Inhibition of PP2A Activity Confers a TRAIL-Sensitive Phenotype during Malignant Transformation

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Abstract

TRAIL is a promising anticancer agent because it induces apoptosis in the majority of human cancer cells but spares the normal cells. To determine the mechanistic nature of how normal cells acquire a TRAIL-sensitive phenotype during the process of malignant transformation, an experimental cell system was developed by sequential introduction of human telomerase reverse transcriptase and SV40 T antigens (large and small) into normal human prostatic epithelial cells (PrEC). This model system demonstrated that inhibition of protein phosphatase 2A (PP2A), either by SV40 small T antigen, okadaic acid, Calyculin A, or PP2A catalytic subunit siRNA, sensitized normal human PrEC and immortalized cells to TRAIL-induced apoptosis. Moreover, sensitization occurred during the premalignant period of tumorigenesis and PP2A exerted its antiapoptotic activity by negatively regulating c-Fos/AP-1. In addition, low-dose okadaic acid treatment sensitized TRAIL-resistant cancer cells to TRAIL, suggesting that PP2A inhibitors could be used as an enhancer of apoptosis induced by TRAIL or TRAIL-like agents. These data indicate that downregulation of PP2A activity is a critical step for normal cells to acquire a TRAIL-sensitive phenotype during tumorogenesis and that the level of PP2A activity may foretell cellular sensitivity to TRAIL-induced apoptosis.

Implications: Inhibition of PP2A is a key determinant in acquiring TRAIL sensitivity during tumorigenesis, with c-Fos/AP-1 as an essential mediator. Mol Cancer Res; 12(2): 1-11. ©2013 AACR.

Introduction

TRAIL/Apo2L, a member of TNF family, possesses strong antitumor activity. Recently, TRAIL-like ligands have shown promising results in phase I and II clinical studies with a very limited cytotoxic profile when used systemically in a variety of cancers (1, 2). TRAIL interacts with specific death domain receptors, DR4 and DR5 (3, 4), and induces intracellular cytoplastic formation of the death-inducing signaling complex (DISC; refs. 5, 6). The formation of DISC triggers proteolytic autoprocessing and activation of caspase-8/10, which in turn can further activate the effector caspases 3, 6, and 7 and subsequent programmed cell death (extrinsic cell death pathway). Activated caspase-8 may also activate the mitochondria-mediated proapoptotic pathways via cleavage of Bid (intrinsic cell death pathway; ref. 7). However, not all cancer cells are sensitive to TRAIL-induced apoptosis. Accumulating results suggest that the processing and signal transductions associated with TRAIL may involve both extrinsic and intrinsic pathways fully depending on the activation of caspases (5, 7), and cellular sensitivity for TRAIL-induced apoptosis in cancer cells can be associated with many important apoptosis-related genes including NF-kB, Akt, Bcl-2, Bax, XIAP, IAPs, Smac/DIABLO, c-Myc (8), and c-FLIP (FLICE-like inhibitory protein; ref. 9). We previously demonstrated that the expression of an antiapoptotic molecule, long form of c-FLIP (c-FLIP(L)), is necessary and sufficient for cancer cells to maintain resistance to TRAIL-induced apoptosis (10). Furthermore, we have reported that one AP-1 family member protein, c-Fos, functions as a proapoptotic molecule by antagonizing c-FLIP(L) (11). More recently, we demonstrated that the c-Fos/AP-1 inducer, 12-O-Tetradecanoylphorbol-13-acetate (TPA), can enhance TRAIL-mediated apoptosis in prostate cancer cells (12).

TRAIL has been reported to specifically kill malignant cells but to be relatively nontoxic to normal cells (4, 13, 14). This brings up an interesting question on how cells acquire TRAIL-sensitive phenotype during tumorogenesis. Cancer is the result of a complex multistep process that involves the accumulation of sequential alterations of certain types of...
genes that play pivotal roles in the cells, which eventually leads to the formation of cancer cells that acquire the following hallmarks: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, reprogramming of energy metabolism, evading immune destruction, and activating invasion and metastasis (15, 16). It has been shown that the combined introduction of the human telomere reverse transcriptase (hTERT), the large T antigen of simian virus 40 (SV40 LT), the small T antigen of simian virus 40 (SV40 ST), and an oncogenic allele of H-RAS is sufficient to convert a wide variety of primary human cells to tumor cells (15). Recent work implicates that the status of telomere is a key regulator of the lifespan of cells and the introduction of hTERT into cells constitutively activates telomerase expression, stabilizes telomere length, and facilitates the immortalization of the cells (17, 18). The role of SV40 LT in transforming human cells seems to lie solely on the inactivation of both the retinoblastoma (pRB) and p53 tumor suppressor genes by directly binding to pRB and p53 (19–21). On the other hand, SV40 ST functions to disrupt the protein phosphatase 2A (PP2A) activity by binding to the PP2A subunits (22, 23). The introduction of hTERT, SV40 LT, and SV40 ST into human primary cells can only immortalize cells. However, cells exert tumorigenic phenotypes after additional introduction of oncogene H-Ras or c-Myc (15). The earlier cell model provides an experimental cell system with definitive genetic background that mimics stepwise progression of human cells from normal to immortalized and then to tumor cells.

PP2A is a major protein serine/threonine phosphatase and is involved in many essential aspects of cellular physiology (24). The loss or inactivation of PP2A is one of the most common events during tumorigenesis. Structurally, PP2A is a large collection of oligomeric enzymes that contain a common catalytic subunit. The most common forms of PP2A contain an active core dimer composed of a catalytic C subunit, also called PP2AC or PPP2C, and a structural A subunit, also called PP1A or PP1C. A third subunit B binds the A-C heterodimer (22, 25, 26). Recent evidence indicates that PP2A is a reluctant tumor suppressor, and suppression of PP2A activity is necessary to push immortalized cells to complete transformation (22, 23, 27). SV40 ST inhibits PP2A activity by interacting with A-C heterodimer, and SV40 ST mutants that cannot bind PP2A also fail to transform some human cells expressing hTERT, SV40 LT, and H-Ras, suggesting that this interaction is required for SV40 ST–mediated transformation (28–30). Similar to SV40 ST, okadaic acid was described as a selective but not a specific inhibitor of PP2A and acted as a tumor promoter during tumorigenesis (31, 32). An additional potent phosphatase inhibitor, Calyculin A, inhibits both phosphatases PP1 and PP2A (33).

To determine how normal human cells acquire TRAIL-sensitive phenotype during the process of malignant transformation, we compared normal human prostatic epithelial cells (PrEC) and derivative cells that were sequentially introduced with hTERT, SV40 LT, and SV40 ST (34). Interestingly, we found that while normal human PrECs and immortalized derivatives that coexpress hTERT and SV40 LT (LH cells) were resistant to TRAIL-induced apoptosis, further introduction of SV40 ST rendered the cells (LHS cells) to be very sensitive to TRAIL-induced apoptosis. We demonstrate that the inhibition of PP2A underlines SV40 ST–mediated acquiring of TRAIL–sensitive phenotype. Furthermore, the activation of c-Fos/AP-1, which is required for normal, immortalized, and tumor cells to be sensitive to TRAIL-induced apoptosis, could be a key negative downstream effector of PP2A. Finally, we show that PP2A activity correlates with the sensitivity to TRAIL-induced apoptosis in multiple prostatic and renal cancer cell lines and that low-dose of okadaic acid treatment can sensitize TRAIL-resistant cancer cells to TRAIL, suggesting that PP2A inhibitors such as okadaic acid can be potentially used as an enhancer of TRAIL or TRAIL-like agents to induce apoptosis in cancer cells. Taken together, these findings suggest that inhibition of PP2A activity is a key step for cells to acquire TRAIL-sensitive phenotype during the process of tumorigenesis, and the level of PP2A activity could be a major determinant for the sensitivity of cancer cells to TRAIL-induced apoptosis.

Materials and Methods
Materials
Recombinant human TRAIL/TNFSF10 was obtained from R&D Systems, Inc. Antibodies to c-Fos, PP2A C, Bcl-2, XIAP, Integrin β1, and PP2A/PP1 inhibitor Calyculin A were obtained from Cell Signaling Technology, Inc. Antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Abcam, Inc. Horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse, goat anti-rabbit) were obtained from Pierce Biotechnology. Okadaic acid was purchased from Sigma. DR4 antibody, PP2A C siRNA and its control siRNA were from Santa Cruz Biotechnology, Inc. Lipofectamine 2000 transfection reagent was obtained from Invitrogen Life Technologies. Antibodies to c-FLIP and DR5 were from Apotech Corp.

Cells and culture
Normal human PrEC and its medium PrECM were purchased from Cambrex Bio Science Walkersville, Inc. Transformed prostatic cell lines LH and LHS were kindly supplied by Dr. William C. Hahn (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). PrEC, LH, and LHS cells were cultured in PrECM according to instructions. PC3, 786-O, A-498, MDA-MB231, and MDA-MB453 were purchased from America Type Culture Collection. PC3-TR was generated by us as described previously (10). All plasticware was from Corning Incorporated. Cells were treated with TRAIL (50 ng/mL) for 24 hours upon 70% to 80% confluence, followed by harvesting and testing. In okadaic acid or Calyculin A experiments, cells were pretreated with okadaic acid or Calyculin A for 1 hour and then cotreated with TRAIL for another 24 hours. In siRNA or A-Fos experiments, siRNA or A-Fos was transfected into cells upon 70% confluence for 24 hours before okadaic acid and/or TRAIL (50 ng/mL) treatment.
Cell viability

Cell viability was determined by MTT method according to the manufacturer’s instructions (Roche Diagnostics). In brief, $5 \times 10^4$ cells were seeded in 96-well plates and cultured for 24 hours before treatment. Cells were then treated with various doses of TRAIL for 24 hours. MTT was added, followed by adding solubilization buffer. Four hours later, absorbance was measured at 590 nm (630 nm as the reference wavelength) using a microtiter plate reader. Viability of untreated cells was set at 100%, and absorbance of wells with medium and without cells was set as zero. All of the results were from at least triplicate experiments.

Apoptosis assay

Apoptosis was detected by using fluorescein isothiocyanate (FITC)–conjugated annexin V (annexin V–FITC) kit according to manufacturer’s protocols (Nanjing KeyGen Biotechnology). Analysis of the apoptotic cells was performed on a FACScan flow cytomter (BD Biosciences; using 488 nm for excitation and 530 nm for detection). All of the results were from at least three independent experiments.

Western blot analysis

Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycholate, 1.0% NP40, and 0.1% SDS] supplemented with a protease inhibitor mixture stock solution (Roche Molecular Biochemicals). After sonication for 15 seconds, cell debris was discarded by centrifugation at 12,000 × g for 15 minutes at 4°C, and the protein concentration was determined by bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology). Plasma membrane protein was prepared following instructions of Plasma Membrane Protein Extraction Kit (Abcam). Equivalent amounts of proteins, as verified by immunoblot analysis against anti-GAPDH, were resolved by 10% to 12% SDS-PAGE and transferred to nitrocellulose membranes by electroblot analysis. Nitrocellulose blots were blocked with 5% (w/v) nonfat milk in TBS/Tween buffer and incubated with the appropriate antibody. Immunostained proteins were visualized on X-ray film after applying the ECL reagents (Pierce Biotechnology).

PP2A activity assay

The PP2A activity of cells was determined by phosphatase assays. The assays were carried out by using a GENMED cellular PP2A activity quantitative kit according to the manufacturer’s protocols. Absorbance was measured at 660 nm using a spectrophotometer. Activity of untreated cells was set at 100%, and PP2A activity of different cells was compared with the untreated control. All the results were from at least three independent experiments.

Transfection of siRNA, A-Fos, or c-FLIP(L)

PP2A C siRNA and its control siRNA were transfected into cells by TransMessenger Kit (Qiagen). A-Fos and its vector CMV 500 was obtained from Dr. Charles Vinson (National Cancer Institute, Bethesda, MD; ref. 35). Full-length human c-FLIP(L) cDNA was constructed into pcDNA3/Zeo(+) vector in our laboratory (10). The plasmids were transfected with Lipofectamine 2000 (Invitrogen Life Technologies) following the manufacturer’s protocols.

Luciferase assay

When the cells seeded in 24-well plates reached 70% to 80% confluence, both AP-1 luciferase reporter (25 ng/well) and Renilla reporter (5 ng/well) from Stratagene were cotransfected into cells. After 24 hours, cells were treated with TRAIL for another 24 hours, or pretreated with okadaic acid or Calyculin A for 1 hour and then treated with TRAIL for another 24 hours, as indicated. In A-Fos experiments, A-Fos, luciferase, and Renilla were cotransfected into cells for 24 hours before okadaic acid and TRAIL treatment. Renilla served as an internal control for normalizing the transfection efficiency, using the Dual-Luciferase Reporter Assay System (Promega). All results represent at least three independent experiments.

Statistical analysis

Data represent the means ± SD and were analyzed with the Student t test by using GraphPad Prism 4.0 software. Statistical significance was considered when $P < 0.05$.

Results

The introduction of SV40 ST into immortalized human PrEC sensitizes cells to recombinant human TRAIL

To track the alterations in TRAIL sensitivity during tumorigenic progression (cellular transformation, immortalization, and carcinogenesis), normal human PrEC, LH (PrEC immortalized by the combination of hTERT and SV40 LT), and LHS (LH cells with additional introduction of SV40 ST) were treated with increasing doses of TRAIL (Fig. 1A). Cell viability assays showed that PrEC and LH cells had reluctant response to TRAIL, whereas LHS cell growth was completely abolished by TRAIL at doses more than 10 ng/mL. Consistent with the results of cell viability, apoptosis (as marked by FITC-conjugated annexin V) assay demonstrated that LHS cells underwent substantial apoptosis with 50 ng/mL of TRAIL treatment, whereas PrEC and LH cells yielded slight responses (Fig. 1B). Consistently, observations made with phase microscopy showed that TRAIL (50 ng/mL) treatment caused minimal damage in PrEC and LH cells viability, whereas the majority of LHS cells underwent cell shrinkage and pyknosis (data not shown). These results suggest that the introduction of SV40 ST is a key step for PrEC to acquire TRAIL-sensitive phenotype during tumorigenesis.

The introduction of SV40 ST decreases the activity of PP2A but has no effects on protein level of PP2A C

Because tumor suppressor PP2A is the only cellular protein known to specifically associate with SV40 ST during infection and transformation (36) and the only genetic
difference between LH and LHS is that LHS has additional ectopic SV40 ST expression, we reasoned that SV40 ST may have conversed the sensitivity of TRAIL in LH cells via its PP2A-inhibiting function. Indeed, we found that the activity of PP2A decreased significantly in LHS cells compared with those in TRAIL-resistant PrEC and LH cells in the absence or presence of TRAIL (Fig. 2A). However, as assessed by immunoblot, we did not observe distinguishable changes at PP2A C (catalytic subunit) protein levels between PrEC, LH, and LHS cells, without or with TRAIL treatment (Fig. 2B). In any case, these results indicate that the introduction of SV40 ST into LHS cells led to compromised PP2A activity, which correlates with a TRAIL-sensitive phenotype.

**Attenuation of PP2A activity by low dose of okadaic acid or Calyculin A sensitizes PrEC and LH cells to TRAIL.**

To further define the correlation between low activity of PP2A and TRAIL sensitivity, we inhibited PP2A activity in PrEC and LH cells by using inhibitors of PP2A, okadaic acid or Calyculin A. Because higher concentrations of okadaic acid or Calyculin A could cause cell death in human cells (37–39), we first determined a suitable dose of okadaic acid or Calyculin A that does not directly cause cell death. We found that 20 nmol/L of okadaic acid or 100 nmol/L of Calyculin A did not induce obvious cell death in our cell models (data not shown). Next, we set to assess the effects of PP2A inhibition on TRAIL sensitivity in resistant cell lines. For this purpose, PrEC and LH cells were pretreated with okadaic acid or Calyculin A for 1 hour, and then treated with TRAIL (50 ng/mL) for 24 hours. As shown in Fig. 3A, combination of TRAIL with okadaic acid or Calyculin A led to marked apoptotic responses in PrEC and LH cells, in contrast with their controls. As expected, okadaic acid or Calyculin A dramatically decreased PP2A activity in both PrEC and LH cells (Fig. 3B). These results are consistent with earlier findings showing that the LHS cells that have introduced SV40 ST and compromised PP2A activity are sensitive to TRAIL. In addition, we examined whether okadaic acid (20 nmol/L) or Calyculin A (100 nmol/L) changed PP2A protein expression. As shown in Fig. 3C, okadaic acid or Calyculin A did not change protein level of PP2A C, similar to the earlier data showing that the introduction of SV40 ST into LH cells (LHS cells) does not affect PP2A protein expression. Together, these results suggest that
persistently activated PP2A is necessary for normal PrEC and immortalized LH cells to be resistant to TRAIL-induced apoptosis, and inhibition of PP2A activity converts their TRAIL-resistant phenotype.

SiRNA of PP2A C inhibits PP2A activity and increases TRAIL sensitivity in PrEC and LH cells

Considering that the chemical antagonists may have off-targets in general and okadaic acid or Calyculin A may confer cellular phenotypes by targeting other phosphoprotein phosphatase family members than PP2A, we carried RNA interference (RNAi) knockdown test to further clarify the specific involvement of PP2A in our study. Because PP2A C is a core component of active PP2A complex, PrEC and LH cells were transfected with PP2A C siRNA for 24 hours, followed by additional treatment with TRAIL for another 24 hours before monitoring TRAIL responsiveness. As shown in Fig. 3D, in both PrEC and LH cells, combined treatment with TRAIL and PP2A C siRNA resulted in significant increases in cell apoptosis, as compared with the treatment with TRAIL or PP2A RNAi alone or cotreatment with TRAIL and the control RNAi. This observation is similar to the earlier findings with PP2A inhibitors. As expected, phosphatase activity analysis (Fig. 3E) and Western blotting (Fig. 3F) demonstrated that both PP2A enzymatic activity and PP2A C protein expression in PrEC and LH cells were markedly attenuated by PP2A C siRNA.

Inhibition of PP2A promotes TRAIL-induced apoptosis

Figure 3. Low-dose okadaic acid (OA), Calyculin A, or siRNA inhibits PP2A activity of PrEC and LH cells, and reverses their TRAIL-resistant phenotype. A, apoptosis analysis of PrEC and LH cells by annexin V assay. Cells were pretreated with okadaic acid (20 nmol/L) or Calyculin A (100 nmol/L) for 1 hour, and then treated with (+) or without (−) TRAIL for another 24 hours. Dimethyl sulfoxide (DMSO) stands for equal amount of vehicle. B, PP2A activity of PrEC and LH cells upon treatments as indicated. PP2A activity control samples without treatment was set at 1. C, PP2A C protein level of PrEC and LH cells upon treatments as indicated. D, measurement of TRAIL-induced apoptosis upon 20 μmol/L of PP2A C siRNA versus control siRNA transfection. E, evaluation of PP2A activity of PrEC and LH cells upon PP2A C siRNA versus control siRNA transfection. F, evaluation of PP2A C protein levels of PrEC and LH cells upon PP2A C siRNA versus control siRNA transfection. For all siRNA tests, PrEC and LH cells were transfected with ramble siRNA (ctl) or specific siRNA of PP2A C for 24 hours and then treated with (+) or without (−) TRAIL for another 24 hours before harvesting. Data, mean ± SD. Asterisks, statistically significant differences between compared values; *, P < 0.05; **, P < 0.01.
(L) (11). The earlier findings that PP2A inhibition can sensitize cells to TRAIL promoted us to hypothesize a mechanistic connection between PP2A and c-Fos/AP-1 pathways. To test this hypothesis, we initially compared endogenous protein expression of c-Fos, c-FLIP(L), TRAIL receptors DR4/DR5, and AP-1 activities in PrEC, LH, and LHS cells. The results demonstrated that both c-Fos expression and AP-1 activity in LHS cells were higher than those of the PrEC and LH cells; c-FLIP(L) expression in LHS cells was lower than those of the PrEC and LH cells; however, both total and plasma membrane DR4 and DR5 expression had no significant change among PrEC, LH, and LHS cells (Fig. 4A and B). To explore whether the mitochondrial pathway plays roles in the process, the expression of Bcl-2 and X-linked inhibitor of apoptosis (XIAP) in PrEC, LH, and LHS cells was also detected. The results demonstrated that PP2A inhibition in LHS cells did not change the protein levels of Bcl-2 and XIAP, however, XIAP level decreased in LHS cells after treating with TRAIL (Fig. 4A), suggesting signals of the mitochondrial pathway such as the inhibitor of apoptosis proteins XIAP are involved in PP2A-regulated TRAIL susceptibility. This finding indicates that the enhanced c-Fos expression, higher AP-1 activity, and decreased c-FLIP(L) expression in LHS cells is derived from SV40 ST–mediated PP2A inhibition. Also, this finding is in the opposite to the lower PP2A activity in LHS cells than that of the PrEC and LH cells (Fig. 2B), indicating a reverse correlation between lower PP2A activity with high c-Fos protein expression and AP-1 activation. To further verify that activated c-Fos/AP-1 is the mediator in TRAIL-elicited apoptosis, we next transfected LSH cells with A-Fos, a dominant negative of c-Fos (35). Importantly, A-Fos overexpression reversed TRAIL-sensitive phenotype of the LHS cells (Fig. 4C) and, as expected, repressed AP-1 activity in this cell line (Fig. 4D). Moreover, overexpressed A-Fos did not change the PP2A activity (Fig. 4E) and PP2A C protein level (data not shown) in LHS cells, indicating that c-Fos/AP-1 is at the downstream of PP2A in signaling cascade.

As a complementary approach, we then substantiated the earlier findings in a reverse direction. We tested and determined that okadaic acid–mediated inhibition of PP2A in both PrEC and LH cells led to dramatic increase of c-Fos expression, AP-1 activity, and decrease of c-FLIP(L), but did not change the expression of DR4/DR5 (Fig. 5A and B). Furthermore, A-Fos overexpression in PrEC and LH cells markedly antagonized okadaic acid–activated AP-1 activity and okadaic acid–sensitized apoptosis upon TRAIL treatment (Fig. 5B and C). However, A-Fos overexpression did not affect PP2A activity activated by okadaic acid (Fig. 5D). Enforced expression of c-FLIP(L) in LHS cells reversed its phenotype from TRAIL-sensitive to TRAIL-resistant (Fig. 5E).

Together, the earlier findings support the notion that PP2A negatively regulates c-Fos expression and AP-1 activity. c-Fos/AP-1 and its downstream target c-FLIP (L) are the essential mediators of TRAIL-induced apoptosis upon PP2A inhibition. Moreover, and conversely, interference of c-Fos/AP-1 does not disturb PP2A expression or activity, supporting that PP2A is a negative upstream regulator c-Fos/AP-1.

**PP2A inhibition by low-dose okadaic acid reverses TRAIL-resistant phenotype in cancer cells**

Although many cancers undergo TRAIL-induced apoptosis, some develop resistance, making TRAIL ineffective as an anticancer agent. The earlier results have demonstrated that inhibition of PP2A is a key factor for normal and transformed prostate cells to acquire TRAIL-sensitive phenotype. To more extensively substantiate our findings, we extended the study into additional cancer types. For this purpose, we examined two additional pairs of cancer cell lines, among which prostate cancer cells PC3 and renal cancer cells A-498 were sensitive to TRAIL and prostatic cancer cells PC3-TR and renal cancer cells 786-O were resistant to TRAIL (Fig. 6A). Significantly, the TRAIL-resistant cell lines (PC3-TR and 786-O) carry higher PP2A activity compared with that of their TRAIL-sensitive counterparts, PC3 and A-498 (Fig. 6B). We got similar results from TRAIL-sensitive MDA-MB231 and TRAIL-resistant MDA-MB453 in breast cancer cells (data not shown). Furthermore, in TRAIL-resistant PC3-TR and 786-O cell lines, PP2A inhibition by okadaic acid alone is not sufficient to induce the apoptotic response, whereas combined treatment with okadaic acid and TRAIL led to marked cellular apoptosis (Fig. 6C). In addition, okadaic acid treatment significantly enhanced AP-1 activity (Fig. 6D) and decreased PP2A activity (Fig. 6E) in the PC3-TR and 786-O cell lines, respectively. These findings are consistent with the observations obtained from the earlier studies on PrEC–LH–LHS system and provide further support for a link between PP2A activity and TRAIL resistance.

Together, our studies in additional cancer models further validated the findings that a reverse relationship exists between PP2A and c-Fos/AP-1 pathways and supported that PP2A inhibition could restore TRAIL sensitivity in resistant tumors via AP-1 activation.

**Discussion**

As a promising apoptosis inducer, TRAIL can potently and selectively elicit apoptosis in a variety of tumor and transformed cells, but sparing most normal cells (4, 13, 14). However, the molecular mechanism behind its functional specificity and selectivity remains unclear. In this study, we demonstrated that alterations in PP2A activity conferred distinct TRAIL responses among normal, transformed, immortalized, and cancer cells. We found that PP2A activity was associated with TRAIL insensitivity and attenuation of PP2A by SV40 ST, okadaic acid, Calyculin A, or siRNA sensitized resistant cells to TRAIL-induced apoptosis. Furthermore, we determined that c-Fos/AP-1 was the critical downstream mediator and effector of the PP2A pathway, which was activated upon PP2A inhibition to promote cellular apoptosis.

It is well accepted, the "multiple hits" hypothesis, that cancer arises from normal cells through the stepwise
accumulation of genetic alterations (40). Most of our knowledge of TRAIL sensitivity in the past was based on comparison of normal cells versus cancer cells. Because of limited resource in cancer cell lines and heterogeneous nature of cancer genetic background, it is always difficult to figure out the specific determinants that contribute to the acquisition of TRAIL-sensitive phenotype. The past attempts to search for mechanisms leading to TRAIL sensitivity rarely yielded dominant effectors. In 2004, Nesterov and colleagues used normal human foreskin fibroblast and normal human embryonic kidney (HEK) cells as models and sequentially immortalized these cells with hTERT, SV40 LT, and oncogenic Ras, which are very similar to our cell models (41). Their results have shown that Ras sensitized normal human cells to TRAIL-induced apoptosis, as both normal and immortalized cells were resistant to TRAIL-induced apoptosis, whereas Ras-transformed cells were susceptible. However, it remains unknown how Ras contributes to TRAIL sensitivity and whether it depends on particular tumorigenic stages. The difference between their studies and ours is that their cell models skipped a common essential step during human tumorigenesis, attenuation of PP2A in tumors. In the present study, we demonstrate for the first time that inhibition of PP2A at normal or premalignant stage is a key step for the cells to be susceptible to TRAIL, and TRAIL susceptibility might be maintained in its malignant

Figure 4. Attenuation of PP2A by SV40 ST activates c-Fos/AP-1, which is essential for PrEC and its transformed counterparts to be sensitive to TRAIL-induced apoptosis. A, protein expression of c-Fos, c-FLIP(L), Bcl-2, XIAP, total, and membranous DR4/DR5 in PrEC, LH, and LHS cells with (+) or without (−) TRAIL treatment. B, AP-1 activity of PrEC, LH, and LHS cells with (+) or without (−) TRAIL treatment. AP-1 activity of PrEC control sample without TRAIL treatment was set at 1. C, TRAIL-induced apoptosis in LHS cells after A-Fos overexpression. D, AP-1 activity of LHS cells after A-Fos overexpression. E, PP2A activity of LHS cells after A-Fos overexpression. AP-1 and PP2A activity of LHS control samples without A-Fos and TRAIL treatment in (D) and (E) was set at 1. GAPDH and integrin β1 are used as a loading control. Bars, SD of at least three replicate experiments. ctl, empty vector (CMV 500) of A-Fos. Data, mean ± SD. Asterisks, statistically significant differences between compared values; *, P < 0.05; **, P < 0.01.
cells after being transformed with oncogenic genes such as Ras.

Here, we used an experimental cell system with genetically defined background to investigate how conversion of human cells from normal to tumorigenic renders them sensitive to TRAIL-induced apoptosis. In this report, we show that both normal and immortalized LH cells are resistant to TRAIL-induced apoptosis, which indicates that concomitant activation of telomere reverse transcriptase and repression of tumor suppression genes such as p53 and Rb are not sufficient to make normal cells sensitive to TRAIL-induced apoptosis. However, the subsequent introduction by SV40 ST renders yielded cells (LHS) susceptible to TRAIL-mediated apoptosis. The tumorigenic activity of SV40 ST is strictly dependent on PP2A, its only known in vivo interacting partner. SV40 ST mutant that loses the interaction with PP2A fails to induce tumorigenic activity (29). SV40 ST may function mainly by inhibiting the phosphatase activity of the PP2A core enzyme, and to a lesser extent by modulating assembly of the PP2A holoenzymes (28). We found that PP2A protein expression was not altered by SV40 ST or inhibitors (okadaic acid and Calyculin A), but...
nevertheless, PP2A activity could be attenuated by these reagents. Our finding pinpoints a significant biologic function of SV40 ST in mammalian cells during tumorigenic transformation and implies that human cells may acquire sensitivity to TRAIL-induced apoptosis during premalignant period.

Because PP2A plays a crucial role in a variety of biologic processes, identifying the downstream targets of PP2A has significant implications in understanding of these processes. In this report, we show that PP2A, in addition to being a well-known tumor suppressor (22, 23), has a novel antiapoptotic function in TRAIL-induced apoptosis during tumorigenesis. Previously, we have shown that activation of c-Fos/AP-1 is an essential effector for TRAIL-induced apoptosis in the prostate cancer cells by suppressing the expression of antiapoptotic protein c-FLIP(L) (11). We assume that c-Fos/AP-1 might also act as a mediator upon PP2A attenuation to confer the TRAIL-resistant phenotype. Indeed, we found a reverse relationship between PP2A and Fos/AP-1 activities in multiple independent nonmalignant and malignant cellular systems. The chemically or biologically antagonized PP2A activated the Fos/AP-1 pathway, decreased the level of c-FLIP(L) and TRAIL-sensitive phenotype. However, inhibition of PP2A has been reported to protect U937 cells from death receptor (including TRAIL)-induced cell death through activating the MARK/ERK signaling pathway (37). The controversial results may be caused by different cell types. Our results support the facts that loss of PP2A is very common during tumorigenesis and tumor cells are more sensitive to TRAIL-induced apoptosis. More recently, it has been reported that bortezomib sensitized hepatocellular carcinoma cells to DR5 antibody CS-1008 through the inhibition of CIP2A, which is an inhibitor of PP2A (38). This result also supports the important role of PP2A on TRAIL-induced apoptosis in cancer cells.

Figure 6. The inhibition of PP2A by okadaic acid (OA) sensitizes cancer cells to TRAIL-induced apoptosis. A, cell viability of prostate cancer cells PC3, PC3-TR and renal cancer cells 786-O, A-498 upon TRAIL treatment over a range of indicated doses. B, comparison of PP2A activity between PC3 and PC3-TR, and between 786-O and A-498. C–E, apoptosis (C), AP-1 activity (D), and PP2A activity of TRAIL-resistant PC3-TR and 786-O cells (E). Cells were pretreated with okadaic acid (20 nmol/L) or the equal vehicle (DMSO; D) alone for 1 hour and then treated with TRAIL (50 ng/mL) for 24 hours. Asterisks, statistically significant differences between compared values; * , P < 0.05; ** , P < 0.01.
However, it remains undetermined by what mechanisms does PP2A negatively regulate c-Fos/AP-1 activity. Recent work has suggested that phosphorylation of c-Fos, an important determinant of its activity, is tightly regulated by a variety of kinases (39). As a major cellular phosphatase, PP2A may decrease expression and activity of c-Fos by dephosphorylating Fos or specific Fos kinases. The complexity in PP2A holoenzyme organization also defers the efforts to pursue the exact regulatory mechanism. In any case, this area is under active investigation in our laboratory at this moment.

In summary, our study first demonstrates that Fox/PP1-mediated an internal connection between PP2A attenuation and TRAIL sensitivity that exists independently of cellular stages during tumorigenesis, suggesting that it is a generalized and conserved mechanism. Moreover, PP2A activity might be used to predict TRAIL sensitivity of cancer cells and PP2A inhibitor can potentially enhance TRAIL-induced apoptosis in cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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