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Prevention of v-Ha-Ras-Dependent Apoptosis by PDGF Coordinates in Phosphorylation of ERK and Akt

Yoshiko Arase,*[,1] Takaki Hiwasa,† Ritsuko Hasegawa,† Jun Nomura,† Hisao Ito,* and Nobuo Suzuki†

**Department of Radiology and †Department of Biochemistry, School of Medicine, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8670, Japan*

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In some v-Ha-*ras*-transfected cell lines, serum deprivation results in apoptosis. Clarification of the molecular mechanisms by which oncogenic Ras controls susceptibility to apoptosis may assist in the development of effective therapies against human cancer with oncogenic *ras* gene. In this report, we established a v-Ha-*ras*-transfected human fibroblast clone, R1. In R1 cells, induction of v-Ha-Ras enhanced susceptibility to cell death under serum-deprived conditions. Ladders of cellular DNA were identified only when oncogenic *ras* was induced under serum-deprived conditions. Platelet-derived growth factor (PDGF) precluded DNA fragmentation of serum-deprived v-Ha-*ras*-transformed cells. Under serum-depleted conditions, the amounts of activated ERK and Akt decreased as compared with those under serum-containing conditions. The decreased levels of activated ERK and Akt were restored by the addition of PDGF. Inhibition of phosphorylated-ERK and Akt resulted in renewed susceptibility to cell death. These results indicate that failure of signal transduction of oncogenic Ras by the deficiency of growth factors such as PDGF causes v-Ha-Ras-dependent apoptosis.

Ras protein is a member of the superfamily of small GTPase and plays roles in the signaling pathways leading to cell proliferation, differentiation and transformation (1–3). Mutations of Ras are found in about 20–30% of human tumors (4), making the *ras* one of the oncogenes most frequently associated with human cancers.

Oncogenic Ras has been shown to alter the susceptibility to apoptosis. In some cell lines, oncogenic Ras represses apoptosis induced by various stimuli: e.g. anti-cancer drugs (5–7), ionizing radiation (8–14) and ultraviolet (UV) radiation (5). On the other hand, oncogenic Ras promotes apoptosis by the stimulation of PKC (15) or the inhibition of PKC (16) and treatment with okadaic acid (17) or 5-FU (18). Under serum-withdrawal conditions, oncogenic Ras prevents apoptosis in some cases (19, 20) via the c-Raf-1 pathway (21) but promotes in other cases (22) via c-Raf-1 (23, 24). Furthermore, c-Raf-1 causes apoptosis in v-Ha-Ras-expressing cells after serum withdrawal throughout the MEK-1-ERK (MAPK) dependent (23) or independent (24) pathways.

Another major downstream molecule of Ras in signal transduction, PI3K-Akt (PKB), has also been investigated well with regard to apoptosis. In NIH3T3 cells, the PI3K-Akt pathway is suggested to suppress apoptosis under serum-deprived conditions (25).

In this report, we examined the effect of v-Ha-Ras on the susceptibility to apoptosis of human cells which are hypersensitive to cell killing induced by several stimuli. Expression of v-Ha-Ras induces apoptosis under serum-deprived conditions, which concurs with the failure in signaling from v-Ha-Ras due to the absence of growth factors such as PDGF.

MATERIALS AND METHODS

Cells and culture conditions. "RSa" cell line was established from human embryonic cells as previously described (26). RSa cells are highly sensitive to UV-induced cell killing and are useful in investigating the mechanism of UV-induced cellular responses (27–29). The cells were cultured with Eagle's MEM supplemented with 10% calf serum and maintained in a 37°C humidified atmosphere containing 5% CO_2 in air.

Agents. Eagle's MEM was purchased from GIBCO/BRL (Grand Island, NY). Calf serum was from GIBCO/BRL (Grand Island, NY).

Abbreviations used: PDGF, platelet-derived growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated/extracellular response kinase kinase; JNK, Jun N-terminal kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; Dex, dexamethasone; DMSO, dimethyl sulfoxide; MMTV-LTR, mouse mammary tumor virus long terminal repeat; UV, ultraviolet.

[1] To whom correspondence should be addressed. Fax: 81-43-226-2101. E-mail: arase-cib@umin.ac.jp.

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v-Ha-*ras* gene (WT) was a kind gift from Dr. B. M. Willumsen (University Institute of Microbiology, Copenhagen). The eukaryotic expression vector, pMSG, was from Amersham Pharmacia Biotech (Uppsala, Sweden). Lipofectin was from Life Technologies Inc. (Gaithersburg, MD). G418 was obtained from GIBCO/BRL. Dexamethasone (Dex) and dimethyl sulfoxide (DMSO) were from WAKO Life Science (Osaka, Japan). Methylene blue and trypan blue dye were from WAKO Life Science (Osaka, Japan). PDGF was from Biomedical Technologies Inc. (Stoughton, MA). PD98059 was from New England Biolabs Inc. (New England, MA) and wortmannin was from Calbiochem (La Jolla, CA). The monoclonal anti-Ras antibody (NCC-RAS-001) was obtained from Funakoshi (Tokyo, Japan). Polyclonal phospho-specific anti-p44/42 MAP kinase (Thr 202/Tyr 204) antibody, polyclonal phospho-specific anti-SAPK/JNK (Thr 183/Tyr 185) antibody and polyclonal phospho-specific anti-Akt (Ser 433) antibody were from New England Biolabs Inc. (New England, MA). Monoclonal anti-Bax antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The chemiluminescent detection system (ECL) was from Amersham Pharmacia Biotech (Buckinghamshire, UK). SYBR Green I nucleic acid gel stain was from FMC Bio Products (Rockland, ME).

Gene transfection. The expression vector, pMSG/v-Ha-*ras*, was constructed by insertion of full-length v-Ha-*ras* gene into a downstream region of MMTV-LTR of pMSG. In this vector, the inserted gene can be induced by the addition of Dex. Cells were co-transfected with pMSG/v-Ha-*ras* and *neo* gene. Transfection was accomplished using lipofectin according to the manufacturer's instructions. After culture for 48 h, cells were replated in the complete medium containing G418 (400 μg/ml) to select neomycin-resistant cells. Cells were pretreated with Dex (1 μM) for 2 days to induce the transfected *ras* gene, while cells were treated with the solvent, DMSO (0.1% v/v) for the control.

Colony formation assay. R1 cells and their vector control cells were pretreated with Dex or DMSO for 2 days and then plated in 100-mm dishes (1×10^3 cells). Cells were incubated for 18 h to allow the cells to attach, irradiated with UV (254 nm, 6, 9 or 12 J/m^2) as described previously (27), and then cultured for a further 2 weeks in the presence or absence of Dex. The cells were stained with 0.2% methylene blue in 30% methanol, and colonies whose diameters were larger than 2 mm were scored.

Viability assay. Cells were plated in 60-mm dishes (1×10^5 cells) and cultured in MEM containing 10% calf serum with or without Dex for 2 days. The medium was then changed to a serum-deprived medium with or without Dex. Viable cells were counted on the days indicated in the legends to Figs. 2 and 6 using 0.04% trypan blue dye.

DNA fragmentation analysis. Cells (5×10^5 cells per 60-mm plate) were pretreated with Dex or DMSO for 2 days and then UV irradiation (10 J/m^2) or mock irradiation was performed as described (27). They were cultured for a further 48 h in medium containing 10% calf serum or with the serum-free medium in the absence or presence of PDGF (4 ng/ml). Cells were then washed three times with phosphate-buffered saline (PBS) and lysed at 37°C for 3 h in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% SDS and 100 μg/ml of proteinase K. After the addition of one-tenth volume of 3 M sodium acetate, nucleotides were extracted with phenol/chloroform and then with chloroform. The high molecular weight DNA was precipitated by addition of seven-tenths volume of 2-propanol followed by centrifugation at 13,000 $\times$ g for 5 s at room temperature. The low molecular weight DNA was recovered from the supernatant and precipitated by incubation overnight at -20°C. After centrifugation (13,000 $\times$ g, 10 min) the precipitate was resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 50 μg/ml of DNase-free RNase A, and incubated for 3 h at 37°C. The samples were applied to 1.5% agarose gel, electrophoresed in 90 mM Tris-borate (pH 8.0) and 2 mM EDTA at 100 V for 3 h, and stained with SYBR Green for 20 min. DNA was visualized by UV illumination.

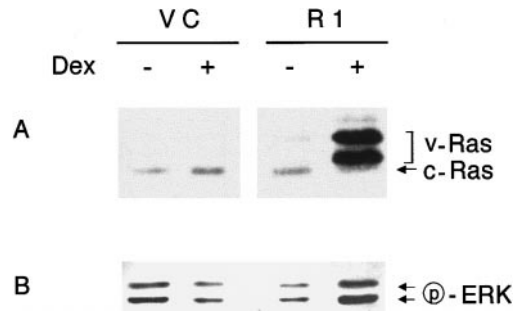

FIG. 1. Induction of v-Ha-Ras protein by treatment of Dex in R1 cells. v-Ha-*ras*-transfected RSa cells (R1) and their vector control cells (VC) were cultured in the absence or presence of Dex for 2 days. The supernatant of cell lysate from these cells was electrophoresed and transferred onto nitrocellulose. (A) The expression of Ras protein was detected by anti-Ha-Ras antibody. Bars indicate transfected v-Ha-Ras and arrow indicates endogenous c-Ras. (B) Western blot analysis was performed with anti-phospho-specific-ERK antibody. Arrows indicate activated-p42 and p44 ERK.

Western blotting. Cells were washed with PBS 4 times and incubated with lysis buffer for 10 min at 4°C. The lysis buffer consisted of 0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, 100 μM antipain, 100 μM pepstatin A, and 100 μM ALLN. The cell lysate was centrifuged at 13,000 $\times$ g for 10 min and the supernatant was used for immunoblot analysis as described elsewhere (30).

RESULTS

Isolation of v-Ha-ras-transfectant by Western blotting. v-Ha-*ras* oncogene was co-transfected with *neo* gene into RSa cells and G418-resistant clones were isolated. Ras proteins were analyzed by Western blot using anti-Ha-Ras antibody. A clone, designated R1, expressed a large amount of v-Ha-Ras only after treatment with Dex (Fig. 1A). Vector control cells, designated VC, did not express v-Ha-Ras either with or without Dex treatment. The two bands in the immunoblot appeared to be v-Ha-Ras since the same profile was obtained for v-Ha-*ras* transfected NIH3T3 cells (31). The faint band of 21 kDa observed in all lanes may correspond to endogenous c-Ha-Ras.

The activation of ERK in R1 cells was then examined to confirm the effect of activated Ras on downstream signaling. Anti-phospho-specific ERK antibody, which recognizes phosphorylated threonine-202 and tyrosine-204 of ERK1 (p42) and ERK2 (p44) was used to detect the activated ERK (32). In R1 cells, the expressed level of activated ERK increased in accordance with the increased level of v-Ha-Ras protein (Fig. 1B). Conversely, activated ERK decreased somewhat after treatment with Dex in VC cells (Fig. 1B).

The effects of v-Ha-Ras expression on the susceptibility to cell death. Using this inducible transfectant, we examined whether oncogenic Ras changes the susceptibility to cell death induced by UV irradiation and serum deprivation. First, the effect of v-Ha-Ras on

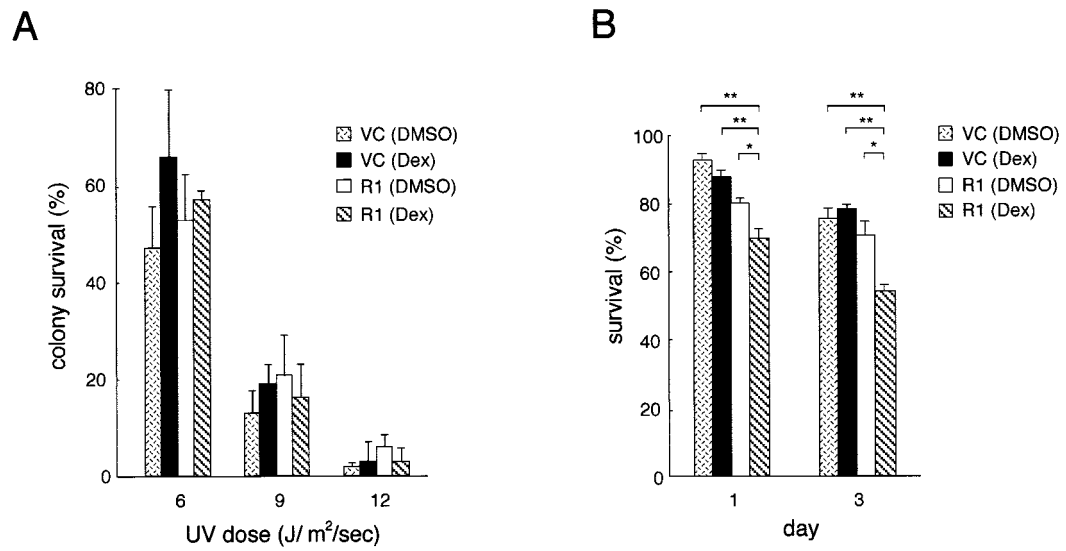

FIG. 2. The effect of v-Ha-Ras expression on susceptibility to cell death. (A) Histogram is demonstrating the effect of v-Ha-Ras expression on susceptibility to cell death induced by UV. Cells were irradiated with UV (6, 9 or 12 J/m^2) or mock-irradiated. Viable cells were examined by a colony formation assay. (B) Histogram is demonstrating the effect of v-Ha-Ras expression on susceptibility to cell death induced by serum deprivation. Viable cells were counted at 1 and 3 days after changing the medium using the dye exclusion method. ** and * represent $P < 0.005$ and $P < 0.01$ respectively.

cellular viability was investigated by a colony formation assay. v-Ha-Ras induced by Dex did not affect the susceptibility to cell death induced by either UV irradiation (Fig. 2A) or ionizing radiation (data not shown). Next, the effect of v-Ha-Ras on cellular viability was examined by measuring the percentage using the dye exclusion method. The expression of v-Ha-Ras caused cell death when cells were cultured in serum-deprived medium (Fig. 2B). In vector-transfected VC cells, the percentage of viable cells after culturing for 72 h in serum-starved conditions was 75% in the absence of Dex and 78% in the presence of Dex ($P = 0.24$). On the other hand, in R1 cells, the percentage of viable cells was 71% in the absence of Dex and fell to 54% in the presence of Dex ($P = 0.008$), indicating that the increased cell death was not due to the side effects of Dex but due to induction of v-Ha-Ras. Thus, it is possible to consider that the cell death under the serum-deprived conditions was caused by v-Ha-Ras.

v-Ha-Ras-dependent-DNA fragmentation under serum-deprived conditions. To investigate v-Ha-Ras-dependent cell death further, typical DNA fragmentation was analyzed. R1 cells were pretreated with Dex (Fig. 3A) or its solvent (Fig. 3B) and then mock-irradiation (Figs. 3A and 3B, lanes 1, 3, and 5) or irradiation with UV (10 J/m^2) (Figs. 3A and 3B, lanes 2, 4, and 6) was performed. These cells were cultured with the medium containing 10% calf serum (Figs. 3A and 3B, lanes 1 and 2) or with the serum-depleted medium in the absence (Figs. 3A and 3B, lanes 3 and 4) or presence (Figs. 3A and 3B, lanes 5 and 6) of PDGF (4 ng/ml) for 48 h. DNA fragmentation was evident when R1 cells were cultured under serum-depleted conditions with the induction of v-Ha-Ras by treatment with Dex (Fig. 3A, lane 3). Treatment with Dex followed by culture with serum-containing medium (Fig. 3A, lane 1) or serum deprivation alone without treatment of Dex (Fig. 3B, lane 3) did not produce ladders of cellular DNA. The presence of PDGF prevented the DNA fragmentation (Fig. 3A, lane 5).

Ladders of cellular DNA were also observed slightly after UV irradiation under serum-containing conditions when v-Ha-Ras was expressed (Fig. 3A, lane 2), but were much less distinct than under serum-free conditions (Fig. 3A, lane 4). PDGF, again, suppressed the DNA fragmentation induced by UV irradiation (Fig. 3A, lane 6). Without induction of v-Ha-Ras, DNA fragmentation induced by UV was not definite (Fig. 3B, lane 2).

Signal molecules relate to v-Ha-Ras-dependent apoptosis. After pretreatment of R1 cells with Dex, cells were cultured for 1, 2, and 4 h. Cells were cultured with medium containing 10% calf serum (Figs. 4A and 4B, lanes 1–3), and with the serum-depleted medium in the absence (Figs. 4A and 4B, lanes 4–6) or presence (Figs. 4A and 4B, lanes 7–9) of PDGF (4 ng/ml). Expression levels of apoptosis-related proteins such as Bax and phosphorylated JNK were not significantly altered by the absence of serum or by the presence of PDGF (Fig. 4A). On the other hand, levels of phosphorylated ERK and Akt decreased when cells were cultured in the serum-free medium (Fig. 4B). The addition of PDGF to the culture medium reinstated the phosphorylation of ERK and Akt (Fig. 4B, lanes 7–9). The expression level

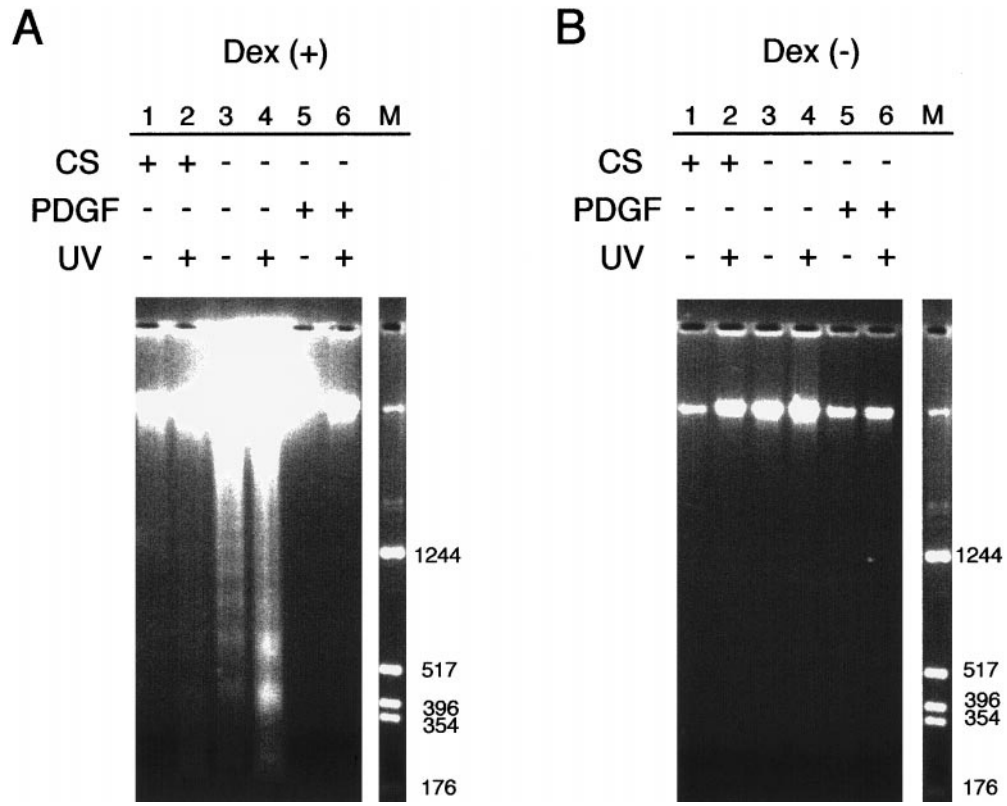

FIG. 3. DNA fragmentation analysis under the serum-depleted conditions. (A) v-Ha-Ras induced by treatment of Dex induced DNA fragmentation. (B) Endogenous c-Ha-Ras did not induced DNA fragmentation. Mock-irradiation (lanes 1, 3, and 5) or irradiation with UV (10 J/m^2) (lanes 2, 4, and 6) was performed. Cells were cultured with medium containing 10% calf serum (lanes 1 and 2) or with the serum-depleted medium (lanes 3–6) in the absence (lanes 3 and 4) or presence of (lanes 5 and 6) of PDGF (4 ng/ml). Following deprivation of serum for 48 h, DNA was extracted. Lane M indicates size marker.

of v-Ha-Ras analyzed by anti-Ha-Ras antibody was hardly effected either by the serum deprivation or the addition of PDGF.

Effects of suppressed phosphorylation of ERK and Akt on the v-Ha-Ras-dependent cell death. To clarify the effects of phosphorylation of ERK and Akt on prevention of v-Ha-Ras-dependent apoptotic cell death, PD98059 (MEK inhibitor; MEK is upstream molecule of ERK) and wortmannin (PI3K inhibitor; PI3K is upstream molecule of Akt) were added in the serum-deprived medium with PDGF. Briefly, cells were cultured with Dex for 2 days to induce v-Ha-Ras protein, and then treated with 20 μM PD98059 or 25 μM wortmannin for 1 h. The medium was then changed to serum-deprived medium with PDGF. Control cells were not pretreated with these inhibitors and their medium was changed to serum-containing or serum-deprived medium with or without PDGF.

Morphological observation showed that R1 cells cultured under medium-containing conditions showed a spindle-like morphology (Fig. 5A). On the other hand, PDGF-untreated R1 cells showed a contracted and round shape 48 h after serum deprivation (Fig. 5B). The majority of the PDGF-treated R1 cells exhibited spindle-like normal morphology under medium-deprived conditions (Fig. 5C). Treatment with PD98059 or wortmannin increased the percentage of contracted and round cells again (Figs. 5D and 5E).

We examined the effect of inhibited phosphorylation of ERK and Akt on the cell viability using the dye exclusion method (Fig. 6). When cells were cultured under the serum-containing conditions, the percentage of viable cells was 91%. Serum deprivation decreased the percentage of viable cells to 43% and PDGF increased it to 87%. When cells were pretreated with 20 μM PD98059, the percentage of viable cells was 43% ($P = 0.0001$) and when cells were pretreated with 25 μM wortmannin, it was 30% ($P = 0.0001$).

DISCUSSION

In this study, we isolated a v-Ha-*ras*-transfected clone, R1, derived from human fibroblast RSa cells. In R1 cells, the expression of v-Ha-Ras was detected only after the cells were treated with Dex (Fig. 1). Using these R1 cells, we studied the effects of oncogenic Ras protein on susceptibility to cell death. RSa cells are so sensitive to UV-induced cell killing that we supposed

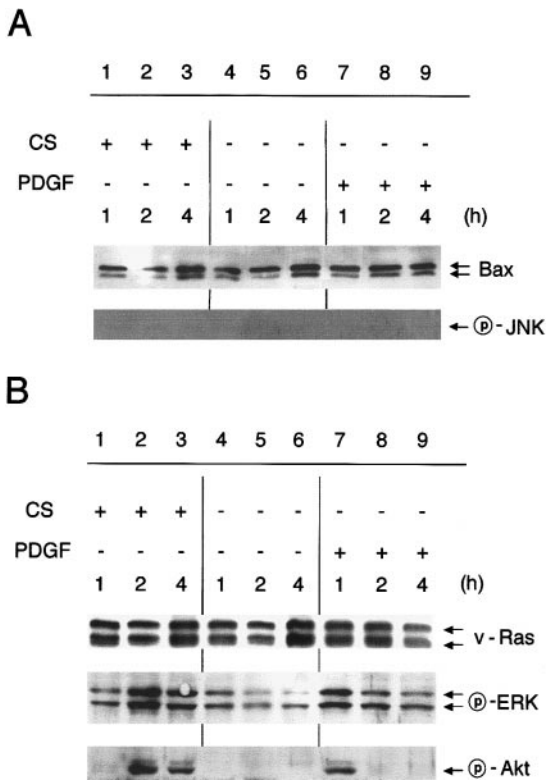

FIG. 4. Effects of serum or PDGF on the expression of downstream molecules of Ras or apoptosis-related molecules. (A) Western blotting was performed using anti-Bax and anti-phospho-specific JNK antibodies. (B) Western blotting was performed using anti-Ha-Ras, anti-phospho-specific ERK and anti-phospho-specific Akt antibodies. Dex-treated-R1 cells were cultured with medium containing 10% calf serum (lanes 1–3) or with serum-depleted medium in the absence (lanes 4–6) or presence (lanes 7–9) of PDGF (4 ng/ml). The cell lysate was extracted at the times indicated (1, 2, and 4 h). Western blot analysis was performed with the indicated antibodies.

that oncogenic Ras would suppress the hypersensitivity to UV-induced cell killing because there are several reports showing oncogenic Ras induces resistance to ionizing or UV radiation (8–14). Contrary to our expectations, the expression of v-Ha-Ras did not affect the susceptibility to cell death induced by UV irradiation (Fig. 2A) but in fact enhanced cell death under serum-deprived conditions (Fig. 2B). DNA fragmentation was also observed only when v-Ha-Ras was expressed under serum-deprived conditions (Fig. 3A, lane 3 versus Fig. 3B, lane 3). This v-Ha-Ras-dependent DNA fragmentation in serum-free medium was prevented by the addition of PDGF (Fig. 3A, lane 5). Similarly to our results, over-expression of c-*myc* induced apoptosis in serum-free medium and PDGF has been reported to suppress the c-*myc*-induced apoptosis in Rat-1 cells (33).

To study the molecular mechanisms of oncogenic Ras-dependent-apoptosis under serum-deprived conditions, we examined the effect of serum or PDGF on the expression of downstream molecules of Ras or apoptosis-related molecules. The expressed levels of apoptosis-related proteins such as Bax and JNK were almost constant (Fig. 4A). On the other hand, the levels of activated ERK and Akt, which were observed under serum-containing conditions, were suppressed under serum-depleted conditions in spite of the highly activated Ras. PDGF reinstated the suppressed expression of activated ERK and Akt (Fig. 4B). This suppressed phosphorylation of ERK and Akt under serum-deprived conditions went along with v-Ha-Ras-dependent apoptosis. Furthermore, the inhibited-phosphorylation of ERK and Akt by PD98059 and wortmannin went along with cell death of v-Ha-*ras*-transformed cells (Figs. 5A–E and Fig. 6), which ensured the relationship between v-Ha-Ras-dependent apoptosis and de-phosphorylation of ERK and Akt. From these findings, we suggest at least two mechanisms of v-Ha-Ras-dependent apoptosis in R1 cells; (a) under PDGF-deprived conditions, v-Ha-Ras activates some factors that trigger an apoptotic pathway, (b) failure of signal transduction of v-Ha-Ras which is due to the absence of PDGF leads to shutting-off of the survival signals. As to (a), the c-Raf-1-MEK-ERK pathway is a candidate as reported previously (23). There is another previous report showing the Ras/Raf-1-dependent and MEK-1-independent apoptosis in the serum-deprived Ha-*ras*-transformed cells (24). They also reported (24) that apoptosis in Ha-*ras*-transformed cells occurred concurrently with an up-regulation in the expression of the pro-apoptotic protein, Bcl-xS, and down-regulation in the expression of the anti-apoptotic protein, Bcl-2. There is also a previous report of Ras-dependent and c-Raf-1-MEK-ERK-independent apoptosis under serum-deprived conditions (34). For (b), activated ERK can protect cells from apoptosis induced by serum deprivation (35) and activated ERK and Akt have been reported to have a role in survival signals (21, 25). In R1 cells, v-Ha-Ras-dependent apoptosis was prevented by PDGF, which coordinated the phosphorylation of downstream molecules such as ERK and Akt. It indicates that the c-Raf-1-MEK-ERK pathway is not the trigger pathway to apoptosis but is necessary to prevent v-Ha-Ras-dependent apoptosis in R1 cells. Therefore, we hypothesize that cells are permitted to avoid cell death only when survival signals transude successfully. We also propose a 'dual signal' hypothesis that mutational activation and/or expression of oncogenes induce cell proliferation in the presence of survival factors but cell death in the absence or deficit of survival factors. Contrary to our results, rodent fibroblastic cells transformed by oncogenic Ras were able to grow in serum-free medium but the cells autocrinally produced growth factors such as PDGF in conditioning medium (36).

Recently, oncogenic *ras* has been reported to trigger cell suicide through the activation of a caspase-independent cell death (37). This cell death is a type of programmed cell death characterized by the early ap-

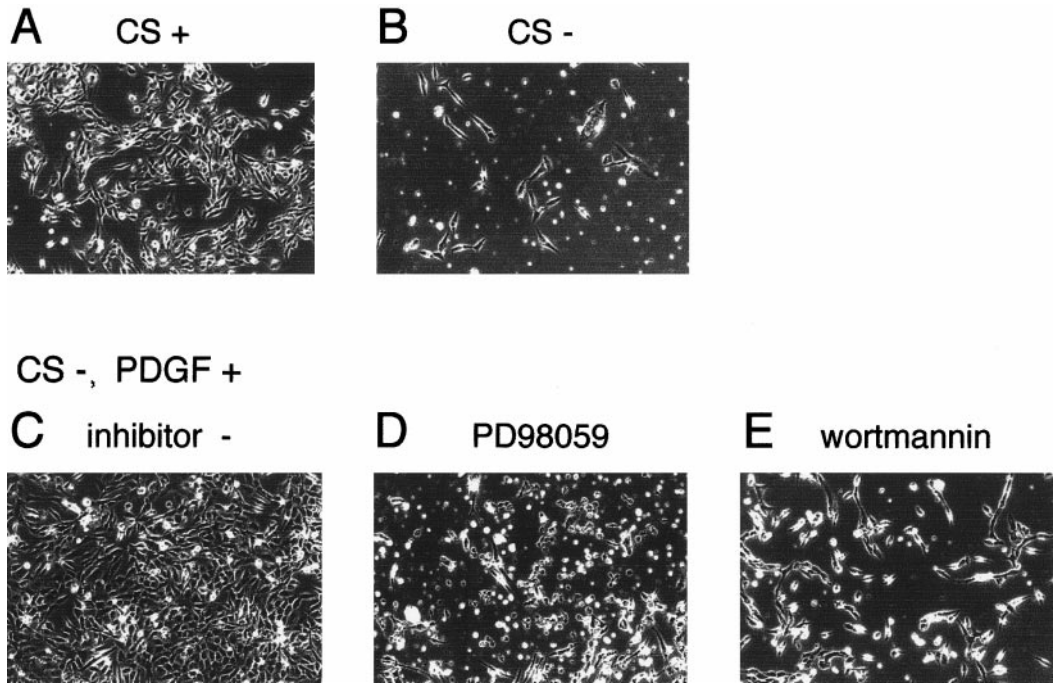

FIG. 5. Effects of phosphorylation of ERK and Akt on v-Ha-Ras-dependent cell death. Subconfluent cells (5×10^4 per 60-mm plate) were cultured in serum-containing medium with Dex for 2 days. Next, 20 μM PD98059, 25 μM wortmannin were pretreated for 1 h. Control cells were not pretreated with these inhibitors. Then, medium was changed to (A) 10% calf serum containing medium, (B) serum-deprived medium, (C, D, and E) serum-deprived medium with PDGF (C) without inhibitor or (D) with 20 μM PD98059 and (E) with 25 μM wortmannin. Cells were photographed at 48 h after changing the medium.

pearance of lysosomally-derived, autophagic vacuoles and by delayed or minimal nuclear collapse. Since small-scale DNA fragmentation was identified in R1 cells, v-Ha-Ras-dependent cell death in R1 cells may not be different from this type of cell death.

In conclusion, v-Ha-Ras-dependent apoptosis in human cells tested here was prevented by PDGF, which coordinated the phosphorylation of downstream molecules such as ERK and Akt. These results suggest that the failure of signal transduction of oncogenic Ras due to the deficiency of growth factors enhances v-Ha-Ras-dependent apoptosis. It is useful to know how the expression of oncogenic genes contributes to programmed cell death because it will assist in the development of effective therapies against human cancer using oncogenic genes.

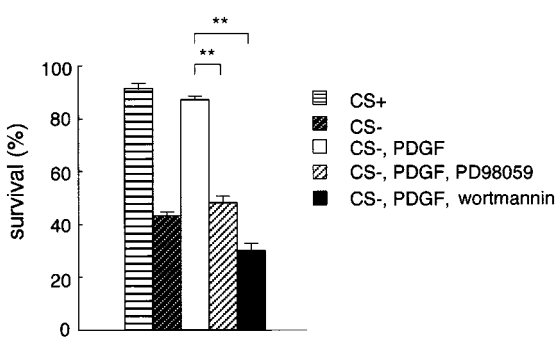

FIG. 6. The effect of inhibitors to susceptibility to cell death induced by serum deprivation in v-Ha-Ras-induced R1 cells. Viable cells were counted 7 days after changing the medium using the trypan blue dye exclusion method. ** represents $P < 0.005$.

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