Elevated expression of ERK 2 in human tumor cells chronically treated with PD98059

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\textit{Abbreviations:} ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; siRNA, small interfering RNA; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEM, Minimal essential medium; NEAA, non-essential amino acids; BrdU, bromodeoxyuridine.
Abstract

We examined the effect of chronic exposure of tumor cells to a mitogen-activated protein kinase/extracellular signal-regulated kinases (ERK) kinase inhibitor, PD98059, on cell proliferation was investigated. Human renal carcinoma cells (ACHN) and prostatic carcinoma cells (DU145) were cultured in the presence of PD98059 for more than 4 weeks (denoted ACHN (PD) cells and DU145 (PD) cells, respectively) and proliferation and signal transduction pathways were examined. PD98059 significantly inhibited the proliferation of parental cells. However, PD98059 failed to inhibit proliferation of ACHN (PD) and DU145 (PD) cells significantly. Expression of ERK 1 and 2 was elevated in these cells. These phenotypes were reversible. Downregulation of ERK 2, but not ERK 1, by small interfering RNA significantly inhibited the proliferation of ACHN (PD) and DU145 (PD) cells. Taken together, chronic exposure of tumor cells to PD98059 induced elevated expression of ERK 2, which was associated with decreased sensitivity of cellular proliferation to PD98059.

Key words: MEK inhibitor, chronic exposure, ERK 2, tumor cells, proliferation
Extracellular signal-regulated kinase (ERK) 1 and 2 are activated through the Ras-Raf-Mitogen-activated kinase (MAPK)/ERK kinase (MEK) pathway and play fundamental roles in a variety of pathophysiological conditions, such as embryonic development, inflammation, tumor growth, and angiogenesis [1, 2]. In particular, their important roles in regulating tumor cell proliferation and migration encouraged us to target these kinases as a potent therapeutic strategy for cancer patients [2-4].

MEK 1 and 2 are the only upstream molecules that are able to activate ERK 1 and 2. Thus, specific inhibition of MEK achieves specific and efficient inactivation of ERK[3, 4]. In human cancer cells, ERK activity is frequently elevated than in normal adjacent cells [5]. It has been shown that MEK inhibitors potently blocked the tumor progression in xenografted animal models [6, 7]. However, MEK inhibitors did not affect human tumors in clinical trials [8, 9].

To address the question of how human tumor cells evade the inhibitory action of MEK inhibition at a cellular level, we examined the effects of chronic exposure to PD98059 on human tumor cells. We show here for the first time that the chronic exposure of tumor cells to a MEK inhibitor upregulated the expression of ERK 2, which was involved in the decreased sensitivity of their proliferation to MEK inhibitor-treatment.
Materials and Methods

*Materials* Anti-MEK 1 polyclonal antibody (12-B), small interfering RNA (siRNA) for human ERK 1 and 2, and non-targeting control siRNA were purchased from Santa Cruz Biotechnologies, Santa Cruz, CA. Anti-phospho-mitogen-activated protein kinase (MAPK) polyclonal antibody was obtained from Cell Signaling Technology, Inc., Beverly, MA. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was purchased from Chemicon International, Temecula, CA, and anti-ERK 1/2 polyclonal antibody was from Upstate Cell Signaling Solutions, Lake Placid, NY. Minimal essential medium (MEM), Ham’s F-12 medium, non-essential amino acids (NEAA), and anti-vinculin monoclonal antibody (VIN-1) were purchased from Sigma Chemical Company, St. Louis, MO. Fetal bovine serum was obtained from Invitrogen Corporation, Carlsbad, CA. HiPerFect transfection reagent was purchased from Quiagen K.K., Tokyo, Japan. PD98059 was obtained from Wako Pure Chemicals, Osaka, Japan. It was dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C until use. After thawing, it was dissolved in culture medium and added to the cells. The final concentration of DMSO was 0.1% and the same amount of DMSO was added to the cells not treated with PD98059.

*Cell culture* A human renal carcinoma cell line, ACHN cells [10], was purchased from Dainippon Pharmaceuticals, Tokyo, Japan. A hormone-resistant human prostate cancer cell line, DU145 cells, was obtained from the American Type Culture Collection. ACHN
cells were cultured in MEM supplemented with NEAA and 10% FBS, and DU145 cells were cultured in Ham's F-12 medium supplemented with 10% FBS. For the chronic exposure of cells to PD98059, cells were cultured in the presence of 50 μM PD98059 for more than 4 weeks. To remove the effect of chronic exposure to PD98059, cells were further cultured without PD98059 for 2 weeks. The culture medium was changed to fresh medium every 3 days. As described below, these cells were examined biologically and biochemically in the presence or absence of PD98059 (the short-period treatment). To avoid confusion between chronic exposure and the short-period treatment with PD98059, unexposed parental cells were denoted ACHN (pa) or DU145 (pa) cells, cells chronically exposed to PD98059 (for more than 4 weeks) were denoted ACHN (PD) or DU145 (PD) cells, and cells exposed to PD98059 for 4 weeks and then cultured without PD98059 for 2 weeks were denoted ACHN (no PD) or DU145 (no PD) cells, respectively. If not specifically stated, ACHN (pa) cells, ACHN (no PD) cells, DU145 (pa) cells, and DU145 (no PD) cells were cultured without PD98059. ACHN (PD) cells and DU145 (PD) cells were cultured with PD98059 in all assays, except when otherwise specified.

Cell proliferation assay Cells were suspended in either MEM with NEAA or Ham's F-12 medium containing 10% FBS and seeded into 24-well plates at a density of $1 \times 10^4$ cells/well in the presence of 0.1% DMSO or 50 μM PD98059. After 3 days, the cells were detached with trypsin and counted with the use of a hemocytometer. Cell number without short-period PD98059-treatment was set to 100.
Labeling index Labeling index was examined to determine the numbers of cells in S-phase using the bromodeoxyuridine (BrdU) In-Situ detection kit® (BD Biosciences Pharmingen, San Diego, CA) as described before [11]. In brief, cells were seeded into wells of 48-well-culture plates. On the following day, medium was changed to fresh medium containing 10% FBS with either 0.1% DMSO or 50 μM PD98059, and culture was continued. After 16 h, cells were pulse-labeled for 4 h with BrdU. Cells were then fixed, treated with 4 M HCl, and uptaken BrdU was visualized with anti-BrdU antibody. At least 500 cells were counted for each well, and labeling indices were determined as labeled nuclei/total nuclei ratios and expressed as percentages.

Treatment of cells with siRNA Cells suspended in MEM with NEAA or Ham’s F-12 medium containing 10% FBS were seeded into 24-well plates (2 × 10⁴ cells/well) and cultured for 20 h. Culture medium was replaced with fresh medium containing 10% FBS. Serum-free medium supplemented with HiPerFect reagent and siRNA at the indicated concentrations were mixed, left for 20 min at room temperature, and then added to cells. The culture was continued for 2 days. Cells from one set of cultures were counted (three wells of each treatment) and total cell lysates, normalized by cell number, were examined for the expression of ERK proteins by immunoblotting as described below.

Immunoblot analyses Cells grown in 24-well plates were serum-starved overnight and then stimulated or left unstimulated with 10% FBS for the indicated periods. Two sets of culture were prepared, one set was used for immunoblotting and the other set was used for
cell counts. Cells were lysed by boiled SDS-sample buffer, and the proteins from $3 \times 10^4$ cells in each lysate were separated on SDS-polyacrylamide gels. Proteins were electrotransferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated with the indicated antibodies, followed by incubation with peroxidase-conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence reagents and exposed on X-ray films. X-ray films were scanned and the densities of particular bands were measured by NIH Image ver. 1.64. Fold activation of ERK 2 was estimated by the ratio of optimal density of phospho-ERK 2/ optimal density of ERK 2 in FBS-treated cells to that in untreated cells. ERK 2/vinculin or ERK 1/GAPDH ratios were calculated as the ratio of optimal density of ERK 2 or 1/optimal density of vinculin or GAPDH.

**Statistical analysis** Values are presented as mean cell numbers ± SD. Differences between two groups were examined by Mann-Whitney’s U test. Differences were considered significant when the P value was less than 0.05.
Results

Effects of chronic exposure to PD98059 on cell proliferation

We first examined whether PD98059 inhibits FBS-induced activation of ERK 1 and 2 in ACHN (pa) cells and DU145 (pa) cells. Proteins from $3 \times 10^4$ cells were examined. As shown in Fig. 1 A, PD98059 at 50 μM efficiently inhibited FBS-induced ERK activation. These cells were cultured in the presence of 50 μM PD98059 for 4 weeks to obtain ACHN (PD) cells and DU145 (PD) cells. ACHN (no PD) cells and DU145 (no PD) cells were derived from ACHN (PD) cells and DU145 (PD) cells, respectively, by culturing them in the absence of PD98059 for 2 weeks. The proliferation of these cells in the presence or absence of PD98059 (short-period treatment) was determined. As shown in Fig. 1 B, the proliferation of ACHN (pa) cells was significantly and dose-dependently inhibited by PD98059 treatment. Proliferation of ACHN (PD) cells was inhibited by PD98059 treatment, but the inhibition was not statistically significant. Proliferation of ACHN (no PD) cells was significantly and dose-dependently inhibited by PD98059 treatment. Similar results were obtained with DU145 cells (Fig. 1 C). Decrease in cell number is affected by the inhibition of proliferation and induction of apoptosis. We did not observe dead, floating cells during the experiments. However, to confirm that the effect of PD98059 was due to the inhibition of cell cycle, we examined the number of cells in S-phase by labeling index. As shown in Fig. 1 D, ACHN (PD) cells and DU145 (PD) cells in S-phase were not significantly decreased by PD98059, indicating that chronic exposure of cells to PD98059 decreased the sensitivity of cell cycle progression.
to PD98059. These results suggest that the sensitivity of the proliferation of ACHN (PD) cells and DU145 (PD) cells to PD98059 was decreased, and that the sensitivity was reversed when PD98059 was removed from the culture medium.

**Effects of chronic exposure to PD98059 on ERK activation**

We next examined the ERK activation by FBS in these cells. ACHN (pa) cells and ACHN (PD) cells were either stimulated or left unstimulated by 10% FBS for 10 min, and protein from $3 \times 10^4$ cells was analyzed. As shown in Fig. 2 A, ERK activation of ACHN (PD) cells by FBS was observed at a level similar to that of ACHN (pa) cells. However, ERK expression was elevated in ACHN (PD) cells, suggesting that the elevated expression of ERK resulted in an amount of activated ERK similar to that in ACHN (pa) cells. Upregulation and activation of ERK was also observed in DU145 (PD) cells. This upregulation required long term exposure to PD98059, because treatment of cells with PD98059 for up to 96 h did not increase the expression of ERK protein (data not shown). The expression and activation of ERK in ACHN (no PD) cells and DU145 (no PD) cells returned to the levels in ACHN (pa) cells and DU145 (pa) cells (Fig. 2 B), suggesting that elevated expression of ERK caused by chronic exposure to PD98059 was also reversible.

**Effects of siRNA for ERK 2 on Erk expression and proliferation**

To examine whether upregulation of ERK is responsible for the decrease in sensitivity of ACHN (PD) and DU145 (PD) proliferation to PD98059, we transfected these cells with ERK 2 siRNA. ERK 2 siRNA specifically and efficiently downregulated
the expression of ERK 2 in both ACHN (PD) cells and DU145 (PD) cells (Fig. 3 A). In ACHN (PD) cells treated with 5 nM siRNA and DU145 (PD) cells treated with 10 nM siRNA, expression of ERK 1 protein was elevated. The proliferation of ACHN (PD) cells treated with control siRNA was not significantly inhibited by PD98059-treatment (Fig. 3 B). On the other hand, the proliferation of ACHN (PD) cells treated with ERK 2 siRNA was significantly and dose-dependently inhibited in response to PD98059-treatment. Similar results were obtained with DU145 (PD) cells (Fig. 3 C). These results indicate that upregulation of ERK 2 was responsible for the decrease in sensitivity of their proliferation to PD98059. When ERK 1 was downregulated by siRNA in ACHN (PD) cells and DU145 (PD) cells (Fig. 4 A), neither the inhibition of their proliferation by PD98059-treatment nor upregulation of ERK 2 was observed (Fig. 4 and B).
Discussion

In the present study, we employed two kinds of human tumor cells, originated from renal cell carcinoma and hormone-resistant prostate carcinoma. These tumors are known to be resistant to conventional chemotherapy, radiation therapy, and hormonal therapy. Therefore, these tumors are good candidates for molecular targeting therapies as well as antiangiogenic therapies. We observed that chronic exposure of these tumor cells to PD98059 decreased the sensitivity of their proliferation to PD98059-treatment. This phenotype was associated with elevated ERK 2 expression, because downregulation of ERK 2 by siRNA restored their sensitivity of proliferation to PD98059 (Fig. 3). Chronic exposure to another MEK inhibitor, U0126 (at 10 μM), also exerted the similar effect on ACHN and DU145 cells (decrease in sensitivity of the proliferation and upregulation of ERK expression), suggesting that these phenotypes may be commonly observed in MEK inhibitor-treated tumor cells. PD98059 and U0126 also inhibit MEK 5, an upstream kinase of ERK 5, at a lower concentration than CI-040 [12]. Thus, decrease in sensitivity to PD98059 may also be due to the upregulation of ERK 5. However, we did not observe the upregulation of ERK 5 in ACHN (PD) cells and DU145 (PD) cells (data not shown). Recently, a CI-1040-resistant clone from colon 26 carcinoma cells has been established and exhibited 100-fold more resistant growth in soft agar to CI-1040 inhibition than the parental cells and 2.8-fold more resistant proliferation in monolayer to CI-1040 inhibition [13]. In this resistant cell line, expression of activated K-Ras was elevated and subsequent upregulation of activated ERK was observed. However, we did not observe the
upregulation of K-Ras in ACHN (PD) cell and DU145 (PD) cells (data not shown). Finally, downregulation of ERK 2, but not ERK 1, by siRNA restored the sensitivity of proliferation (Fig. 3 and 4), suggesting that upregulated ERK 2 was responsible for the decreased sensitivity to PD98059 in ACHN (PD) cell and DU145 (PD) cells and these phenotype was different from the CI-1040-resistant colon 26 cell line. Although treatment with CI-1040 downregulated the activated ERK in both xenograft animal models [6] and clinical trials [8], the ERK assays used in these studies were normalized by the amount of protein, not by the cell number. Thus, in vivo, upregulation of ERK expression by tumor cells may occur after several weeks of treatment. Indeed, the re-growth of xenografted MDA-MB-231 breast tumors treated with CI-1040 was observed on the 24th day of treatment [14]. Pancreatic BxPc3 xenografts started to grow after the completion of CI-1040-treatment [15]. Furthermore, another MEK inhibitor PD184161 failed to suppress MEK activity in hepatocellular carcinoma xenografts following long term (24 days) and to regress established xenograft tumors [16]. These in vivo studies are consistent with our data (Fig. 1 B and C). It is therefore possible that chronic exposure of tumor cells to MEK inhibitors may decrease the sensitivity of their proliferation in vivo as well.

The mechanism underlying upregulation of ERK in ACHN (PD) cells and DU145 (PD) cells is not known. However, downregulation of ERK 2 protein by siRNA significantly restored the sensitivity of proliferation of these cells to PD98059-treatment (Fig. 3, B and C). In these experiments, the expression of ERK 1 protein was elevated. However, elevated ERK 1 did not restore the sensitivity of proliferation. Conversely,
efficient downregulation of the expression of ERK 1 by siRNA neither affected the proliferation nor upregulation of ERK 2 (Fig. 4 A, B). In gene-targeted mouse models, ERK 2−/− mice died in utero, whereas ERK 1−/− mice were viable, fertile, and grew normally [17, 18]. The proliferation and sustained activation of ERK 2 displayed by embryonic fibroblasts from ERK 1−/− mice were similar to those of fibroblasts from wild type embryos, suggesting that ERK 2 compensates for ERK 1 deficiency. Taken together, the amount of ERK 2 expression may be critical for the proliferation of tumor cells in the presence of a MEK inhibitor.

Our data provide evidence that chronic exposure to a MEK inhibitor affects signal transduction pathways, leading to decrease in sensitivity of tumor cell proliferation to MEK inhibition. Treatment of ACHN (PD) cells and DU145 (PD) cells with 1 μg/ml of actinomycin D for 16 h significantly repressed ERK expression in association with decreased proliferation (data not shown). Thus, treatment of patients with a MEK inhibitor in combination with inhibitors of protein synthesis or translation could be an important option for future clinical studies.
Acknowledgements

We are grateful to Takumi Shimogama for his skilled outstanding help. This work was supported by Grants-in-Aid for Japan Society for the Promotion of Science.
References


Figure legends

Fig. 1. (A) Effect of transient treatment of parental ACHN cells and DU145 cells with PD98059 on FBS-mediated activation of ERK. Cells grown in 24-well plates were serum-starved overnight and PD98059 (50 µM) or DMSO (0.1%, vehicle) was added to the cells. Sixty minutes later, cells were stimulated with 10% FBS or left unstimulated for the indicated periods. Two sets of culture were prepared: one set was used for immunoblotting and the other was used for cell counts. Cells were lysed by boiled SDS-sample buffer and the proteins from $3 \times 10^4$ cells in each lysate were separated on SDS-PAGE gels. Proteins transferred to PVDF membranes were incubated with the indicated antibodies and visualized by chemiluminescence. Reproducible results were obtained in two independent experiments. (B) Effect of chronic exposure to PD98059 and removal of PD98059 on the proliferation of ACHN cells. Parental ACHN cells—denoted ACHN (pa) cells—were cultured in the presence of 50 µM PD98059 for more than 4 weeks. These cells were then denoted ACHN (PD) cells. ACHN (PD) cells cultured for 2 weeks in the absence of PD98059 were denoted ACHN (no PD) cells. Cells were suspended in MEM containing NEAA and 10% FBS, and seeded into 24-well plates at a density of $3 \times 10^4$ cells/well with either 0.1% DMSO (PD98059; 0 µM) or indicated concentration of PD98059. After 3 days, cell number was counted with a hemocytometer. Cell number without short-period PD98059-treatment was set to 100. Bars represent means ± SD for triplicate wells. Reproducible results were obtained in two independent experiments. (C) Effect of chronic exposure to PD98059 and removal of PD98059 on the
proliferation of DU145 cells. Parental DU145 cells—denoted DU145 (pa) cells—were cultured in the presence of 50 μM PD98059 for more than 4 weeks. These cells were denoted DU145 (PD) cells. DU145 (PD) cells cultured for 2 weeks in the absence of PD98059 were denoted DU145 (no PD) cells. Cells were suspended in Ham’s F-12 medium containing 10% FBS and proliferation of these cells in the presence or absence of PD98059 was examined as described above. Cell number without short-period PD98059-treatment was set to 100. Bars represent means ± SD for triplicate wells. Reproducible results were obtained in two independent experiments. (D) Labeling indices of ACHN and DU145 cells in the presence or absence of PD98059. Cells were seeded into wells of 48-well plates and cultured for 24 h in the presence (PD cells) or absence (pa and no PD cells). Medium was changed to fresh medium containing either 0.1% DMSO (PD98059; 0 μM) or 50 μM PD98059 and cultured for 16 h. Cells were pulse-labeled with BrdU for 4 h and labeling indices were determined as labeled nuclei/total nuclei ratios. Values are expressed as percentages ± SD for triplicate wells.

Fig. 2. (A) Effect of the chronic exposure of ACHN cells and DU145 cells to PD98059 on FBS-mediated activation of ERK. ACHN (pa) cells, ACHN (PD) cells, DU145 (pa) cells, and DU145 (PD) cells grown in 24-well plates were serum-starved overnight and stimulated with 10% FBS or left unstimulated for 10 min. Proteins from $3 \times 10^4$ cells were examined for ERK activation by immunoblotting as described in the legend of Fig. 1 A. Immunoblotting with anti-vinculin antibody was performed to assess loaded proteins. Reproducible results were obtained in two or three independent experiments. (B) Effect
of the removal of PD98059 from the culture medium of ACHN (PD) cells and DU145 (PD) cells on FBS-mediated activation of ERK. ACHN (pa) cells, ACHN (no PD) cells, DU145 (pa) cells, and DU145 (no PD) cells grown in 24-well plates were serum-starved overnight and were stimulated with 10% FBS or left unstimulated for 10 min. Proteins from $3 \times 10^4$ cells were examined for ERK activation by immunoblotting. Immunoblotting with anti-vinculin antibody was performed to assess loaded proteins. Reproducible results were obtained in two independent experiments.

**Fig. 3. (A)** Effect of ERK 2 siRNA on ERK expression of ACHN (PD) cells and DU145 (PD) cells. Cells grown in 24-well plates were treated with the indicated reagents. Two days later, cells were lysed and proteins from $3 \times 10^4$ cells were examined for ERK expression by immunoblotting. Immunoblotting with anti-vinculin antibody was performed to assess loaded proteins. Protein bands were scanned and the densities of particular bands were measured by NIH Image version 1.62. The ERK 2/vinculin ratio was calculated as described in “Materials and Methods”. **(B)** Effect of ERK 2 siRNA on the proliferation of ACHN (PD) cells. Cells grown in 24-well plates were treated with the indicated reagents. Two days later, the cell number was counted. ACHN (pa) cells were examined as control. Bars represent means ± SD for triplicate wells. Reproducible results were obtained in two independent experiments. **(C)** Effect of ERK 2 siRNA on the proliferation of DU145 (PD) cells. Cells grown in 24-well plates were treated with the indicated reagents, and 2 days later, the cell number was counted. DU145 (pa) cells were examined as control. Bars represent means ± SD for triplicate wells. Reproducible results
were obtained in two independent experiments.

**Fig. 4. (A)** Effect of ERK 1 siRNA on ERK expression in ACHN (PD) cells and DU145 (PD) cells. Cells grown in 24-well plates were treated with the indicated reagents. Two days later, proteins from $3 \times 10^4$ cells were examined for ERK expression by immunoblotting. Immunoblotting with anti-GAPDH antibody was performed to assess loaded proteins. The ERK 1/GAPDH ratio was calculated as described above. **(B)** Effect of ERK 1 siRNA on the proliferation of ACHN (PD) cells and DU145 (PD) cells. Cells grown in 24-well plates were treated with the indicated reagents, and 2 days later, the cell number was counted. Bars represent mean cell numbers/well ± SD for triplicate wells. Reproducible results were obtained in two independent experiments.
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IB : anti-phospho-MAPK

IB : anti-ERK 1/2

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C

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ACHN | DU145

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