentrations of the agents, as indicated. Left: Cell proliferation was determined by [^{3}H]thymidine uptake for 1 h at the final hour of the 2-day treatment. The control uptake values (2.6, 0.7 and 2.4 pmol/h/well for K562, HL60 and HL60/ADR, respectively) in the absence of the added agents were taken as 100%. Right: Cell viability was determined by trypan blue exclusion using the same cell samples for thymidine uptake shown above. The data presented are means of triplicate incubations, with S.E. of less than ± 5% (not shown). Similar results were obtained in two other experiments.

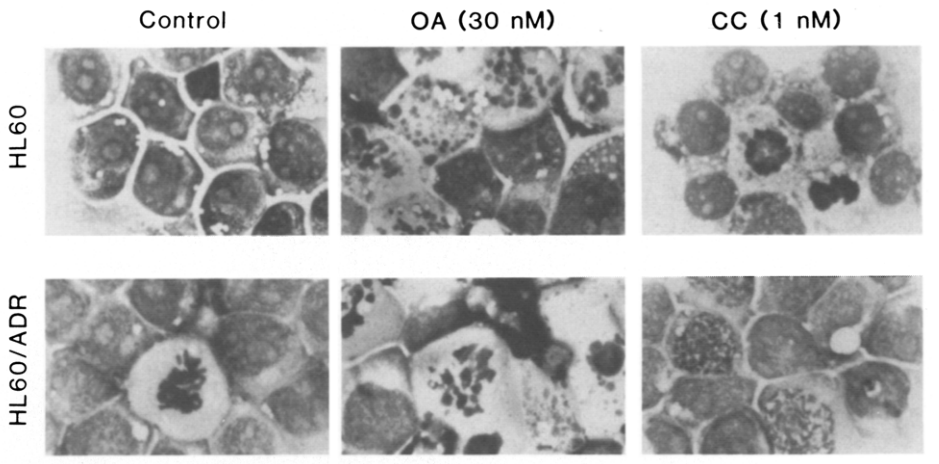

Fig. 2. Micrographs showing mitotic arrest of HL60 and HL60/ADR cells by okadaic acid (OA) and calyculin A (CC). The cells (2 x 10^5/ml/well) were incubated without (control) or with the indicated concentrations of the agents for 1 day. The proportion of mitotic cells shown was not strictly representative of those in overall cell population. Similar results were obtained in three other experiments. Original magnification, x 1,000.

Okadaic acid (30 nM) and calyculin A (1 nM) caused pronounced mitotic arrest of HL60 and HL60/ADR cells, characterized by chromatid scattering/overcondensation (Fig. 2). Mitotic cells in the absence of toxins (control), such as the one seen for HL60/ADR (Fig. 2), were observed rarely and represented < 3% of total cell population for both cell lines at a given time point. In the presence of 30 nM okadaic acid or 1 nM calyculin A, the mitotically arrested cells, however, were increased to 55 and 15% respectively for HL60 cells, and 75 and 13% respectively for HL60/ADR. We reported recently that okadaic acid caused similar mitotic arrest in K562 cells (8).

The findings (Fig. 2) that okadaic acid or calyculin A caused mitotic arrest are consistent with the role of PP1/PP2A in normal mitotic progression (4-7). Axton et al. (6) reported that neuroblasts from PP1-deficient drosophila exhibited marked mitotic arrest and abnormal mitotic spindles. In the present immunocytochemical studies (Fig. 3), we observed that microtubular structures were clearly identifiable in control HL60 and HL60/ADR cells, but not in the mitotically arrested cells caused by colchicine due to disassembly of microtubules in the present of the tubulin-interacting agent. In the okadaic acid-treated cells, while many cells exhibiting scattered/overcondensed chromatids, mitotic spindles appeared present but disorganized and multipolar, phenomena similar to those reported by Axton et al. for the PP1-deficient neuroblasts (6). Effects similar to okadaic acid were also noted for calyculin A in K562 cells (micrographs not shown).

Next, we examined the effects of TPA and calyculin A on the in situ nuclear protein phosphorylation in intact cells (Fig. 4). Although TPA stimulated phosphorylation of several proteins (notably p130, p80 and p66) in both HL60 and HL60/ADR, calyculin A was more

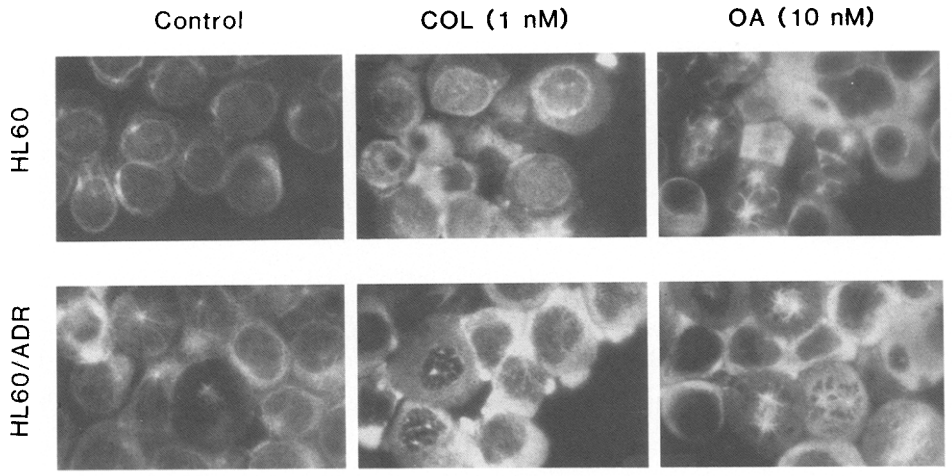

Fig. 3. Immunofluorescence micrographs showing mitotic spindles in HL60 and HL60/ADR incubated without (control) or with colchicine (COL) or okadaic acid (OA). The cells (1 x 10^5/ml/well) were incubated for 1 day. See "Experimental Procedures" for further details. Similar results were obtained in another set of experiments. Original magnification, x 1,000.

effective in stimulating the same (e.g. p130 and p80) and additional (e.g. p60 and p40) proteins. Identities of these and other phosphoproteins are still unknown; p66 possibly is lamin B, a nuclear PKC substrate (11), p80 possibly is MARCKS, the ubiquitous PKC substrate (12) and

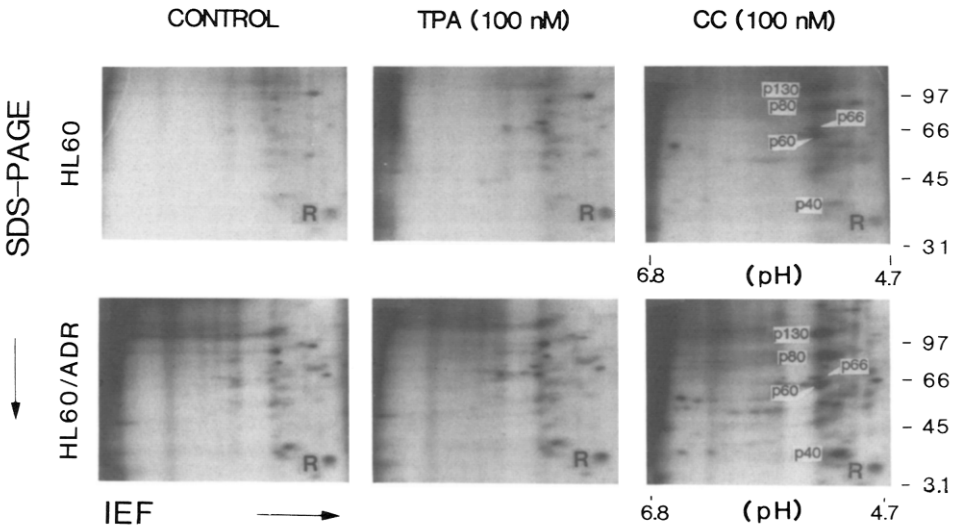

Fig. 4. Two-dimensional electrophoresis showing effects of TPA and calyculin A (CC) on phosphorylation of nuclear proteins in HL60 and HL60/ADR cells. See "Experimental Procedures" for further details. R, reference protein whose phosphorylation was not altered by the agents. Similar results were obtained in another set of experiments.

p130 is possibly vinculin, a PKC substrate although its phosphorylation in intact cells was not demonstrated (13). It appeared that phosphorylation of p66 was more specifically stimulated by TPA, and p60 and p40 were more specifically stimulated by calyculin A. Because TPA has no effect on mitotic arrest and calyculin A does not induce differentiation of HL60 cells, it is suggested that p66 might be more specifically related to differentiation whereas p60 and p40 might be critical for mitotic arrest. In addition, we noted that overall phosphorylation of cellular proteins was qualitatively and quantitatively higher in HL60/ADR than in HL60 cells (Fig. 4), in line with the earlier report by others (13). Enhanced expression of drug-pumping activity is characteristic of HL60/ADR, the adriamycin resistant variant of HL60 cells (9,14). It appeared that enhanced phosphorylation of general and specific proteins by calyculin A (Fig. 4) and increased resistance to the cytotoxicity of the PP1/PP2A inhibitors (Fig. 1) might be also related to phenotypic characteristics of drug resistant cells. This notion was also supported by our observations that the typical multidrug-resistant KB-V1 cells are (up to 35-fold) more resistant to okadaic acid and calyculin A than the drug-sensitive parental KB-3 cells (T.C. Chambers and J.F. Kuo, in preparation).

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