

Ras Mutation, Irrespective of Cell Type and p53 Status, Determines a Cell's Destiny to Undergo Apoptosis by Okadaic Acid, an Inhibitor of Protein Phosphatase 1 and 2A

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ABSTRACT

Okadaic acid (OA), a toxin from the black sponge *Halicondria okadae*, is a specific inhibitor of serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A). OA is a tumor promoter but also induces apoptosis in some tumor cell lines. In this study, we determined whether *ras* mutation and/or p53 status are characteristics associated with the cell's sensitivity to the induction of apoptosis by OA. Several cell lines that differed in *ras* and p53 mutations were treated with OA (10–100 nM). At 24 to 48 h after treatment, the percentage of cells undergoing apoptosis was quantitated. The cell lines with mutations in either H-*ras* (human bladder carcinoma cell line T24 and mouse keratinocyte cell line 308), or K-*ras* (human colon carcinoma cell lines DLD-1 and HCT116; human prostate cancer cell lines LNCaP and PC-3; human lung cancer cell lines Calu-6 and SKLU-1; and human pancreatic cancer cell line MIAPaCa2) were more sensitive to OA-induced apoptosis (3- to 10-fold) than the cell lines that lacked the *ras* mutation (mouse epidermal cell lines C50 and JB6; murine fibroblast cell line NIH3T3; human colon cancer cell line HT29; human kidney epithelial cell line Hs715.K; and human pancreatic cancer cell line Bx-PC3).

Similarly, using isogenic cell lines we found that overexpression of mutated H-*ras* in NIH3T3 and in SV40 immortalized human uroepithelial cells (SVHUC) enhanced their sensitivity to undergo apoptosis in response to OA treatment. The T24, DLD-1, SKLU-1, Calu-6, and MIAPaCa2 cell lines express mutated p53. The SVHUC as well as their *ras*-transfected counterparts have inactive p53 due to complex formation between large "T" antigen and p53. Taken together, these results imply that OA-induced apoptosis may involve a p53-independent pathway. The transfectants (NIH3T3-*ras* and SVHUC-*ras*), which express mutated H-*ras*, have up-regulated PP2A activity. OA treatment inhibited *in vivo* the levels of PP1 and PP2A activity, and induced apoptosis in SVHUC-*ras* and other cell lines. We conclude that OA-induced cell death pathway in *ras*-activated cell lines may involve a cross talk between PP1 and PP2A and *ras* signaling pathways. In light of the present results, the current theory that OA promotes mouse skin tumor formation by selective expansion of initiated cells that harbor *ras* mutations needs reevaluation.

The reversible phosphorylation of proteins, catalyzed by protein kinases and protein phosphatases (PP), is the key mechanism for the regulation of diverse cellular functions (Cohen, 1997). The serine/threonine PPs are encoded by the PPP and PPM gene families. The PPP family includes PP1, PP2A, and PP2B (calcineurin and Ca²⁺-calmodulin-regulated PP), whereas the PPM family consists of Mg²⁺-dependent PPs, which include PP2C and pyruvate dehydrogenase phosphatase. The catalytic subunit of PP1 (PP1c) is a 37-kDa protein that forms heterodimers with several regulatory units. PP2A is a family of trimeric holoenzymes composed of a 36-kDa catalytic subunit (PP2Ac) bound to a constant regulatory subunit of 65 kDa (PR654) and one of the number of variable subunits (52–130 kDa). The PPP family of PPs are

involved in many cellular processes including glycogen metabolism, calcium transport, muscle contraction, protein synthesis, regulation of cell cycle, and RNA splicing (Cohen, 1997; Fischer, 1997). A number of naturally occurring agents such as okadaic acid (OA), microcystin, tautomycin, and calyculin A (Fujiki and Suganuma, 1993; Cohen, 1997), have been reported to inhibit the members of the PPP family (Table 1).

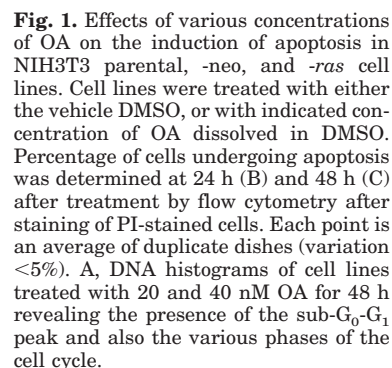
OA is the most commonly used inhibitor of PPs 1 and 2A. OA, a toxic polyether compound of a C₃₈ fatty acid, was first isolated from two sponges, *Halicondria okadae* and *H. melanodocia* (Fujiki and Suganuma, 1993). OA is a mouse skin tumor promoter that stimulates gene expression (Rosenberger and Bowden, 1996) and has the ability to induce a pseudomitotic effect. OA has also been shown to induce apoptosis in several cell lines such as human breast cancer

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ABBREVIATIONS: PP, protein phosphatase; TPA, 12-O-tetradecanoylphorbol-13-acetate; OA, Okadaic acid; TNF α , tumor necrosis factor- α ; UEPC, uroepithelial cell.

observed genetic changes during tumorigenesis in humans (Bos, 1989; Vojtek and Der, 1998). Approximately 30 to 40% of all human cancers elicit mutations in the *ras* allele (Bos, 1989). *Ras*, a 21-kDa membrane-associated GTP-binding protein, regulates diverse extracellular signals controlling cellular processes (Vojtek and Der, 1998; Marshall, 1995). The mammalian *ras* gene family consists of four homologous

Inhibitor	IC ₅₀
OA	PP2A > PP1 > PP2B (PP2A, 0.1 nM; PP1, 10–15 nM; PP2B, 5 μM)
Calyculin	PP2A = PP1 > PP2B (PP2A, 0.5–1.0 nM; PP1, 2 nM)
Microcystin-LA	PP1, 2nM; PP2A, 4 pM (does not enter some mammalian cells)
Tautomycin	PP1 > PP2A > PP2B (PP1 = 1 nM; PP2A, 10 nM)
Dinophysitoxin (35-methylokadaic acid)	PP2A > PP1 > PP2B
OTA	Inactive analog of OA



members (*H-ras*, *K-ras*, *N-ras*, and *R-ras*) and each encodes a 21-kDa protein (McCormick, 1995). A series of proteins involving protein-protein interactions as upstream molecules are responsible for the control of *ras* activation. *Ras* activation, for its downstream signaling, involves its association with the plasma membrane. The association of *ras* with the outer plasma membrane is mediated by a 15-carbon farnesyl group that is covalently linked to *ras* (Lerner et al., 1997). The plasma membrane location of *ras* switches *ras* protein from the inactive GDP-bound to the active GTP-bound state. Mutations in *ras* genes localize in the N-terminal region controlling GTP-binding. Mutations lock *ras* in the GTP-bound state. Guanosine nucleotide exchange factors promote the formation of active GTP-bound forms of *ras*, whereas GTPase activating proteins promote the formation of the inactive GDP-bound form of *ras*. The mammalian *ras* gene family acquires oncogenic activity by somatic mutations pre-

dominantly localized to amino acids 12, 13, and 61 (Vojtek and Der, 1998). The role of *ras* oncogenes in tumorigenesis has been well documented in various animal models (Stanely, 1995). Mutations in one of the three *ras* genes has been associated with specific human cancers. Transitional cell carcinoma of human urinary bladder have predominately *H-ras* mutations, whereas 95% of pancreatic adenocarcinomas, and other human tumors including colon cancers contain mutation in the *K-ras* gene. *N-ras* mutations have been observed in some cases of acute myeloid leukemia (Boss, 1989).

We determined whether chronic activation of *ras* due to mutations contributes to the cell's sensitivity to the induction of apoptosis by OA. We present data indicating that 1) cell lines that have *ras* mutation (*H* or *K*), irrespective of p53 status (von Kleist et al., 1975; Taparowsky et al., 1982; Strickland et al., 1988; Shirasawa et al., 1993; Pergolizzi et al., 1993; Aoki et al., 1997), can be induced to undergo apo-

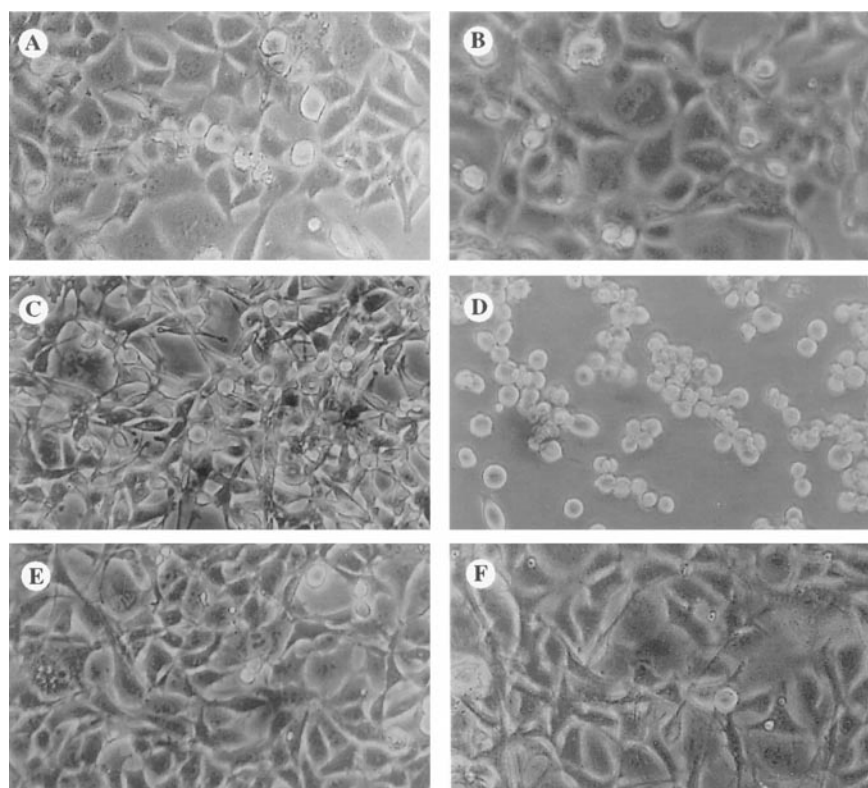
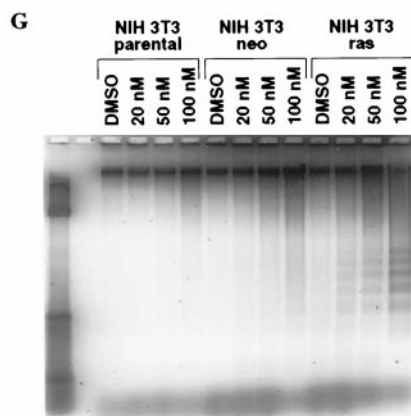


Fig. 2. OA-induced apoptosis of NIH3T3 cell lines. A, C, and E are phase-contrast photomicrographs of DMSO-treated NIH3T3 parental, *-ras*, and *-neo* cell lines, respectively; whereas B, D, and F reveal the typical morphology of NIH3T3 parental, *-ras*, and *-neo* cell lines after treatment with 20 nM OA after 48 h. G, DNA fragmentation analysis at 48 h after treatment of NIH3T3 parental, *-ras*, and *-neo* cells with the indicated concentrations of OA.



ptosis by treatment with the inhibitors of PP1 and PP2A; 2) the *H-ras* mutation up-regulated the levels of PP2A activity; and 3) OA-induced apoptosis accompanied inhibition of *ras*-induced levels of PP2A activity.

Materials and Methods

Materials

OA was purchased from Alexis Laboratories (San Diego, CA). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was purchased from Life Systems (Newton, MA). All other chemicals, including propidium iodide (PI), RNase H, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatase kit was purchased from Life Technologies/BRL (Gaithersburg, MD). Annexin V-staining kit was purchased from Clontech (Palo Alto, CA).

Anticyclin D1 was obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Anticyclin B1 was from Pharmingen (San Diego, CA).

Cell Lines

NIH3T3 cells, stably transfected with either *E/J-ras* or the neoexpression vector, were obtained from Dr. Terry Oberley, Department of Pathology, Veterans Administration Hospital, University of Wisconsin (Madison, WI). Human uroepithelial cell (UEPC) lines (SVHUC and SVHUC-*ras*) were obtained from Dr. Catherine Reznikoff, Department of Human Oncology, University of Wisconsin (Madison, WI). Human pancreatic ductal adenocarcinomas cell lines [BxPC3 and MIAPaCa2; originally from the American Type Culture Collection (Rockville, MD)] were obtained from Dr. Pamela L. Crowell, Department of Biology, Indiana University-Purdue University at Indianapolis (Indianapolis, IN). BxPC3 cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS). The MIAPaCa2 cells were grown in Dulbecco's modified Eagles' medium (DMEM) containing 10% FBS. Lung cancer cell lines (Calu-6 and SKLU-1) were obtained from Dr. Joan Schiller, University of Wisconsin (Madison, WI) and were grown in MEM medium supplemented with 8% FBS. Colon carcinoma cell lines DLD-1 and COLO 201 were maintained in RPMI 1640 supplemented with 10 and 20% FBS, respectively, whereas HCT116 and HT29 cell lines were maintained in DMEM

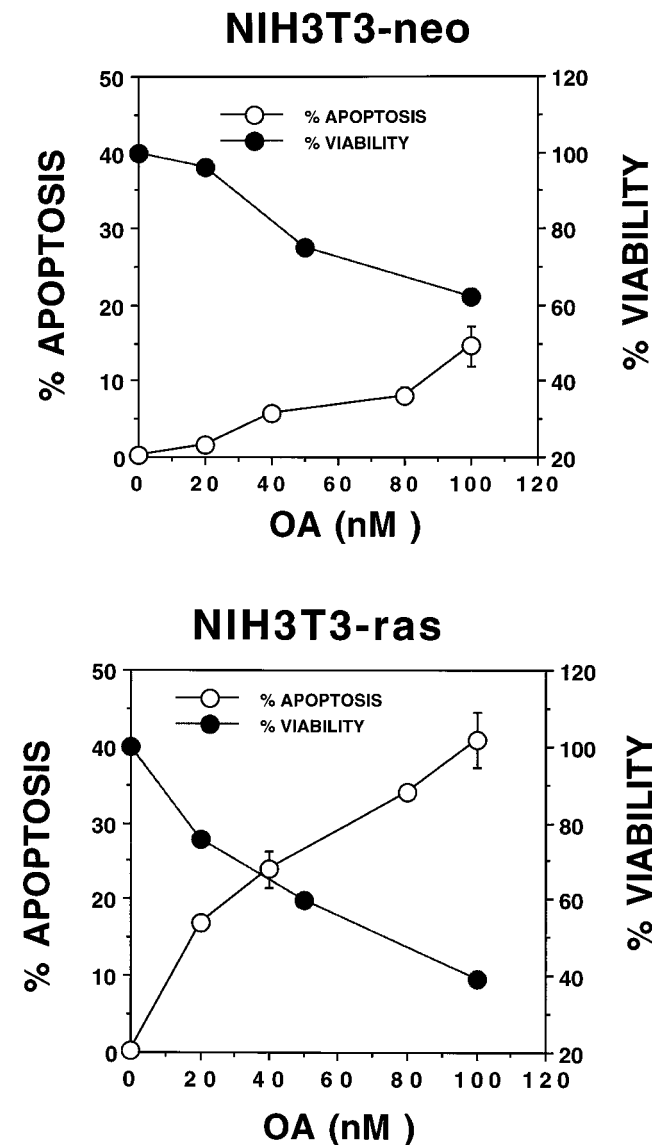


Fig. 3. Correlation between OA-induced loss of cell viability and apoptosis. At 48 h after treatment of NIH3T3-*ras* and -*neo* cells with the indicated doses of OA, cell viability and apoptosis (sub- G_0 cells) were determined by MTT assay and flow cytometric analysis, respectively. Each apoptosis value represents means \pm S.E. from three separate dishes, whereas each viability value is an average of six readings from six wells.

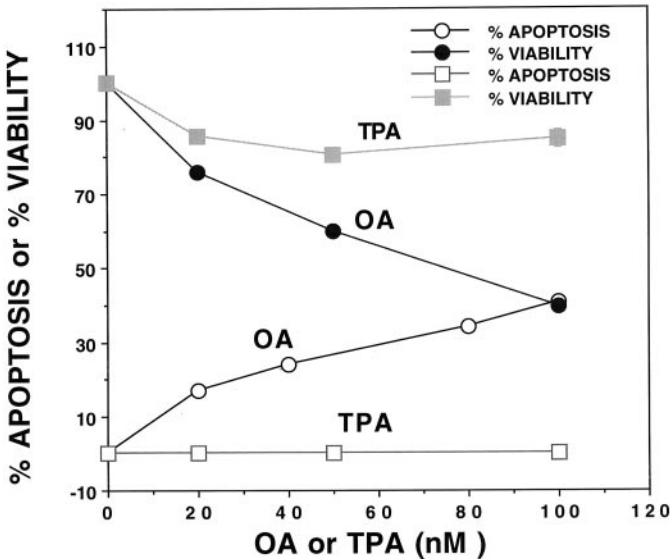


Fig. 4. Comparison of OA and TPA for their ability to affect cell viability and induce apoptosis in NIH3T3-*ras* cell line. NIH3T3-*ras* cells were treated with the tumor promoter TPA or OA (20–100 nM) for 48 h, and percentage of viable cells was assessed by MTT assay and a parallel experiment was set up to determine the sub- G_0 population by flow cytometric analysis. Each point in the graph represents average values from duplicate plates (variation <5%) for flow cytometric analysis and means of six replicates for the MTT assay.

TABLE 2

Cytotoxicity of PP inhibitors

NIH3T3-*neo* and NIH3T3-*ras* cells were treated with either the vehicle, DMSO, or with various concentrations of the indicated inhibitor (0.01, 0.1, 1, 5, 10, 20, 50, and 100 nM). At 48 hours after treatment, cell viability was determined by MTT assay. Concentrations of inhibitors required to induce 50% cell death are shown.

	IC ₅₀	
	Ras	Neo
	nM	
OA	34	100
Calyculin	1.0	NA
Microcystine	100	NA
Tautomycin	30	NA

NA, no effect.

medium supplemented with 10% FBS. Human prostate cancer cell line PC3 was cultured in RPMI 1640 medium supplemented with 5% FBS. T24 cells were maintained in MacCoys 5A medium supplemented with 10% FBS. The LNCaP and NIH3T3 cells were maintained in DMEM supplemented with 5% FBS. SVHUC and SVHUC-*ras* and mouse keratinocytes cell lines 308 and C50 [provided by Dr. Jill Pelling, University of Kansas Medical Center (Kansas City, KS)], were cultured in Ham's F12 medium supplemented with 250 U/ml

insulin, 5 $\mu\text{g/ml}$ Transferrin, 15 mM dextrose, 0.1 mM nonessential amino acids, 1 $\mu\text{g/ml}$ hydrocortisone, 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 1% FBS. Mouse epidermal cell line JB6 [provided by Dr. Nancy M. Colburn, Cell Biology Section, Laboratory of Viral Carcinogenesis, National Cancer Institute (Frederick, MD)] was maintained in MEM containing 5% FBS, whereas the normal human kidney cell line HS 715.K was grown in the above medium with 10% FBS.

Cell Treatments

The cells were allowed to grow in their respective media until they were 70% confluent. Stock solution of OA was prepared in ethanol and was further diluted in DMSO. Cells were treated with various concentrations of OA. Control dishes were treated with the vehicle DMSO (final concentration: <0.1%).

Cell Viability Assay

The viability of the cells were determined using the MTT assay (Mosmann, 1983).

Apoptosis Assays

The cells undergoing apoptosis were identified and quantitated as follows.

Phase-Contrast Microscopy. The cells exhibiting characteristic morphological features associated with apoptosis were viewed under a microscope with a 40 \times objective and then photographed.

Flow Cytometric Analysis. At appropriate times after treatment, the cells were harvested by trypsin-EDTA treatment, washed twice with ice-cold PBS and subsequently stained with antibody to Annexin V-fluorescein isothiocyanate (FITC) and PI. This dual staining technique revealed the viable (annexin V-/PI-), preapoptotic [annexin V+/PI-], apoptotic [annexin V+/PI+], and damaged [annexin V-/PI+] cells (Darzynkiewicz et al., 1996). For analysis of cell cycle markers, the harvested cell pellet was permeabilized using 0.25% Triton-X-100 solution in PBS for 5 min at 4°C. The cell pellet was washed and stained with anticyclin D1 and anticyclin B1 antibodies followed by incubation with the secondary antibody conjugated to FITC. The cell pellet was washed and stained with staining solution containing PI (5 $\mu\text{g/ml}$) and RNase H (200 $\mu\text{g/ml}$). This staining technique allowed the immunochemical detection of cyclins in relation to cell cycle position (DNA content) by mutiparameter flow cytometric analysis. The percentage of cells undergoing apoptosis were also quantitated by the method described by Darzynkiewicz et al. (1994). The cell pellet obtained after harvesting the treated and untreated cells was resuspended in ice-cold PBS and fixed in 95% ethanol. The fixed cells were pelleted, resuspended in a PI staining solution, filtered through a 40- μm pore nylon mesh, and analyzed using a Becton-Dickinson FACStar plus flow cytometer with excitation 488 nm. The DNA histograms elicited the sub-G₀-G₁ cells as the apoptotic peak in the various phases of the cell cycle.

DNA Fragmentation. To detect the DNA ladder typical of the apoptotic cells, DNA fragmentation analysis was performed. At appropriate times after treatment, the genomic DNA was isolated (Gentra Systems, Minneapolis, MN) and subjected to agarose gel electrophoresis (1.8%). The DNA in the gel was visualized with the Fluoroimager SI (Molecular Dynamics, Sunnyvale, CA) after staining for 1 h with SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene, OR).

PP Assay. PP activity was assayed using ³²P-labeled phosphorylase α as a substrate which detects both PP1 and PP2A activities. We used the PP kit supplied by Life Technologies/BRL. At appropriate times after treatment, the cell pellet was homogenized in the extraction buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 15 mM β -mercaptoethanol, 0.25 M sucrose, 0.3% Triton X-100, 5 $\mu\text{g/ml}$ leupeptin, and 5 $\mu\text{g/ml}$ aprotinin and centrifuged (40,000g) to give a soluble supernatant. The PP activity in the clear supernatant was determined by measuring the trichloroacetic acid-

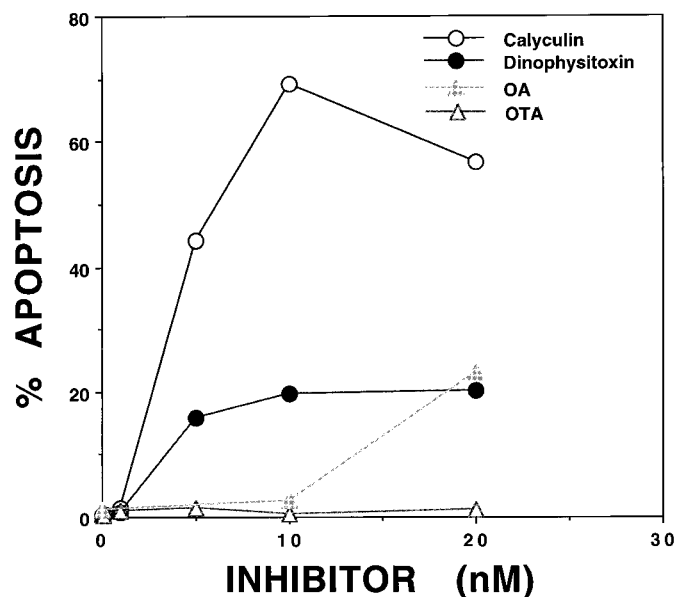


Fig. 5. Induction of apoptosis in NIH3T3-*ras* by PP inhibitors. NIH3T3-*ras* cells were treated with the indicated dose of the phosphatase inhibitors (calyculin, dinophysitoxin, OTA, and OA) and percentage of sub-G₀ cells were determined by flow cytometry after staining the cells with PI. Each point is an average of determinations from duplicate plates (variation <5%).

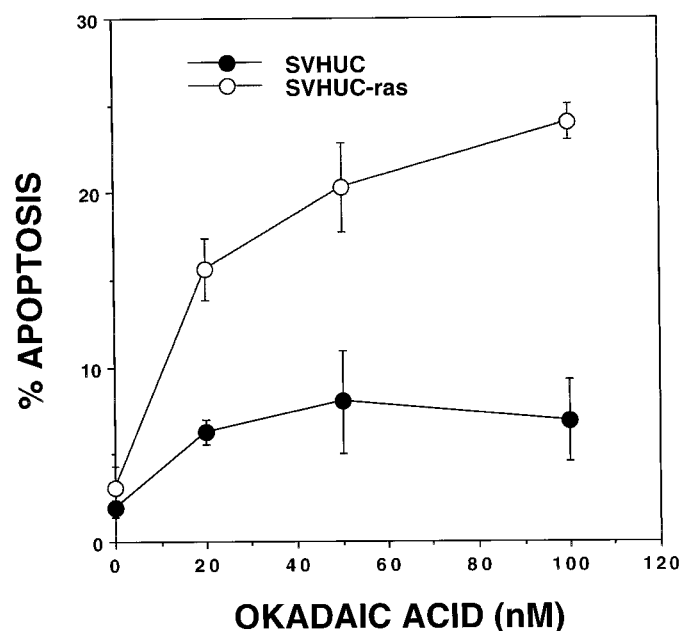


Fig. 6. OA-induced cell death in an isogenic pair of human UEPs. E/J-*ras* was transfected into SVHUC to yield SVHUC-*ras* cells. Cells were treated with either the vehicle, DMSO, or the indicated concentrations of OA in DMSO for 24 h. Percentage of cells undergoing apoptosis were analyzed by flow cytometry after staining with PI. Each point represents apoptosis represents means \pm S.E from three separate dishes.

soluble counts released after incubation of the ^{32}P -labeled phosphor-lyase α in the cell extract. The PP activity was linear up to assay times of 10 min and 5 μg protein of the cell extract. Routinely, incubation for PP activity was carried out for 10 min with an extract containing 5 μg of protein as determined by the Bio-Rad assay (Bio-Rad, Hercules, CA).

Results

Ras Mutation Imparts Sensitivity to Cells for Induction of Apoptosis by OA. To determine whether *ras* mutation is the key genetic event that is associated with the susceptibility of cells to undergo apoptosis in response to treatment with the PP inhibitor OA, we used genetically identical cell lines that differed only in the expression of mutated *ras*. In our initial experiments, we used NIH3T3 and human UEPC stably transfected with either pSV2-neo or PREJ *ras* (mutated c-H-*ras* with 12th codon mutation).

Subconfluent cultures of the parental, NIH3T3-neo, and NIH3T3-*ras* cells were treated with OA (10–40 nM) for a period of 24 or 48 h. The percentages of cells undergoing apoptosis were quantitated by flow cytometric analysis after staining of cells with PI (Fig. 1A). OA-induced apoptosis was observed only in NIH3T3-*ras* transfectants but not in parental NIH3T3 and NIH 3T3-neo cells at 24 h after OA treatment at 10, 20, or 40 nM (Fig. 1B). OA treatment at 40 nM for 48 h also resulted in a small increase in the apoptotic response of the parental NIH3T3 and NIH3T3-neo cell lines (Fig. 1C).

The effects of OA on the morphology of NIH3T3 transfectants

are shown in Fig. 2A. The addition of OA caused the cells to round up and detach from the substratum, which are preparatory events leading to apoptosis. The NIH3T3-*ras* cells were more sensitive to OA-induced detachment than the parental NIH3T3 and NIH3T3-neo cells (Fig. 2, A–F). OA-induced apoptosis of NIH3T3 cell lines was further confirmed by DNA fragmentation analysis. In this experiment, DNA was isolated from the parental NIH3T3-*ras* and neotransfected cells at 48 h after treatment with 20, 50, and 100 nM OA. As shown in Fig. 2G, internucleosomal cleavage was not observed in the parental and neotransfected NIH3T3 cell lines, whereas it was clearly observed in the *ras*-transfected NIH3T3 cells. The effects of OA treatment on the viability of the cells was analyzed by the MTT assay. As shown in Fig. 3, there appears to be a good correlation between the onset of apoptosis and the cell viability. The effect of OA on the induction of apoptosis in NIH3T3-*ras* cells was specific. As shown in Fig. 4, the potent mouse skin tumor promoter TPA failed to induce apoptosis in NIH3T3-*ras* cells.

We also evaluated the cytotoxicity of a series of inhibitors of PP1 and PP2A (Table 1) toward NIH3T3-neo and NIH3T3-*ras* cell lines. The results are shown in Table 2. In these experiments, the NIH3T3 cells were treated with various concentrations of the indicated inhibitor (0, 0.01, 0.1, 1, 5, 10, 20, 50, and 100 nM), and at 48 h after treatment, cell viability was determined by the MTT assay. Shown in Table 2 are the concentrations which sacrificed 50% of the cells (IC_{50}). Again, the NIH3T3-*ras* cells were more sensitive than the NIH3T3-neo cells to killing by the PP inhibitors; calyculin was the

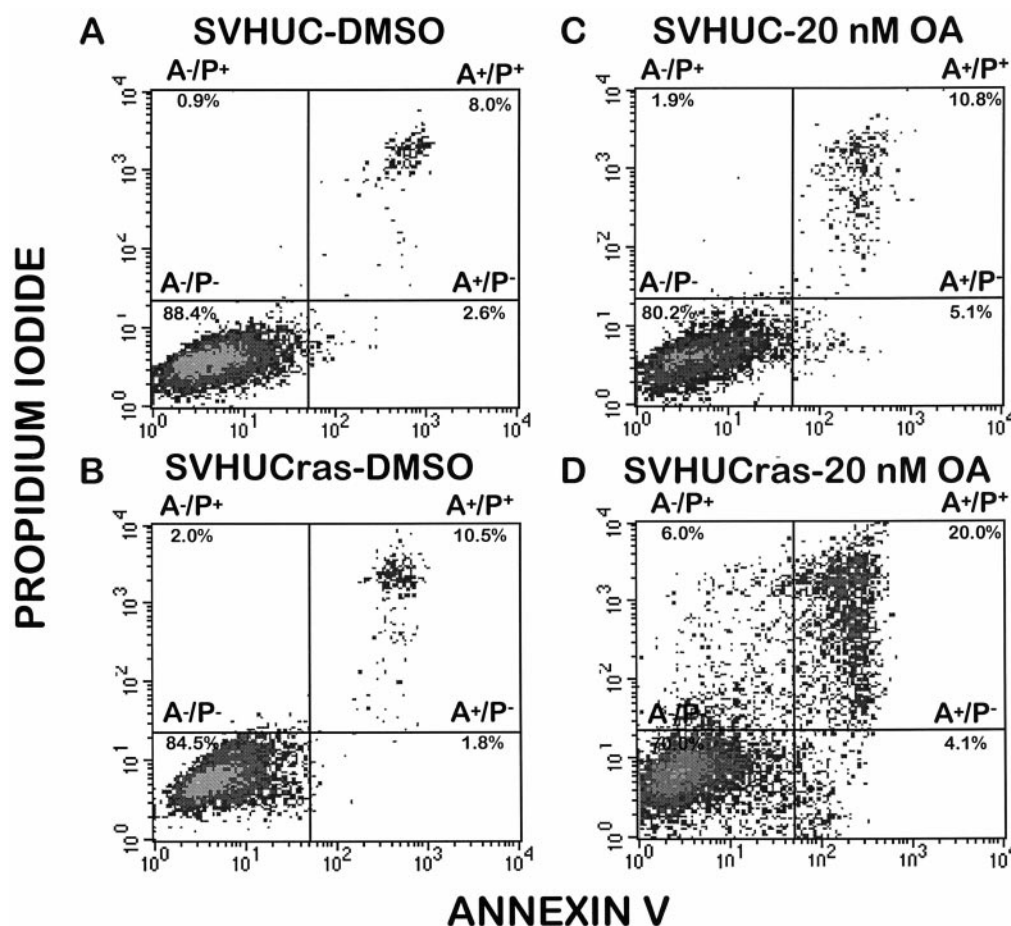


Fig. 7. OA-induced apoptosis of SVHUC and SVHUC-*ras* cell lines as determined by dual-colored (PI and Annexin V) flow cytometry. Shown are the cytograms of annexin V binding (ordinate) versus PI uptake (abscissa) in DMSO- and OA-treated cells. Cell lines were treated with either the vehicle, DMSO (A and B), or with 20 nM OA (C and D) for 24 h. The cells were harvested by trypsinization, washed with PBS, and subsequently stained with antibody to annexin V conjugated to FITC and with PI (10 $\mu\text{g}/\text{ml}$). Viable [annexin V $^-$ /PI $^-$], preapoptotic [annexin V $^+$ /PI $^-$], apoptotic [annexin V $^+$ /PI $^+$], and the residual damaged [annexin V $^-$ /PI $^+$] cells are shown in the respective quadrants. A, annexin V; P, PI.

most cytotoxic among the PP inhibitors. In a separate experiment, we compared the cytotoxicity of OA with calyculin, dinophysitoxin, and OA tetracetate (OTA) on NIH3T3-*ras* cells. In this experiment (Fig. 5), apoptotic cells (sub-G₀ cells) were determined by flow cytometric analysis of PI-stained cells. Calyculin and dinophysitoxin were more cytotoxic than OA. OTA, an inactive analog of OA, did not induce apoptosis in NIH3T3-*ras* cells (Fig. 5).

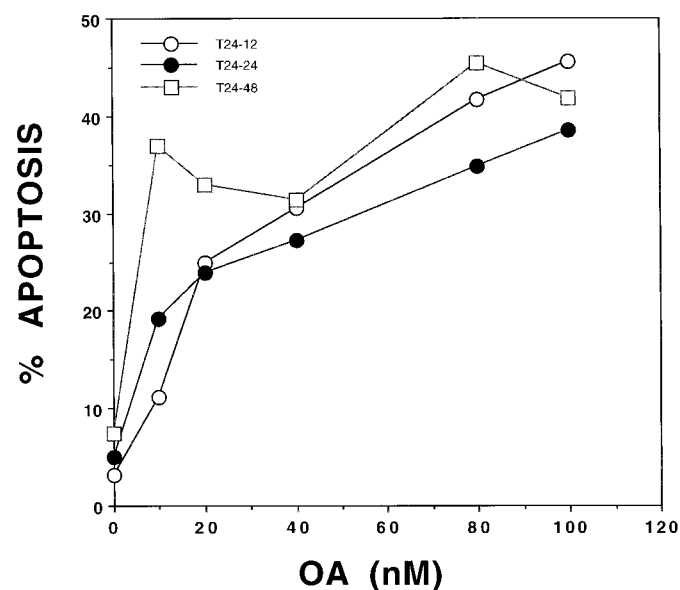


Fig. 8. Effect of OA-dose and time of treatment on the induction of apoptosis in T24 cells. T24 cells were treated with either the vehicle, DMSO, or with varying doses of OA (10–100 nM). Cells were harvested at 12, 24, and 48 h after treatment and were stained with PI to determine percentage of cells undergoing apoptosis. Each point in the graph is an average of duplicate dishes (variation <5%).

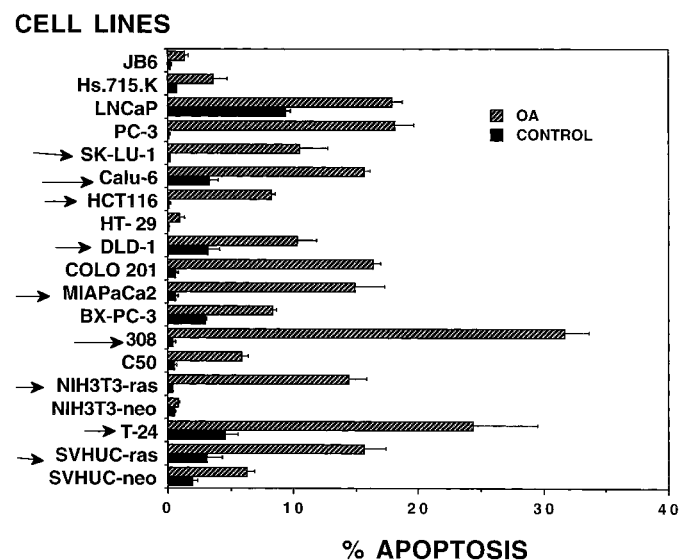


Fig. 9. OA-induced apoptosis in cell lines with or without mutated *ras*. Semiconfluent cultures of all the indicated cell lines were exposed to 20 nM OA in DMSO for a period of 24 h except PC-3 and LNCaP, SK-LU-1, and Calu-6 cells, which were treated for 48 h, after which cells were harvested, fixed in ethanol, and then stained with PI. Percentage of cells undergoing apoptosis was analyzed by flow cytometry. Each value is mean \pm S.E. of determinations from three separate plates for determining apoptosis. Similar results were observed in a repeat experiment. Arrow points to the cell lines with mutations in *ras* oncogene.

We further compared the effects of OA on the induction of apoptosis in the isogenic UEPC (Pratt et al., 1992). As shown in Fig. 6, the UEPC with mutation in H-*ras* (SVHUC-*ras*) were more sensitive than the parental cells (SVHUC) to OA-induced apoptosis. OA-induced sensitivity of SVHUC-*ras* cells was further confirmed by dual-colored (PI and Annexin V) flow cytometric analysis (Fig. 7). We also found that human bladder carcinoma cell line T24, which is known to harbor H-*ras* mutations, was highly sensitive to OA for induction of apoptosis (Fig. 8). In this experiment (Fig. 8) about 25% of cells underwent apoptosis as early as 12 h after treatment with 20 nM OA.

OA Induces Apoptosis in a Variety of Cell Types with Mutations in Either H- or K-*ras*. To further obtain evidence that *ras* mutations determine a cell's sensitivity to OA-induced apoptosis, we used several established cell lines with or without *ras* mutations. As shown in Fig. 9, we observed a distinct difference in the percentage of cells undergoing apoptosis between cell lines with and without mutations in the *ras* oncogene. Cell lines harboring mutations in either H-*ras* [e.g., T24 (Taparowsky et al., 1982) and 308 (Strickland et al., 1988) cells or in K-*ras* (DLD1 and HCT116; Shirasawa et al., 1993); PC3 and LNCaP (Pergolizzi et al., 1993); Calu-6 and SKLU-1 (Lehman et al., 1991); and MIAPaCa2 (Aoki et al., 1997)] were more sensitive to OA-induced apoptosis (Fig. 9) than cell lines with wild-type *ras* oncogene [mouse epidermal cell lines C50, and JB6 (Sun et al., 1993); murine fibroblast cell line NIH3T3, human kidney epithelial cell line Hs715.K, human UEPC line SVHUC human pancreatic cancer cell line BxPC3 (Aoki et al., 1997); and human colon cancer cell line HT29 (von Kleist et al., 1975)].

OA-Induced Apoptosis Is Independent of p53 Status. The tumor suppressor p53, a nuclear phosphoprotein, plays a key role in the induction of apoptosis. Post-translational modification by phosphorylation of p53, mediated by several kinases, is essential for its transcriptional activity (Mowat, 1998). p53 is dephosphorylated by PP1 and PP2A. The findings that OA has been shown to induce hyperphosphorylation of p53 (Yatsunami et al., 1993) prompted us to determine whether OA-induced apoptosis involves a p53-dependent pathway. In these experiments, we determined the ability of OA to induce apoptosis in various cell lines with altered p53 status. At first we determined the effects of OA on the induction of apoptosis in SVHUC cells. SV40 encodes two proteins, large "T" antigen and "t" antigen. The large "T" antigen complexes with p53 and pRb family of proteins (Kao et al., 1993). As shown in Fig. 6, OA-induced apoptosis in SVHUC in the absence of active p53 due to complex formulation with the large T antigen. We also found that cell lines with or without mutations in p53 were susceptible to OA-mediated apoptosis (Fig. 9). Cell lines sensitive to OA-induced apoptosis included the prostate cancer lines PC3 and LNCaP. PC3 is androgen independent and is defective for both its alleles for the p53 gene, whereas LNCaP is androgen sensitive and expresses the wild-type p53 gene (Israel et al., 1995). Human bladder transitional carcinoma T24 cells carry an inframe deletion of tyrosine 126 in the p53 gene (Cooper et al., 1994). The colon cancer cell line HCT 116 expresses wild-type p53 gene (Take et al., 1996), whereas DLD-1 cells harbor a mutation at position 241 (resulting a change from serine to phenylalanine) in p53. The lung cancer cell line Calu-6 has mutations at positions 196 and 213 whereas SKLU-1 cells

exhibits mutation at position 193 in the p53 gene. These results imply that OA-induced apoptosis may involve a p53-independent pathway.

OA-Induced Apoptosis Accompanies Changes in Cell Cycle Progression. Because OA has been reported to in-

duce the hyperphosphorylation of cell cycle regulator proteins pRb and p53 (Yatsunami et al., 1993), we determined whether OA-induced apoptosis accompanies alterations in the cell cycle profile. Asynchronously growing NIH3T3 parental, NIH3T3-*ras*, and NIH3T3-*neo* cells were treated with

TABLE 3

Effect of OA on cell cycle progression

NIH3T3-parental, -*ras* and -*neo* cells were treated with 20 nM OA for 48 h whereas SVHUC and SVHUC-*ras* were treated for 24 h, and percentage of cells in various phases of the cell cycle was determined by flow cytometric analysis of PI-stained cells.

Cell Line	Treatment	Apoptosis	G ₀ -G ₁	G ₂ -M	S
		%	%	%	%
NIH3T3-parental	DMSO	0.79	77.23	16.59	6.18
	20 nM OA	2.83	74.65	22.06	3.59
NIH3T3- <i>neo</i>	DMSO	0.25	77.22	19.41	3.37
	20 nM OA	3.71	72.21	26.88	0.91
NIH3T3- <i>ras</i>	DMSO	1.25	76.79	16.49	6.72
	20 nM OA	23.18	62.01	37.99	0
SVHUC	DMSO	0.23	24.9	49.6	25.4
	OA	0.4	15.3	47.2	37.5
SVHUC- <i>ras</i>	DMSO	0.6	37.5	34.1	28.4
	OA	24.22	15.6	47.5	36.9

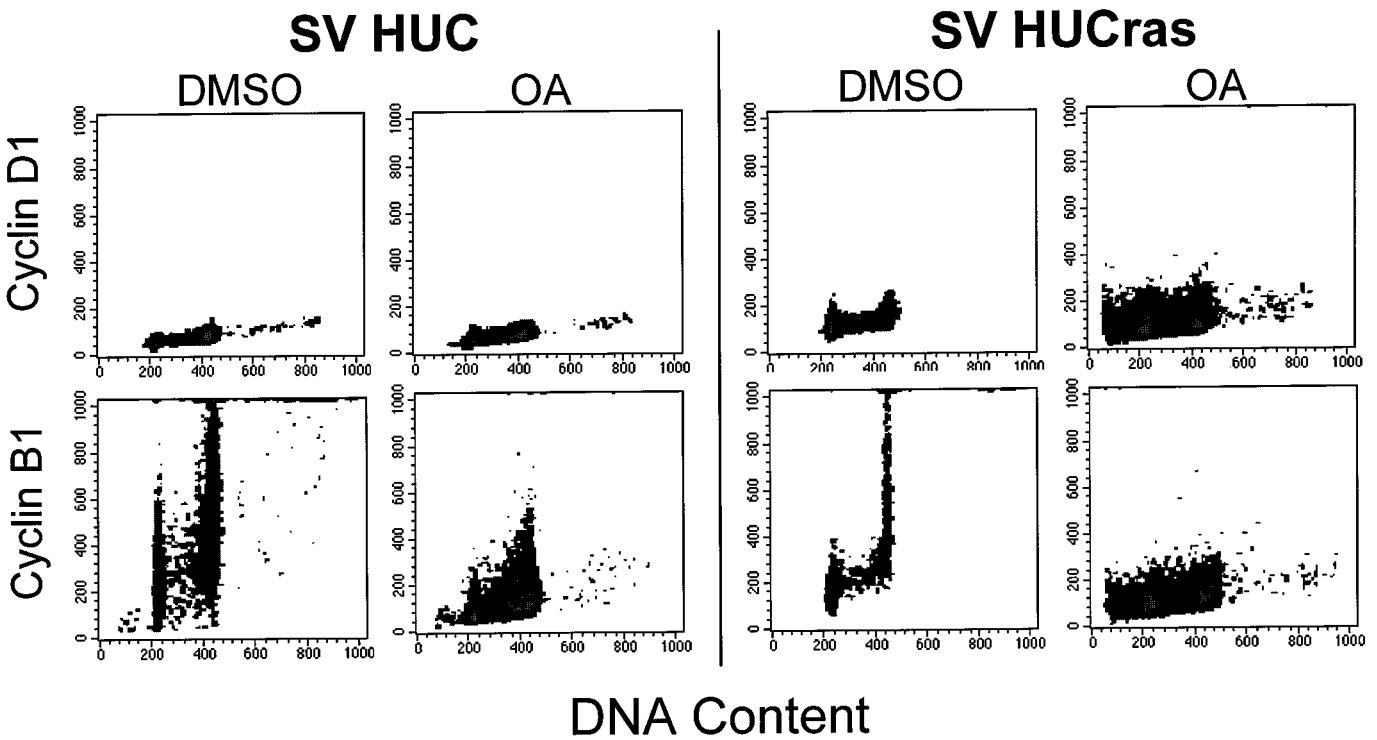


Fig. 10. Analysis of cell cycle markers and DNA content by flow cytometry in OA-treated SVHUC and SVHUC-*ras* cells. Human UEPs with (SVHUC-*ras*) and without (SVHUC) mutated H-*ras*, were treated with either DMSO or 20 nM OA for 24 h. Cells were fixed, permeabilized, incubated with the indicated anticyclin antibodies, and stained using a secondary antibody conjugated to FITC. Cells were also stained with PI for DNA content and analyzed on a FACScan flow cytometer.

TABLE 4

Effects of OA on cell cycle markers and induction of apoptosis

Quantitation of the data illustrated in Fig. 10 is shown. Values represent fold increase in level of cyclin staining over background staining of isotype control.

Cell Line	Treatment	Cyclin D1 and B1 Levels at Various Phases of Cell Cycle					
		D1			B1		
		G ₀ -G ₁	G ₂ -M	S	G ₀ -G ₁	G ₂ -M	S
SVHUC	DMSO	1.45	1.42	1.47	6.89	6.90	9.04
	OA	1.09	1.24	1.22	1.94	2.53	2.68
SVHUC- <i>ras</i>	DMSO	1.94	1.84	1.52	3.24	6.79	3.24
	OA	1.53	1.48	1.47	1.57	1.54	1.63

20 nM OA for 48 h, whereas SVHUC and SVHUC-*ras* cells were treated with 20 nM OA for 24 h. Both cell lines were stained with PI to determine the percentage of cells in various phases of the cell cycle. The NIH3T3-*ras* and SVHUC-*ras* cells treated with OA for 48 h displayed a decrease in the G₀-G₁ phase of the cell cycle accompanied with an increase in the sub-G₂-M phase of the cell cycle as well as in the sub-G₀-G₁ phase of the cell cycle. The parental NIH3T3, the neotransfected NIH3T3 cells and SVHUC cells did not reveal a distinct G₂-M block in their cell cycle progression (Table 3).

We also determined the effects of OA (20 nM) on the expression of cyclins D1 and B1 in SVHUC and SVHUC-*ras* cells. OA treatment did not alter the level of expression of cyclin D1, whereas B1 expression was decreased in both cell lines. However, OA treatment led to a marked increase in the G₂-M block in SVHUC-*ras* cells (Fig. 10 and Table 4).

H-Ras Mutation Up-Regulates PP2A Activity. A possibility was explored to determine whether *ras* mutations result in alterations in the basal PP1 and PP2A activities. In this experiment (Fig. 11), the indicated cell lines were grown to subconfluency and then were used to assay PP activity. PP2A and PP1 activities were distinguished by their sensitivity to 2 and 20 nM OA, respectively (Huang et al., 1995; Murata et al., 1996). The PP1 and PP2A activities were detected, although at different levels, in all the cell lines evaluated. A novel observation is the finding that H-*ras* mutation leads to an increase in the PP2A activity. As shown in Fig. 11, NIH3T3 and human UEPs after transfection with the mutated H-*ras* displayed an increase in PP2A activity. It is noteworthy that SVHUC, which are immortalized after transfection with the wild-type SV40, which has both large T and small t antigens (Pratt et al., 1992), had reduced or no PP2A activity. This is because of the fact that small t antigen specifically inhibits PP2A activity (Yang et al., 1991; Howe et al., 1998). As shown in Fig. 10, SVHUC-*ras* up-

regulated PP2A activity. Similar results were observed in other H-*ras* transfected cell lines (Fig. 11).

We also determined the effects of OA treatment on in vivo PP1 and PP2A activity. In this experiment (Fig. 12), the indicated cell lines were treated with 20 nM OA. At 24 h after treatment, cells were harvested to assay PP1 and PP2A activity. OA, which induced apoptosis in several cell lines, inhibited in vivo both the PP1 and PP2A activity.

Discussion

Our results that show that the cell lines that harbor *ras* mutations are more sensitive than cell lines with wild-type *ras* to OA-induced apoptosis, were unexpected. OA is a mouse skin tumor promoter (Fujiki and Suganuma, 1993). A majority of the mouse skin papillomas and carcinomas, elicited by initiation with 7,12-dimethylbenz[a]anthracene and by promotion with TPA, contain a point mutation (A to T) in the Ha-*ras* oncogene at codon 61 (Pelling et al., 1988; Fujiki et al., 1989; Kim et al., 1997). Similarly, Fujiki et al. (1989) reported similar codon 61 mutations in the Ha-*ras* gene in mouse skin tumors developed by initiation with 7,12-dimethylbenz[a]anthracene and promotion with OA class tumor promoters (OA, dinophysistoxin-1, and calyculin A) and TPA-type tumor promoters (teleocidin and aplysiatoxin). These results led to the current hypothesis that tumor promoters selectively cause the clonal expansion of a population of initiated cells, which harbor Ha-*ras* mutations, to form skin papillomas and carcinomas. Our cell lines, which express Ha-*ras* mutations, were insensitive to TPA for the induction of apoptosis (Fig. 4). However, the question of how OA, which selectively may expand cells with Ha-*ras* mutations in intact mouse skin in vivo, induces apoptosis in vitro of cell lines that contain *ras* mutations, is critical to the interpretation of present results (Hennings et al., 1992; Fujiki and Suganuma, 1993). The role of *ras* mutations in mouse skin tumor formation by the inhibitors of PP 1 and 2A needs reevaluation. We hypothesize that the initiated cells, which escape OA-induced apoptosis and proliferate to form papillomas and carcinomas, have other genetic lesions in addition to Ha-*ras* mutations. All epidermal cells, which have only Ha-*ras* mutations, undergo apoptosis during tumor promotion with OA. We now present results indicating that the proliferative signals of *ras*-activated cells can be diverted toward apoptosis by inhibition of PP1 and PP2A by OA.

OA-induced cell death was due to apoptosis and not the results of necrosis as confirmed by several criteria such as morphological characteristics, analysis of preapoptotic cells entering apoptosis by dual-colored (PI and Annexin V) flow cytometry, and DNA fragmentation by agarose gel electrophoresis (Figs. 1–3 and 6–8). OA-induced apoptosis appears to involve mitochondrial changes and DNA fragmentation (Fig. 2). It is clear from the results, using isogenic cell lines, that *ras* mutation imparts sensitivity to cells to OA-induced apoptosis (Figs. 1–3 and 6 and 7). To obtain additional evidence to support the conclusion that *ras* mutation imparts sensitivity to cells to OA-induced apoptosis, we screened several established cell lines harboring mutations in either H-*ras* (T24 and 308) and K-*ras* genes (DLD-1, HCT116, Calu-6, SK-LU-1, and MIA PaCa2). The results illustrated in Fig. 9 clearly indicate that the sensitivity of several murine and human cell lines to OA-induced apoptosis correlated with the

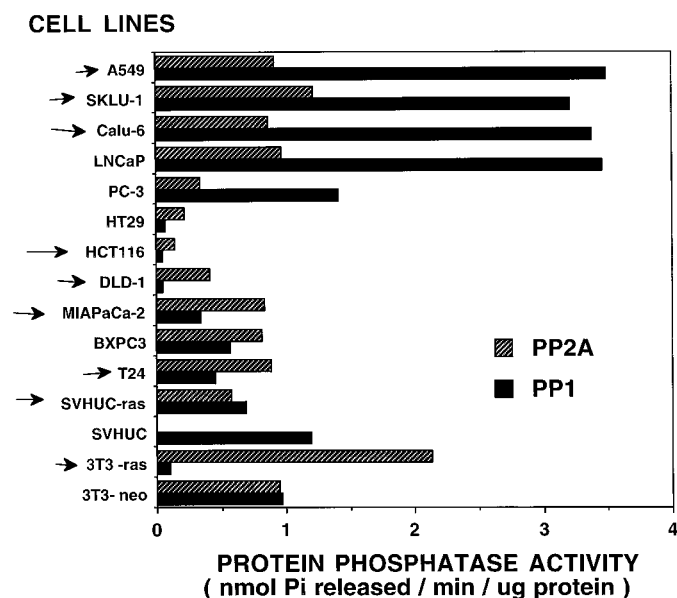


Fig. 11. Basal PP1 and PP2A activities in various cell lines. Each cell extract was prepared from untreated subconfluent cultures. Cell extract was treated either with 2 or 20 nM OA in vitro to distinguish between PP1 and PP2A activities. Phosphatase activity was measured using ³²P-labeled phosphorylase *a* as a substrate as described in *Materials and Methods*.

presence of mutation in the *ras* oncogene H or K. Because the concentrations of OA used in the present study inhibited both PP1 and PP2A (Figs. 11 and 12), the onset of apoptosis appears to link to the sustained phosphorylation of PP1 and PP2A substrates.

The role of functional p53 in determining cell's sensitivity to OA-induced apoptosis has been inconsistent. In this context the reports by Sheikh et al. (1996), and Boe et al. (1991) are noteworthy. They observed the induction of apoptosis by OA in breast cancer cell lines with disrupted p53 function. However, others have proposed both p53-independent and p53-dependent pathways in OA-induced apoptosis in NIH3T3 (p53^{-/-}) (Yan et al., 1997) and rabbit lens epithelial cells (Li et al., 1998). Interestingly, we found that cell lines with or without mutations in p53 were equally susceptible to OA-induced apoptosis (Figs. 6 and 9), implying that OA-induced G₂-M arrest and apoptosis involves p53-independent pathways.

The mechanisms by which the expression of H-*ras* up-regulates PP2A activity (Fig. 11) are unknown. The native form of PP2A consists of A and C subunits that are considered to form the core of the enzyme. The C unit binds stably to the carboxyl terminal region of the A subunit. The B subunit binds to the amino terminal region of the A subunit. The B family has three members (B α , B β , and B δ) (~55 kDa). The B' family consists of several isoforms (~54–68 kDa), whereas the B'' family has two members (72 and 130 kDa). The combination of B subunits to the core PP2A heterodimeric complex gives rise to the several PP2A variants that exhibit substrate specificity. The B subunits act as targeting subunits that direct PP2A to specific subcellular locations (Strack et al., 1998). *Ras*-induced up-regulation of PP2A activity may be the result of increased synthesis of PP2A subunits (A, C, and B) due to increased transcription or translation. PP2A activity is also regulated by post-translational modification (methylation) of the catalytic subunit of PP2A (Turowski et al., 1995; Kloeker et al., 1997). *Ras* activation may up-regulate PP2A activity by increasing the production of ceramide catalyzed by sphingomyelinase. The cer-

amide 2 has been shown to activate PP2A activity (Galadari et al., 1998).

The cell lines, treated in vivo with OA, inhibited both PP1 and PP2A activity (Fig. 12). The mechanisms by which inhibition of PP1 and PP2A activity lead to the induction of apoptosis in *ras*-activated cells remains to be defined. OA-induced apoptosis preceded arrests cells in the progression of G₂-M phase (Table 3). Entry into mitosis is triggered by the maturation promoting factor, which is composed of cdk2 and cyclin B. In early G₂, the human cdc2 protein is phosphorylated on tyrosine 15 and threonine 14 by Weel and Mik1 to inhibit its activation. G₂ to M progression requires activation of cdc2 by dephosphorylation of cdc2 by the dual specificity PP cdc25 (Draetta and Eckstein, 1997). Cdc25C activity in G₂ phase is negatively regulated by PP2A. In human cells, there are three cdk activating phosphatases (cdc25A, cdc25B, and cdc25C). Cdc25A is expressed early in the G₁ phase of the cell cycle, whereas cdc25B and cdc25C are expressed at the G₂-M boundary (Draetta and Eckstein, 1997). It is likely that OA-induced G₂-M arrest may be the result of inhibition of cyclin B1 accumulation by OA (Table 4). However, the roles of other cell cycle regulatory proteins such as other cyclins and cyclin-dependent kinase inhibitors in OA-induced G₂-M arrest remain to be explored.

OA treatment leads to the production of tumor necrosis factor- α (TNF α) in BALB/c 3T3 cells (Komori et al., 1993). TNF α is a cytokine (molecular mass 17 kDa) and is produced mainly by monocytes and/or macrophages. TNF α is implicated in various biological processes such as immunoregulation, inflammation, cachexia and mitogenesis. OA and TNF α have been shown to share similar effects such as the induction of protein phosphorylation and the expression of early response genes. TNF α , like OA, was found to be a tumor promoter (Guy et al., 1992; Fujiki et al., 1997). We also found that OA-induced apoptosis of human cell lines accompanied release of TNF α in the medium (D.R. and A.K.V., unpublished observations). The role of TNF α in OA-induced apoptosis is currently being investigated.

In summary, we have shown, using isogenic cell pairs (Figs. 1–3, 6, and 7) and other established cell lines (Fig. 9), that OA and other inhibitors of PP1 and PP2A (e.g., calyculin, tautomycin, dinophysitoxin, and microcystin) induce apoptosis in cell lines that harbor either H- or K-*ras* mutations. OA-mediated apoptosis does not appear to require the presence of wild-type p53 (Fig. 9). An overall survey of the basal levels of PP1 and PP2A indicates the presence of both enzymes at different levels in different cell lines (Fig. 11). However, H-*ras* mutation appears to affect the level of PP2A activity (Fig. 11). The results presented indicated that the cell survival signals imparted by the oncogenic *ras* can be diverted to cell death signals by inhibition of PP1 and PP2A activities. Thus, the PP1 and PP2A and their associated signaling molecules (e.g., hyperphosphorylated proteins and genes) may be potential targets for human cancer treatments.

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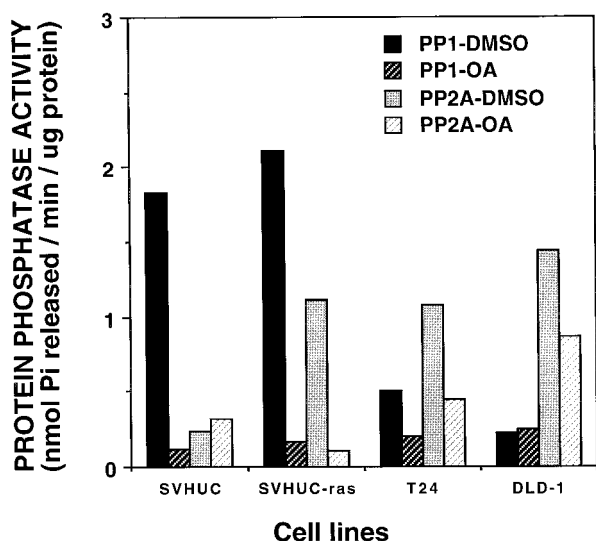


Fig. 12. PP1 and PP2A activities of SVHUC, SVHUC-*ras*, T24, and DLD-1 cells treated with OA in vivo. Indicated cell lines were treated with 20 nM OA for 24 h and the level of PP1 and PP2A activities were measured as described in *Materials and Methods*.

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