Differential Responses of Human Papillary Thyroid Cancer Cell Lines Carrying the RET/PTC1 Rearrangement or a BRAF Mutation to MEK1/2 Inhibitors

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Objectives: To examine the effects of 2 mitogen-activated protein kinase kinase (MEK1/2) inhibitors on papillary thyroid carcinoma (PTC) cell lines carrying the RET/PTC1 rearrangement or a BRAF mutation. In PTC, RET/PTC1 rearrangement or BRAF mutations results in constitutional activation of RET kinase or BRAF, respectively. Along the RET or BRAF signaling cascades, the activated RET kinase or BRAF activates MEK1/2, and then mitogen-activated protein kinases (extracellular signal-related kinase 1/2 [ERK1/2]) is activated. Activated ERK1/2 enters the nucleus and phosphorylates a variety of transcription factors, resulting in cancer cell proliferation. The MEK1/2 inhibitors, PD98059 and U0126, have been shown to inhibit cell growth in other cancers.

Design: In vitro study.

Subjects: Papillary thyroid carcinoma cell lines carrying the RET/PTC1 rearrangement (BHP2-7) or a BRAF mutation (BHP5-16).

Intervention: We treated PTC cells carrying the RET/PTC1 rearrangement or a BRAF mutation with 2 MEK1/2 inhibitors (PD98059 and U0126).

Main Outcome Measures: Using Western blot analysis, we detected the expression of phosphorylated ERK1/2 and expression of cleaved poly(ADP-ribose) polymerase (PARP) in cells after treatment with either inhibitors. Growth inhibition was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results: Using Western blot analysis, we detected the dephosphorylation of ERK1/2 in PTC cells carrying the RET/PTC1 rearrangement or a BRAF mutation after treating the cells with 2 MEK1/2 inhibitors (PD98059 and U0126). In addition, both PD98059 and U0126 completely inhibited the growth of the PTC cells carrying a BRAF mutation but partially inhibited the growth of the PTC cells carrying the RET/PTC1 rearrangement. Finally, we observed PARP cleavage only in cells with a BRAF mutation in the Western blot analysis.

Conclusion: These data suggested that treatment with MEK1/2 inhibitors can be used as tools for inhibiting the growth of PTC cells.


HYPOCRISY CANCER HAS SEVERAL HISTOLOGIC SUBTYPES, INCLUDING PAPILLARY THYROID CARCINOMA (PTC), FOLLICULAR THYROID CARCINOMA, MEDULLARY THYROID CARCINOMA, AND ANAPLASTIC THYROID CARCINOMA. Of these subtypes, PTC is by far the most common, accounting for at least 80% of thyroid carcinomas. Two major types of mutations are detected in PTC cells, RET/PTC rearrangements and BRAF mutations. RET/PTC rearrangements are unique to thyroid cancer, with RET/PTC1 and RET/PTC3 being the most common types of this rearrangement. These rearrangements result from the generation of chimeric oncogenes with the 3' end of the kinase domain from the RET kinase fused with the H4 gene (RET/PTC1), regulatory subunit Rα of cyclic adenosine monophosphate–dependent protein kinase A (RET/PTC2), or the RFG gene (or ELE1 gene) (RET/PTC3). These chimeric oncogenes retain the kinase activity of RET and result in ubiquitous expression (normally, the thyroid cells do not express the RET receptor) and constitutive activation of RET.

BRAF, a component of the RET/RAF/mitogen-activated protein kinase kinase (MEK1/2)/mitogen-activated protein kinase or extracellular signal-regulated kinase (ERK) signal transduction pathway, belongs to the RAF family of serine-threonine kinases that also includes ARAF and c-raf. The most common BRAF mutation in cancer is a T→A substitution at nucleotide 1799 that results in conversion of a valine to glutamic acid at codon 600 (V600E) of the BRAF protein.
on exon 15.14 The negative charge introduced by glutamic acid at position 600 mimics the effect of phosphorylation at an adjacent site when BRAF is activated and results in constitutive activation of BRAF.14 Others have detected the V600E mutation of BRAF in various human cancers, including melanoma (up to 66%),15,16 ovarian carcinoma (up to 40%),15,17 and colorectal carcinoma (up to 20%).15,18 In PTC, the incidence of RET/PTC rearrangements ranges from 2.5% to 67.0%,8,19 and the incidence of BRAF mutations ranges from 29% to 83% depending on the cohort being studied.20 No reports of rearrangements ranges from 2.5% to 67.0%,8,19 and the incidence of BRAF mutations ranges from 29% to 83% depending on the cohort being studied.20 No reports of

The PTC cell line carrying the RET/PTC1 rearrangement (BHP2-7) or a BRAF mutation (BHP5-16) was kindly provided by Jerome Hershman, MD (VA Greater Los Angeles Healthcare System, Los Angeles, California).33,34 The cells were maintained in RPMI1640 medium containing 10% fetal bovine serum, nonessential amino acid mixture, 1mM sodium pyruvate, and 2mM L-glutamine in a 37°C incubator supplied with 95% air and 5% carbon dioxide.

CELL CULTURE

PD98059 and U0126 were purchased from VWR (West Chester, Pennsylvania). PD98059 was dissolved in dimethyl sulfoxide (DMSO) as a 50mM stock solution and stored in the dark at −20°C. Fresh U0126 was made in DMSO as a 10mM solution before each use.

CHEMICALS

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POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

Protein extracts from PTC cells were prepared in lysis buffer containing 20mM Tris–hydrochloric acid, pH 7.4, 1% octyl phe-noxy polyethoxyethanol (Triton X-100; Sigma-Aldrich, St Louis, Missouri), 300mM sodium chloride, 1mM phenylmethylsulfonyl fluoride, 50mM sodium fluoride, 1mM sodium vanadate, and proteinase inhibitor cocktail III (Calbiochem, San Diego, California). Total protein concentrations were estimated using the Bradford assay (Bio-Rad, Hercules, California) with bovine serum ovalbumin as a standard. For Western blot analysis, proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels using a miniprotein II electrophoresis system (Bio-Rad). The proteins were then transferred to Hybond-ECL membranes (Amersham Bioscience, Piscataway, New Jersey) using a mini-transblot electrophoretic transfer cell (Bio-Rad) at 80 V for 1 hour at room temperature. After transfer, the membranes were blocked and probed with antibodies at 4°C overnight as indicated by the manufacturer. Phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2, and PARP antibodies (Cell Signaling Technology, Danvers, Massachusetts) were used at a dilution of 1:1000 and a monoclonal antibody against actin (Sigma-Aldrich) was used at a dilution of 1:2000.

CELL PROLIFERATION ASSAY

To determine the optimal concentration of MEK1/2 inhibitors (PD98059 and U0126) for the dephosphorylation of PTC cells, we treated cells with 50µM to 150µM PD98059 or 1µM to 50µM U0126 for 24 hours. Protein extracts were prepared after these treatments and p-ERK1/2 was examined using Western blot analysis (Figure 1A). Treatment with 50µM PD98059 or 10µM U0126 significantly decreased the expression of p-ERK1/2 whereas it did not affect the expression of total ERK1/2 (Figure 1A). We selected 75µM PD98059 and 10µM U0126 in the ensuing studies to observe the maximum effects of these inhibitors.

MEKI/2 INHIBITORS DECREASE THE PHOSPHORYLATION OF ERK1/2

We performed a time course study to determine the duration of ERK1/2 inactivity in the cells. The PTC cells were treated with 75µM PD98059 or 10µM U0126 for 1 hour to 72 hours, and the p-ERK1/2 proteins were detected using Western blot analysis (Figure 1B). The amount of p-ERK1/2 decreased after treatment with PD98059 for 1 hour compared with that in untreated cells, and the expression of p-ERK1/2 returned to normal at 6 hours after treatment. Also, the expression of p-ERK1/2 decreased dramatically after only 1 hour of treatment with U0126 and remained low for up to 48 hours; expression
Expression of phosphorylated extracellular signal-related kinase 1/2 (p-ERK1/2) in papillary thyroid carcinoma (PTC) cells after treatment with PD98059 or U0126 for 1 hour. Cells treated with DMSO only (−) were used as positive controls. The concentrations used in the experiments conducted hereafter (B and C) are indicated by asterisks. B, Cells with a BRAF mutation (BHP5-16) were treated with 50µM, 75µM, 100µM, or 150µM PD98059 or 1µM, 5µM, 10µM, or 50µM U0126 for 24 hours. The expression of p-ERK1/2 was detected using Western blot analysis. Total ERK1/2 was used as a loading control and cells treated with dimethyl sulfoxide (DMSO) only were used as positive controls. The concentrations used in the experiments conducted hereafter (B and C) are indicated by asterisks. C, Cells with an RET/PTC1 rearrangement (BHP2-7) or a BRAF mutation (BHP5-16) were treated with PD98059 or U0126 for 24 hours first and then incubated in regular medium without the inhibitors for 24 hours (24 hours + 24 hours). Cells treated with DMSO only were used as positive controls. Cells treated with DMSO only (−) were used as positive controls. Cells treated with DMSO only (−) were used as positive controls.

INHIBITION OF CELL GROWTH BY PD98059 AND U0126

We performed MTT assays on PTC cells with the RET/PTC1 rearrangement or with a BRAF mutation using 75µM PD98059 or 10µM U0126. After treatment for 4 days, we observed dramatic inhibition of growth of cells with a BRAF mutation (88% reduction in cell growth, P < .001) compared with that of the control cells on day 4. We detected a more modest inhibition of growth of cells with the RET/PTC1 rearrangement (61% and 68% for PD98059 and U0126, respectively; P < .001).

INHIBITION OF CELL GROWTH BY PD98059 OR U0126 IS CAUSED BY PARP CLEAVAGE IN PTC CELLS WITH A BRAF MUTATION

To determine the mechanism of cell-growth inhibition by PD98059 and U0126, we evaluated the expression of p-ERK1/2.
the caspase 3 substrate PARP as an indicator of apoptosis using Western blot analysis. After treatment with PD98059, cells with a BRAF mutation showed cleaved PARP as early as 1 day after treatment, and the expression remained detectable for up to 4 days (Figure 3A). The cleaved PARP was undetectable for up to 4 days in cells with the RET/PTC1 rearrangement. We observed similar results in cells treated with U0126 (Figure 3B): cells with a BRAF mutation showed cleaved PARP as early as 1 day after treatment, and the expression remained detectable for up to 4 days (data not shown). No cleaved PARP was detectable for up to 4 days in cells with the RET/PTC1 rearrangement treated with U0126.

**COMMENT**

In the present study, we evaluated the commercially available MEK1/2 inhibitors PD98059 and U0126 for their effects on PTC cells with the RET/PTC1 rearrangement or with a BRAF mutation. Both inhibitors were able to decrease the phosphorylation of ERK1/2. Also, they both inhibited the growth of PTC cells, although to a different extent in cells with the RET/PTC1 rearrangement and cells with a BRAF mutation. Cleaved PARP appeared to be the mechanism of growth inhibition in cells with a BRAF mutation only after treatment with PD98059 or U0126. The mechanism of growth inhibition in cells with the RET/PTC1 rearrangement remains to be determined.

Different types of cancer cells exhibit different levels of sensitivity to PD98059 and U0126 in terms of the concentration necessary to dephosphorylate ERK1/2. For example, in studies of breast carcinoma cell lines treated with PD98059 at 10µM and with U0126 at 2µM, the dephosphorylation of ERK1/2 was observed using Western blot analysis. Investigators also treated melanoma cells with 40µM PD98059 and 5µM U0126 and colon cancer cells with 50µM PD98059 and 20µM U0126, the ERK1/2 was dephosphorylated using Western blot analysis. Namba et al. treated PTC cells with 5µM U0126 for 24 hours and found that it inhibited cell growth by 40% in NPA cells (PTC cell line with a BRAF mutation) but not in TPC-1 (a PTC cell line with the RET/PTC1 rearrangement). In addition, Specht et al. treated PTC cells with 1-25µM of U0126 for 3 days and found that inhibition of the growth of NPA cells was statistically significant at 10µM. Our data confirmed the results obtained by Namba et al. and Specht et al. When we treated PTC cells with 1µM to 5µM U0126 for 24 hours, the p-ERK1/2 was still detectable using Western blot analysis but was undetectable with 10µM U0126. When we treated PTC cells with 10µM U0126 for up to 4 days, we observed greater than 90% inhibition of growth of PTC cells with a BRAF mutation. The increased growth inhibition in our study compared with that in the study by Namba et al. was probably a result of our use of a higher concentration of U0126 (10µM vs 5µM). For PD98059, Ouyang et al. treated rat thyroid cells carrying a BRAF mutation with 75µM PD98059 and found dephosphorylation of ERK1/2 using Western blot analysis. No information on the concentration of PD98059 used to treat human PTC cells was available in the literature. In most of our experiments, we treated cells with 75µM PD98059, which is the highest concentration that PTC cells can tolerate before crystallization and cytotoxic effects occur. Although treatment with 50µM PD98059 resulted in a decrease in the expression of p-ERK1/2, our time-course study showed that even with treatment at 75µM, the dephosphorylation of ERK1/2 was only transitory (up to 6 hours for p-ERK2 and 24 hours for p-ERK1).

Inactivation of the ERK1/2 by PD98059 and U0126 appeared to be a rapid and reversible process as shown in Western blot analysis. After only 1 hour of treatment with either inhibitors, expression of p-ERK1/2 decreased dramatically in cells with the RET/PTC1 rearrangement and cells with a BRAF mutation. The duration of suppressed p-ERK1/2 expression appeared to be longer with treatment with U0126 than with treatment with PD98059. Nevertheless, both PD98059 and U0126 totally inhibited the growth of cells with a BRAF mutation but only partially inhibited the growth of cells with the RET/PTC1 rearrangement.

The apoptosis pathway is mediated by a cascade of caspases. First, pro-caspase 9 is activated and results in the cleavage of pro-caspase 9. Then the cleaved caspase 9 activates caspase 3. The substrate of caspase 3 is PARP.
and activation of PARP results in the cleavage of PARP. Cleaved PARP is known to be involved in DNA cleavage and apoptosis execution. Others have reported induction of apoptosis by PD98059 and U0126 using PARP cleavage as an indicator in breast cancer cells,51 melanoma cells,29 colon cancer cells,39 and luteinized granulose cells.40 In the present study, we also detected PARP cleavage in cells with a BRAF mutation as early as 1 day after exposure to either PD98059 or U0126. The PARP cleavage in cells with a BRAF mutation was detected up to 4 days after treatment with PD98059 or U0126 (data not shown). We did not detect PARP cleavage in cells with the RET/PTC1 rearrangement after treatment with either inhibitor. These results suggest that cells with a BRAF mutation may be more sensitive to some MEK1/2 inhibitors than cells with the RET/PTC1 rearrangement in terms of induction of apoptosis. Furthermore, cells with the RET/PTC1 rearrangement may use pathways other than those used by cells with a BRAF mutation to obtain cellular growth inhibition by these same inhibitors. Despite the inhibitory effects of these inhibitors to PTC cells, both PD98059 and U0126 were used for in vitro study only due to the poor solubility of PD98059 and inactivity of U0126 in vivo.52 Several new inhibitors have been developed and approved for human use.41,42 BAY 43-9006, developed by Bayer Pharmaceuticals Corp, West Haven, Connecticut, is a multifunction kinase inhibitor inhibiting both BRAF and RET kinase activity.43 CI-1040 (PD0184352), developed by Pfizer Inc, New York, New York, is a specific inhibitor of MEK1/2 and has been shown to inhibit tumor growth in other type of tumors as well.44 These new and less toxic therapies are clearly needed for PTC, a disease that is increasing in incidence and prevalence of MEK activity. Future studies should examine these new inhibitors that specifically inhibit the BRAF/MEK/ERK pathway in patients with PTC.

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