Regulation of TRAIL-mediated Apoptosis by PKCK2 in Cancer Cell Lines

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Regulation of TRAIL-mediated Apoptosis by PKCK2 in Cancer Cell Lines

Directed by professor **Kunhong Kim**

The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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Hyangtae Choi

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ABSTRACT

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Protein kinase casein kinase 2 (PKCK2) is a serine/threonine kinase that has been known to play important roles in cell cycle control, cellular differentiation, and proliferation. Recently, it was reported that intracellular high PKCK2 activity prevents cancer cells from undergoing apoptosis through phosphorylating procaspase-2. When PKCK2 activity is down-regulated by specific inhibitor, procaspase-2 is activated, procaspase-8 is processed by active caspase-2, and then, TRAIL-resistant cancer cells become primed for TRAIL-mediated apoptosis. To examine whether PKCK2-dependent mechanism for TRAIL resistance of cancer cells

could be generalized, endogenous PKCK2 activity and TRAIL sensitivity were correlated using 17 human cancer cell lines originated from stomach and liver. Among the 17 cancer cell lines, 11 cell lines (64.7%) corresponded with the mechanism while 6 cell lines (35.3%) did not: 5 cell lines (MKN-45, MKN-74, SNU484, SNU719, SNU886) showed 'low PKCK2 activity - high TRAIL sensitivity', 6 cell lines (AGS, MKN-1, MKN-28, Hep3B, SK-HEP-1, SNU739) showed 'high PKCK2 activity - low TRAIL sensitivity', 4 cell lines (NCI-N87, HepG2, SNU761, SNU878) showed 'low PKCK2 activity - low TRAIL sensitivity' and 2 cell lines (SNU638, SNU668) showed 'high PKCK2 activity – high TRAIL sensitivity'. 6 cell lines (AGS, MKN-1, MKN-28, Hep3B, SK-HEP-1, SNU739) that showed 'high PKCK2 activity - low TRAIL sensitivity' were sensitized to TRAIL by inhibiting PKCK2 activity. The expression profiles of pro- or anti-apoptotic molecules of cancer cell lines were examined to get insights why PKCK2 activity and TRAIL sensitivity did not correlated in some cancer cell lines. Some cancer cells expressed pro- or anti-apoptotic molecules at too low or too high level to induce apoptosis. Among the molecules, procaspase-8 was focused especially. PKCK2 phosphorylated procaspase-8 at threonine-373, and the phosphorylation inhibited processing of procaspase-8 by active caspase-2.

Taken together, intracellular PKCK2 activity determines TRAIL sensitivity in several cancer cell lines and PKCK2 regulates TRAIL sensitivity of cancer cells by phosphorylating procaspase-8.

Key words: TRAIL, protein kinase CK2, procaspase-2, procaspase-8, apoptosis, cancer cell lines.

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Ⅰ. INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent inducer of apoptosis of cancer cells but not of most normal cells[1,2]. However, the fact that many cancer cells develop a TRAIL-resistance phenotype limits the clinical efficacy of TRAIL[3,4]. Caspases play important roles in the initiation and execution of apoptosis and their activation can be regulated by phosphorylation[5,6]. Pro-apoptotic molecules like Bid can be also regulated by phosphorylation[7]. Protein kinase casein kinase 2 (CK2) has been classified traditionally as a messenger-independent protein serine/threonine kinase that is typically found in tetrameric complexes consisting of

two catalytic (α and/or α') subunits and two regulatory β subunits[8-10]. It plays key roles in cell cycle control, cellular differentiation, and proliferation[8,11]. Since not much attention has been paid to caspase-2 because caspase-2 deficient mice showed only subtle phenotypes[12,13], little is known about the mechanism by which caspase-2 becomes activated or the role of caspase-2 in particular apoptotic pathways. Recently, it was reported that PKCK2 phosphorylates procaspase-2 at serine-157 residue, thereby preventing its dimerization and activation[14]. When PKCK2 activity is down-regulated by a specific inhibitor, procaspase-2 is dephosphorylated, dimerized, and activated, and then procaspase-8 is cleaved by active caspase-2. When TRAIL is engaged into cell surface TRAIL-death receptors, the cleaved procaspase-8 is then recruited into death inducing signaling complex (DISC), and becomes fully activated followed by apoptosis.

In this study, endogenous PKCK2 activity and TRAIL sensitivity were correlated using 17 human cancer cell lines originated from stomach and liver to examine whether PKCK2-dependent mechanism for TRAIL resistance of cancer cells could be generalized. Among the 17 cancer cell lines, 11 cancer cell lines (64.7%) corresponded with the mechanism while 6 cancer cell lines (35.3%) did not. The expression profiles of pro- or anti-apoptotic molecules of cancer cell lines were examined to get insights why PKCK2 activity and TRAIL sensitivity did not correlated in some cancer cells. Some cancer cells expressed pro- or anti-apoptotic molecules at too low or too high level to induce apoptosis. The previous reports that

procaspase-2 activation is inhibited by PKCK2 mediated phosphorylation at serine-157[14] and that Bid cleavage at aspartic acid-59 by caspase-8 is inhibited by PKCK2 mediated phosphorylation at threonine-58[7] led us to search for PKCK2 phosphorylation site(s) that may inhibit procaspase-8 processing. This study showed that PKCK2 phosphorylated procaspase-8, thereby inhibiting procaspase-8 processing by active caspase-2.

Ⅱ. MATERIALS AND METHODS

1. Cell lines and culture conditions

The human stomach cancer cell line AGS and the human hepatoma cell line HepG2 were cultured in MEM (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen). The human hepatoma cell lines Hep3B and SK-HEP-1 were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The human stomach cancer cell lines (MKN-1, MKN-28, MKN-45, MKN-74, NCI-N87, SNU484, SNU638, SNU668, and SNU719), and the human hepatoma cell lines (SNU739, SNU761, SNU878 and SNU886) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All the cell lines were grown at 37°C in 5% CO_2.

2. Cell viability assay

Cells were seeded in the 96-well plate and incubated for 16 hr. The cells were co-treated with 30 μM TBB[15] (SIGMA-Aldrich, St-Louis, MO, USA) and 500 ng/ml TRAIL[16] (ATGen, Sungnam, Korea) for 3 hr. For the measurement of cell viability, MTT assay was performed. Briefly, cells were incubated with 2 mg/ml MTT (SIGMA-Aldrich) for 2 hr. The supernatants were then removed and 100 μl DMSO (Duchefa, BH Haarlem, The Netherlands) was added to the 96-well plate. Absorbance

was recorded at 570nm using SpectraMax ELISA reader[17]. The data are expressed as mean±s.d. for quardruplicate, and similar results were obtained from two independent experiments.

3. PKCK2 *in vitro* kinase activity assay

PKCK2 *in vitro* kinase activity assay was carried out as described previously[18]. Briefly, 3 μg of bacterially expressed GST-CS (CK2 substrate) proteins were incubated with glutathione sepharose 4B beads for 1 hr, then they were washed twice using 1x kinase buffer [4 mM MOPS, pH 7.2, 5 mM β-glycerolphosphate, 1 mM EGTA, 200 μM sodium orthovanadate, and 200 μM DTT]. Then the beads were incubated either with 100 ng recombinant active PKCK2 (ATGen) or 100 μg of cell lysates in final 50 μl of kinase reaction buffer [10 μl of 5x kinase buffer, 10 μl of magnesium/ATP cocktail solution (90 μl of 75 mM $MgCl_2$/500 mM ATP plus 10 μl (100 μCi) of [γ-^{32}P]-ATP)] for 20 min at 30°C. Reactions were stopped by washing three times using 1x kinase buffer. Samples were resuspended with 30 μl of 2x SDS sample loading buffer and subjected to SDS-PAGE followed by staining with coomassie brilliant blue and autoradiography.

4. Western blot analysis

Cells were lysed in RIPA buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EGTA, and 0.25% sodium deoxycholate]. Lysates were incubated for 15 min at 4°C and

cleared by centrifugation at 12,000 rpm for 15 min at 4°C. Supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amount of proteins was subjected to electrophoresis on SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The blotted membranes were immunostained with antibodies specific for the following antigens: Bcl-xL, caspase-2 (BD biosciences Pharmingen, San Jose, CA, USA), caspase-3 (Santa Cruz, Santa Cruz, CA, USA) , caspase-8, cIAP-1, cIAP-2, phosphor-Threonine, survivin (Cell signaling Technology, Beverly, MA, USA), Bcl-2 (Stressgen, Victoria,BC, Canada), PKCK2α (Millipore). The signals were developed by a standard enhanced chemiluminescence (ECL) method according to the manufacture's protocol (Roche, Indianapolis, IN, USA)

5. Immunoprecipitation (IP)

Cells were collected and lysed in 1 ml IP lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40] with 1x complete protease inhibitor cocktail (Roche). The cell lysates were pre-cleared and then incubated with the indicated antibodies for overnight at 4°C. The complexes were precipitated with Protein A/G-Sepharose beads (Santa Cruz), washed, and re-suspended in 30 μl 1x SDS-loading buffer. Non-immune mouse IgG (Santa Cruz) served as a negative control.

6. Expression and purification of recombinant proteins

Human caspase-8 P1 (a.a 360-479), caspase-8 P1 T373A (a.a 360-479), caspase-8 P2 (a.a 170-240) were cloned into MBP-pRSET. For protein expression, plasmids were transformed into the *E.coli* BL21 (DE3) pLysS. *E .coli* were grown overnight at 25°C in LB media containing 0.5 mM IPTG. *E. coli* were lysed by sonication and recombinant proteins were purified using Amylose resin affinity chromatography. Purified proteins were then resolved with SDS-polyacrylamide gel to quantitate and to assess purity. Human full-length caspase-8 was expressed in SF9 insect cells using The Bac-to-Bac® Baculovirus Expression System (Invitrogen) according to the manufacture's protocol and purified using NTA-Nickel resin affinity chromatography.

7. Caspase *in vitro* cleavage assay

Recombinant full-length human caspase-8 protein purified from SF9 was incubated in the presence or absence of 20 μg of recombinant PKCK2 (ATGen) in final 40 μl of kinase reaction buffer [10 μl of 5x kinase buffer, 1 mM cold ATP] for 20 min at 30˚C. 15μl of reactants were transferred to a new tube containing either with or without 10 units of recombinant human caspase-2 enzyme (Alexis, San Diego, CA, USA) in final 50 μl of caspase reaction buffer [50 mM HEPES, pH 7.2, 50 mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% glycerol and 10 mM DTT], and then incubated for 18 hr at 37˚C. Endogenous procaspase-8 obtained from HCE4 esophageal cancer cells treated with or without DRB 40 μM for 24 hr was incubated with or without 10 units of

recombinant human caspase-2 enzyme in final 50 μl of caspase reaction buffer for 1 hr. Reactions were stopped by adding 5x SDS sample loading buffer and subjected to Western blot analysis.

Ⅲ. RESULTS

1. TRAIL cytotoxicity toward human cancer cell lines derived from stomach and liver.

To generalize previous observation that PKCK2 activity determines TRAIL sensitivity (Fig. 1), 17 cancer cell lines originated from stomach and liver were used. To examine TRAIL cytotoxicity toward cancer cell lines, cell viability was measured using MTT assay. Among 17 cell lines, 7 cancer cell lines (MKN-45, MKN-74, SNU484, SNU638, SNU668, SNU719, SNU886) showed cell viability lower than 60% in case of TRAIL treatment could be classified as ‘TRAIL-sensitive’ cell lines, the other 10 cancer cell lines (AGS, MKN-1, MKN-28, NCI-N87, HepG2, Hep3B, SK-HEP-1, SNU739, SNU761, SNU878) showed cell viability higher than 60% could be classified as ‘TRAIL-resistant’ cell lines (Fig. 2, Table. 1).

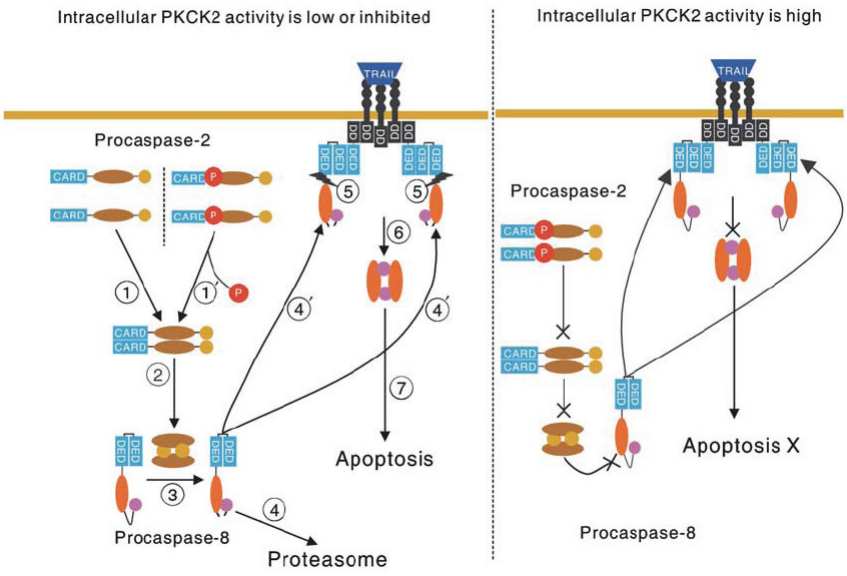


Figure 1. Proposed mechanism of PKCK2 involvement in TRAIL-mediated apoptosis. When PKCK2 activity is high (right), TRAIL-mediated apoptosis cannot occur. When PKCK2 activity is low or inhibited (left), procaspase-2 is dephosphorylated(①), dimerized and activated(②). Activated caspase-2 cleaves procaspase-8 between the large and small subunits(③). When TRAIL is engaged, cleaved procaspase-8 is recruited to DISC(④'). Once cleaved, further cleavage between the prodomain and large subunit can occur by induced proximity(⑤), and procaspase-8 can be fully activated(⑥). Finally, TRAIL-mediated apoptosis can occur(⑦).

Table 1. Origin of cancer cell lines, their TRAIL sensitivity, and PKCK2 activity.

Origin	Cell line	TRAIL sensitivity*	PKCK2 activity**
Stomach	AGS	R	H
	MKN-1	R	H
	MKN-28	R	H
	MKN-45	S	L
	MKN-74	S	L
	NCI-N87	R	L
	SNU484	S	L
	SNU638	S	H
	SNU668	S	H
	SNU719	S	L
Liver	HepG2	R	L
	Hep3B	R	H
	SK-HEP-1	R	H
	SNU739	R	H
	SNU761	R	L
	SNU878	R	L
	SNU886	S	L

* TRAIL sensitivity S : TRAIL-sensitive R : TRAIL-resistant

** PKCK2 activity L : Low PKCK2 activity H : High PKCK2 activity

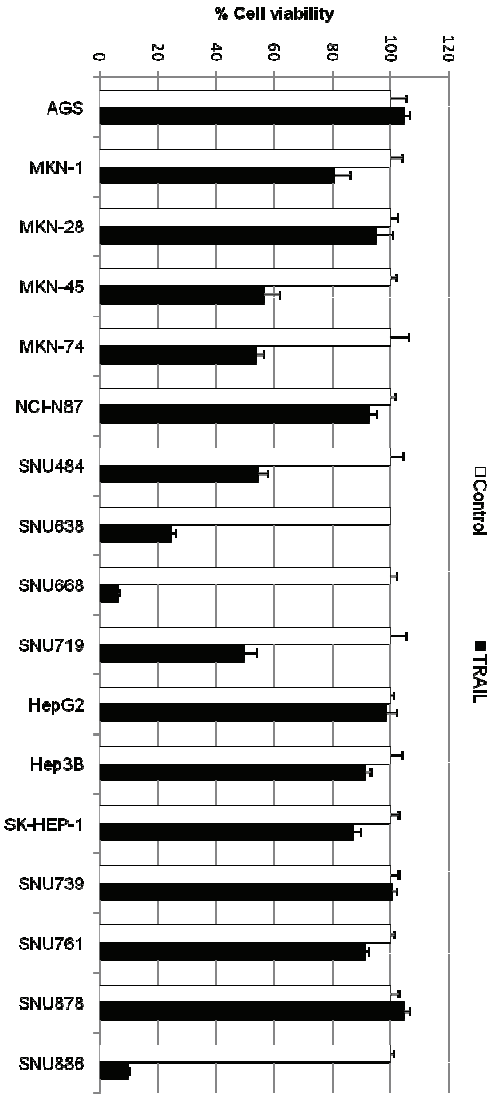
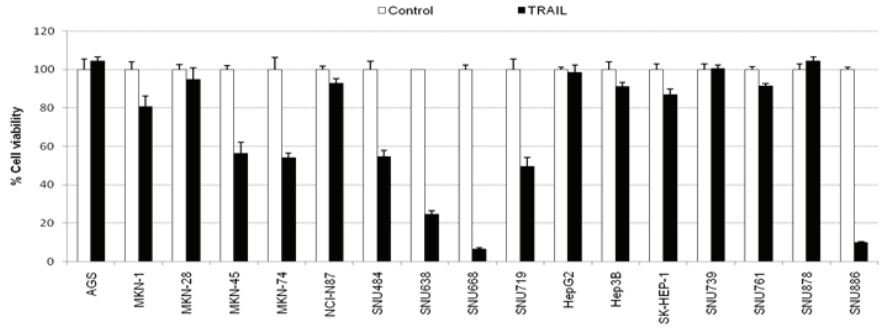

% Cell viability
0
20
40
60
80
100
120
AGS
MKN-1
MKN-28
MKN-45
MKN-74
NCI-N87
SNU484
SNU638
SNU668
SNU719
HepG2
Hep3B
SK-HEP-1
SNU739
SNU761
SNU878
SNU888
□Control
■TRAIL

Figure 2. TRAIL sensitivity of cancer cell lines originated from stomach and liver. Cell viability was measured by MTT assay using cancer cells incubated with or without 500ng/ml TRAIL for 3 hr. The data are expressed as mean ± SD for quardruplicate, and similar results were obtained from two independent experiments.

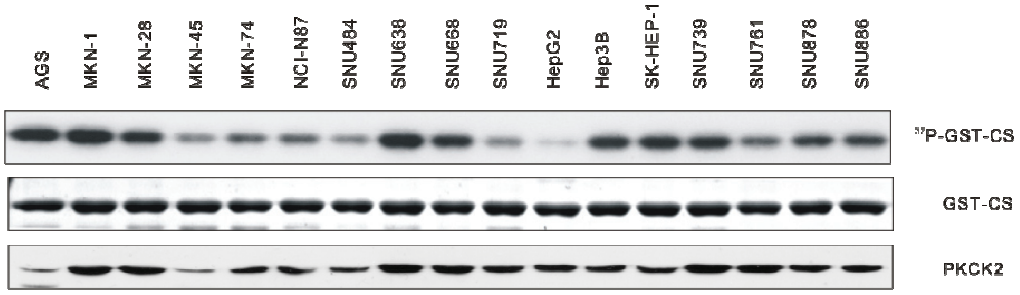
2. Correlation between TRAIL sensitivity and their intracellular PKCK2 activity.

Then, intracellular PKCK2 activity of cancer cell lines was evaluated by PKCK2 *in vitro* kinase assay. Relative PKCK2 activity, compared to the activity of HepG2 that had the lowest PKCK2 activity, was calculated. Cancer cell lines that showed relative PKCK2 activity lower than 2 could be classified as 'low PKCK2 cell lines', and that showed relative PKCK2 activity higher than 2 could be classified as 'high PKCK2 cell lines' (Fig. 3). Cancer cell lines were grouped based on both TRAIL sensitivity and PKCK2 activity. Among 17 cell lines, 5 cell lines (MKN-45, MKN-74, SNU484, SNU719, SNU886) showed 'low PKCK2 activity - high TRAIL sensitivity', 6 cell lines (AGS, MKN-1, MKN-28, Hep3B, SK-HEP-1, SNU739) showed 'high PKCK2 activity - low TRAIL sensitivity', 4 cell lines (NCI-N87, HepG2, SNU761, SNU878) showed 'low PKCK2 activity - low TRAIL sensitivity' and 2 cell lines (SNU638, SNU668) showed 'high PKCK2 activity – high TRAIL sensitivity' (Table. 1).

A.


Control
TRAIL
% Cell viability
120
100
80
60
40
20
0
AGS
MKN-1
MKN-28
MKN-45
MKN-74
NCI-N87
SNU484
SNU638
SNU668
SNU719
HepG2
Hep3B
SK-HEP-1
SNU739
SNU761
SNU878
SNU886

B.


AGS
MKN-1
MKN-28
MKN-45
MKN-74
NCI-N87
SNU484
SNU638
SNU668
SNU719
HepG2
Hep3B
SK-HEP-1
SNU739
SNU761
SNU878
SNU886
32P-GST-CS
GST-CS
PKCK2

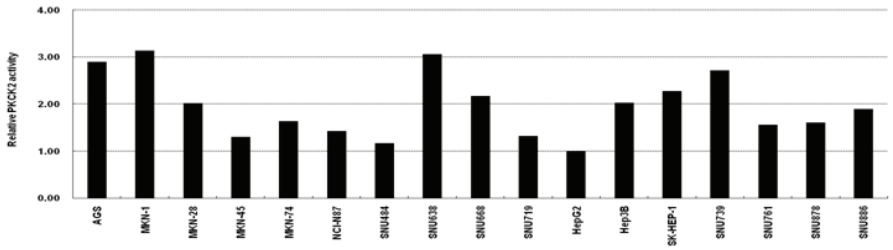

Relative PKCK2 activity
4.00
3.00
2.00
1.00
0.00
AGS
MKN-1
MKN-28
MKN-45
MKN-74
NCI-N87
SNU484
SNU638
SNU668
SNU719
HepG2
Hep3B
SK-HEP-1
SNU739
SNU761
SNU878
SNU886

Figure 3. Correlation between TRAIL sensitivity and their intracellular PKCK2 activity. A. TRAIL sensitivity was measured using MTT assay. B. PKCK2 activity was evaluated using PKCK2 *in vitro* kinase assay. Expression level of PKCK2α catalytic subunit was examined using Western blot analysis (upper panel, PKCK2). Relative PKCK2 activity, compared to the activity of HepG2 that had the lowest PKCK2 activity, was calculated. (lower panel).

3. Sensitization of TRAIL-resistant cancer cell lines to TRAIL by inhibiting intracellular PKCK2 activity.

To confirm the assumption that PKCK2 is a major determinant of TRAIL-resistance of cancer cells, 6 cancer cell lines (AGS, MKN-1, MKN-28, Hep3B, SK-HEP-1, SNU739) that showed 'high PKCK2 activity - low TRAIL sensitivity' were co-treated with TBB, a specific PKCK2 inhibitor and TRAIL. All 6 cancer cell lines were sensitized to TRAIL by TBB co-treatment (Fig. 4).

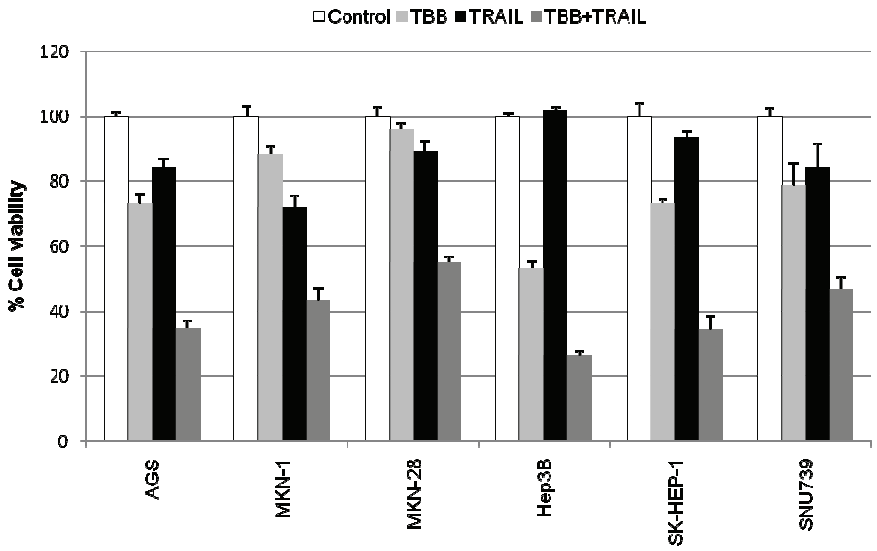


Figure 4. Sensitization of TRAIL-resistant cancer cell lines to TRAIL by inhibiting intracellular PKCK2 activity.

Cell viability was evaluated by the MTT assay using cancer cells incubated with TRAIL (500 ng/mL) for 3 hr in the presence or absence of TBB (30 μM).

The data are expressed as mean ± SD for quardruplicate, and similar results were obtained from two independent experiments.

4. Expression patterns of pro- or anti-apoptotic molecules in cancer cell lines.

To examine why 6 cancer cell lines (NCI-N87, HepG2, SNU761, SNU878 – low PKCK2 activity and low TRAIL sensitivity, SNU638, SNU668 – high PKCK2 activity and high TRAIL sensitivity) showed no correlation between PKCK2 activity and TRAIL sensitivity, expression profiles of pro- or anti-apoptotic molecules in cancer cell lines were checked using Western blot analysis. There were some clues to explain the mismatch between intracellular PKCK2 activity and TRAIL sensitivity. For example, NCI-N87 expressed procaspase-2 at low level, and cIAP-2 that is known to inhibit caspase activation at medium level, SNU668 expressed Bcl-xL that is known to inhibit apoptosis at low level, cIAP-2 low level, and SNU878 expressed procaspase-3 at low level, Bcl-2 that is known to inhibit apoptosis at low level (Fig. 5).

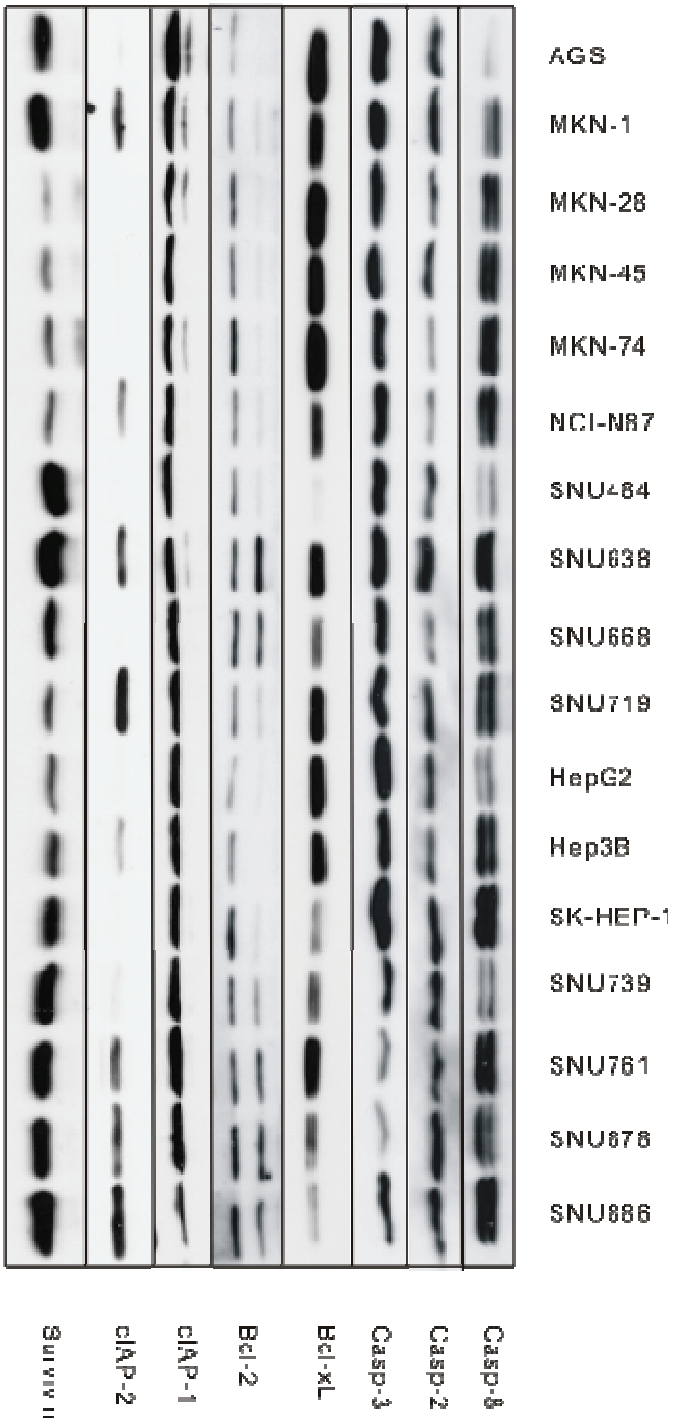

AGS
MKN-1
MKN-28
MKN-45
MKN-74
NCI-N87
SNU484
SNU638
SNU668
SNU719
HepG2
Hep3B
SK-HEP-1
SNU739
SNU761
SNU878
SNU886
Survivin
cIAP-2
cIAP-1
Bcl-2
Bcl-xL
Casp-3
Casp-2
Casp-8

Figure 5. Expression patterns of pro- or anti-apoptotic molecules in cancer cell lines. To check the expression profiles of pro-or anti-apoptotic molecules between cancer cell lines, Western blot analysis was performed using indicated antibodies.

5. PKCK2 phosphorylates procaspase-8 at threonine-373.

Previous reports that PKCK2 regulates procaspase-2 activation and that PKCK2 regulates Bid cleavage prompted us to examine whether procaspase-8 is another substrate for PKCK2. HCE4 cells were co-transfected with caspase-2 siRNA and HA-tagged caspase-2 mutant (HA-S157A/C2Sil) to express constitutively active caspase-2[14]. Two days after transfection, the cells were treated with or without DRB, a PKCK2 inhibitor. Western blot analysis showed that procaspase-8 is only processed by caspase-2 in the presence of DRB, suggesting that PKCK2 may phosphorylate procaspase-8, thereby inhibiting its processing by active caspase-2 (Fig. 6B). On computational search for potential PKCK2 phosphorylation site(s) in procaspase-8, five potential PKCK2 consensus motives for phosphorylation were found. (Fig. 6A). To determine the phosphorylation site within procaspase-8, MBP-fusion human caspase-8 proteins MBP-P1 (a.a 360-479) or MBP-P2 (a.a 170-240) were expressed in bacteria and purified. Each protein contained two or three potential PKCK2 consensus motives (Fig. 6A). PKCK2 *in vitro* kinase assay was performed using each protein as a substrate, and MBP-P1 was extensively phosphorylated by PKCK2 (Fig. 6C). MBP-P1 contained two potential consensus motives at threonine-373 and serine-375 and thus, MBP-P1 mutant (T373A) was produced by converting threonine-373 into alanine. PKCK2 *in vitro* kinase assay showed that PKCK2 phosphorylated procaspase-8 at threonine-373 (Fig. 6D).

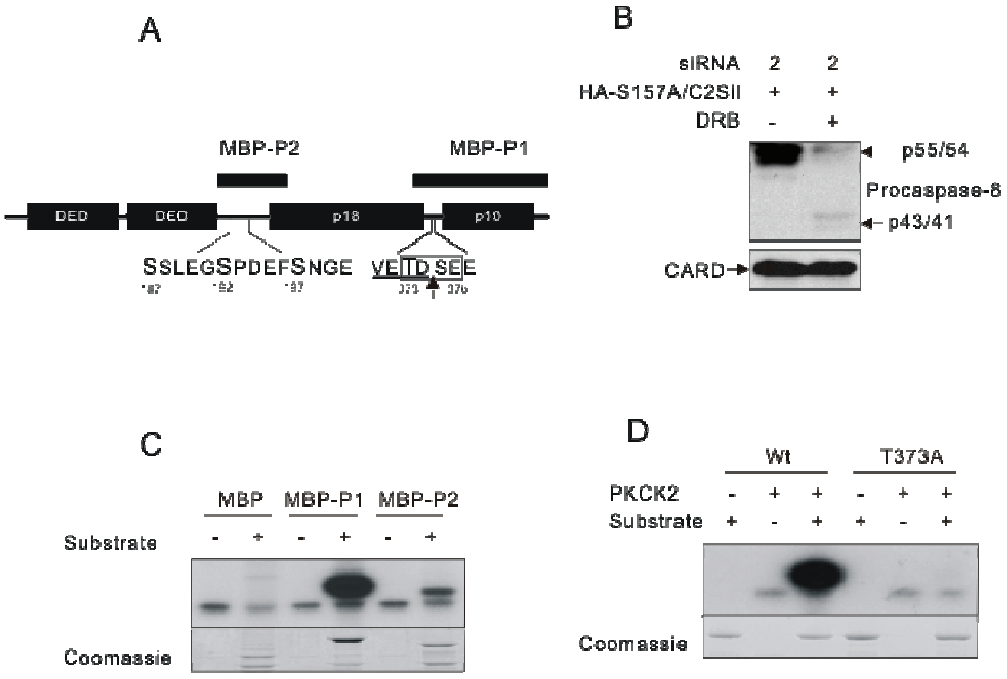


Figure 6. PKCK2 phosphorylates procaspase-8 at threonine-373. A. Schematic representation of procaspase-8. DED, death effector domain; p18 and p10, the large and small subunits that form mature caspase-8. Threonine-373, the potential PKCK2 phosphorylation site is shown in *boldface*. An arrow indicates the caspase-2 cleavage site and a box represents the minimal consensus sequence that is the target of PKCK2-mediated phosphorylation. B. Caspase-2 cleaves procaspase-8 in DRB treated cancer cells. HCE4 cells were co-transfected with caspase-2 siRNA (2) and 1 μg of pCMV-HA-S157A/C2sil (C2sil; caspase-2 mutant that is introduced two silent mutations to prevent the destruction of mRNA by siRNA). Two days after transfection, the cells were treated with or without 40 μM DRB for 24 hr. Western

blot analysis with anti-procaspase-8 is shown. The expression of exogenous procaspase-2 was confirmed by Western blot analysis with anti-HA. "CARD" indicates the position of the HA-tagged CARD that is generated after processing of the HA-tagged S157A/C2sil. C. PKCK2 phosphorylates procaspase-8. PKCK2 *in vitro* kinase assay was performed using MBP-P1 or -P2 protein as a substrate. D. PKCK2 phosphorylates procaspase-8 at threonine-373. PKCK2 *in vitro* kinase assay was performed using MBP-P1 or MBP-P1 T373A protein as a substrate.

6. Phosphorylation of procaspase-8 by PKCK2 regulates its cleavage by caspase-2

To examine whether PKCK2 phosphorylates procaspase-8 *in vivo*, an esophageal cancer cell line HCE4 was used. To prevent activation of procaspase-2 in the presence of a PKCK2 inhibitor, DRB, HCE4 cells were pre-treated with z-VAD-fmk for 2 hr, and then treated with DRB for 24 hr. Immunoprecipitation followed by Western blot analysis revealed that procaspase-8 is phosphorylated at threonine resisdue by PKCK2 (Fig. 7A) *in vivo*. The interaction between endogenous PKCK2 and procaspase-8 was confirmed using immunoprecipitation followed by Western blot analysis (Fig. 7B). To examine whether the phosphorylaton could affect the cleavage of procaspase-8 by active caspase-2, full-length recombinant human procaspase-8 was expressed in SF9 insect cells and purified. Purified procaspase-8 was phosphorylated using cold ATP and recombinant PKCK2 *in vitro* and then, incubated with active recombinant human caspase-2. Consistent with a previous result (Fig. 6B), un-phosphorylated procaspase-8 was cleaved by active caspase-2, but phosphorylated procaspase-8 was not (Fig. 7C). To confirm this again, HCE4 cells were transfected with siRNA against caspase-2, and then were treated with DRB. Cell lysates were immunoprecipitated with anti-procaspase-8 (C8) and incubated with active recombinant human caspase-2. In the presence of DRB, immunoprecipitated endogenous procaspase-8 was cleaved (Fig. 7D). Taken together, these results suggest that PKCK2 phosphorylates procaspase-8 at threonine-373, and the phosphorylation

protects procaspase-8 from cleavage by caspase-2.

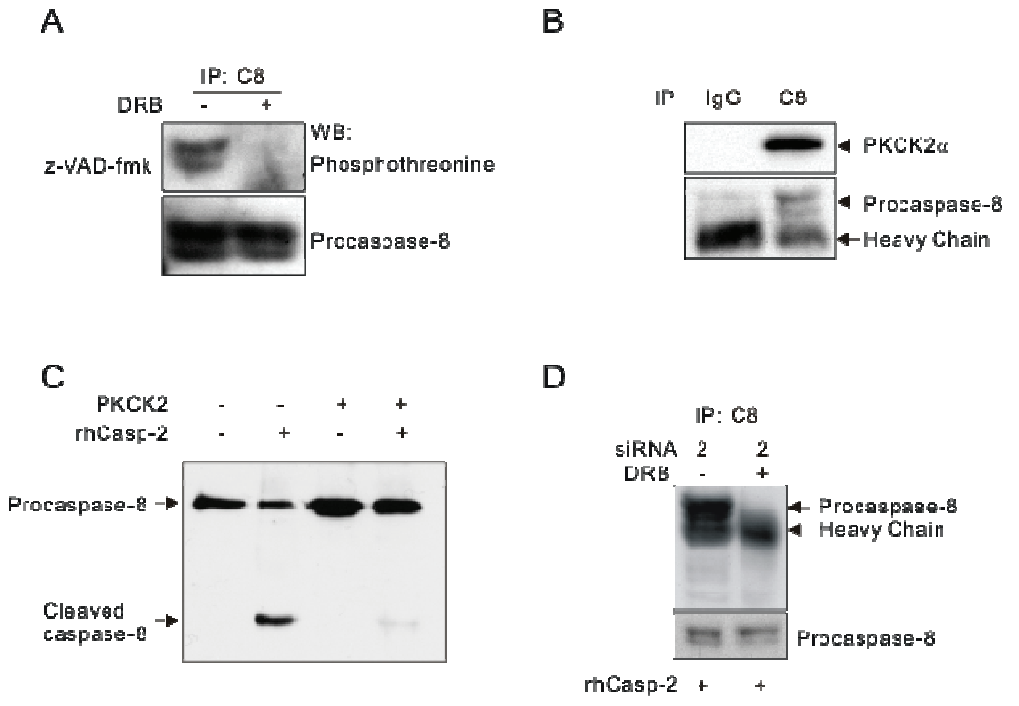


Figure 7. Phosphorylation of procaspase-8 by PKCK2 inhibits its cleavage by caspase-2. A. PKCK2 phosphorylates procaspase-8 *in vivo*. The pancaspase inhibitor, z-VAD-fmk pretreated HCE4 cells were treated with or without DRB. Immunoprecipitation using anti-procaspase-8 (C8) was followed by Western blot analysis with anti-phosphothreonine or anti-procaspase-8. B. Procaspase-8 interacts directly with PKCK2 *in vivo*. The lysates obtained from HCE4 cells were immunoprecipitated with anti-procaspase-8 (C8) or non-immune IgG (IgG) followed by Western blot analysis using anti-PKCK2. The same blot was re-probed with anti-procaspase-8. C. Phosphorylation of full-length recombinant human procaspase-8

by PKCK2 inhibits its cleavage by caspase-2. Full-length procaspase-8 purified from SF9 insect cells was incubated with or without 20 μg of recombinant human active PKCK2α for 20 min at 30°C, and incubated with or without 10 units of recombinant human active caspase-2 for 18 hr at 37°C. rhCasp-2, recombinant human active caspase-2. D. Phosphorylation of endogenous procaspase-8 inhibits its cleavage by caspase-2. HCE4 cells were transfected with caspase-2 siRNA (2) and, two days later, treated with or without DRB. Cell lysates were immunoprecipitated with anti-procaspase-8 (C8) and incubated with or without 10 units of recombinant human active caspase-2 for 1 hr at 37°C. Samples were Western blotted with anti-procaspase-8 antibody. Immunoblotting for procaspase-8 is shown below.

Ⅳ. DISCUSSION

Recently, a mechanism by which cancer cells acquire a resistance against TRAIL-mediated apoptosis was revealed[14]. PKCK2 phosphorylates procaspase-2 at serine-157, thereby preventing its dimerization and activation. However, when PKCK2 activity is inhibited by a specific inhibitor, procaspase-2 is dephosphorylated, dimerized, and activated. The activated caspase-2 then processes procaspase-8 monomers between the large and small subunits, thereby priming cancer cells for death-receptor-mediated apoptosis. To examine whether PKCK2 dependent mechanism for TRAIL resistance of cancer cells can be generalized, intracellular PKCK2 activity was evaluated and correlated with TRAIL sensitivity in 17 human cancer cell lines originated from stomach and liver. Among 17 cancer cell lines, 11 cell lines (64.7%) corresponded with the mechanism. However, 6 cell lines (35.3%) showed no correlation between intracellular PKCK2 activity and TRAIL sensitivity. Pro- or anti-apoptotic molecules expression profiles in cancer cell lines were checked to examine why they showed no correlation. There were some clues to explain the mismatch between intracellular PKCK2 activity and TRAIL sensitivity. It is possible that some sort of cancer cells would express pro- or anti-apoptotic molecules at too low or too high level to induce apoptosis.

Among the several pro- or anti-apoptotic molecules, caspase-8 was regulated by

PKCK2. PKCK2 phosphorylated procaspase-8 at threonine-373, and the phosphorylation protected it from cleavage by caspase-2. Previous reports showed that Bid cleavage is regulated by PKCK2[5] and that procaspase-2 cleavage is regulated by PKCK2[14]. This study showed that procaspase-8 cleavage was also regulated by PKCK2. These observations suggest that PKCK2 regulates TRAIL-mediated apoptosis in cancer cells through phosphorylating several pro-apoptotic molecules. Interestingly, recent publications suggest that caspase-8 promotes cell migration and cell-matrix adhesion[19-22], and the promotion may be regulated by non-receptor tyrosin kinase Src through phosphorylating procaspase-8 at tyrosine-380[23]. Noteworthy, tyrosine-380 is almost next to threonine-373, the residue that is phosphorylated by PKCK2. It would be interesting to investigate the possibility that PKCK2 regulates cell migration and adhesion by phosphorylating procaspase-8 as non-receptor tyrosin kinase Src does.

In summary, PKCK2 activity determines TRAIL sensitivity of various cancer cell lines, and PKCK2 regulates TRAIL-mediated apoptosis in cancer cells by not only phosphorylating Bid or procaspase-2 but also phosphorylating procaspase-8. This mechanism may not be applicable to some cancer cells that have epigenetic or genetic changes in the molecules involved in apoptosis such as a mutation of DR5 found in head and neck cancer, and non-small cell lung cancers[24,25], a homozygous deletion[26], mutations[27], or polymorphism[3,27] found in the death domain region of DR4, inactivation of caspase-8 by DNA methylation or gene deletion[28-30]. However, it is

clear that inhibiting PKCK2 activity is beneficial in cancer therapy.

Ⅴ. CONCLUSION

1. Among 17 cancer cell lines originated from stomach and liver, 11 cell lines (64.7%) showed correlation between intracellular PKCK2 activity and TRAIL-sensitivity.

2. PKCK2 inhibition sensitized TRAIL-resistant cancer cells to TRAIL.

3. PKCK2 phosphorylated procaspase-8 at threonine-373

4. PKCK2 regulated TRAIL-mediated apoptosis in cancer cells by phosphorylating procaspase-8.

5. Taken together, PKCK2 activity determines TRAIL sensitivity of various cancer cell lines, and PKCK2 regulates TRAIL-mediated apoptosis in cancer cells by phosphorylating procaspase-8.

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ABSTRACT (in Korean)

암세포에서 PKCK2에 의한 TRAIL 매개 세포사멸 조절

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최 향 태

프로틴 카이네이즈 케이신 카이네이즈 2 (PKCK2)는 serine/threonine 카이네이즈로서 세포주기조절, 세포분화 그리고 세포증식에 중요한 역할들을 한다고 알려져 있다. 세포 내 높은 PKCK2 활성이 procaspase-2를 인산화시킴으로써 암세포가 세포사멸 하는 것을 예방한다는 것이 최근에 보고되었다. PKCK2 활성이 특이적 저해제에 의해 낮아지면 procaspase-2는 활성화되고 활성화된 caspase-2에 의해

procaspase-8이 processing되며 TRAIL저항성 암세포는 TRAIL매개 세포사멸에 준비된 상태가 된다. 이러한 암세포의 TRAIL 저항성을 가지는 PKCK2 의존적인 기전이 일반화 될 수 있는지 알아보기 위하여, 위와 간에서 유래한 17개 암세포주의 PKCK2 활성과 TRAIL 민감도의 연관성을 알아보았다. 17개의 암세포주 중에서 11개 암세포주 (64.7%)는 기존에 보고된 기전에 부합된 반면, 6개 암세포주 (35.3%)는 그렇지 않았다. 5개 암세포주 (MKN-45, MKN-74, SNU484, SNU719, SNU886)는 '저 PKCK2 활성 - 고 TRAIL 민감성'을 보였고, 6개 암세포주 (AGS, MKN-1, MKN-28, Hep3B, SK-HEP-1, SNU739)는 '고 PKCK2 활성 - 저 TRAIL 민감성'을 보였고, 4개 암세포주 (NCI-N87, HepG2, SNU761, SNU878)는 '저 PKCK2 활성 - 저 TRAIL 민감성'을 보였으며 2개 암세포주 (SNU638, SNU668)는 '고 PKCK2 활성 - 고 TRAIL 민감성'을 보였다. '고 PKCK2 활성 - 저 TRAIL 민감성'을 보인 6개 암세포주 (AGS, MKN-1, MKN-28, Hep3B, SK-HEP-1, SNU739)는 PKCK2 활성을 저해함으로써 TRAIL에 감작되었다. 몇 개의 암세포주가 PKCK2 활성도와 TRAIL민감도의 연관성을 가지지 않는 이유를 설명하기 위하여 세포사멸을 유도, 또는 저해한다고 알려진 물질들의 발현 경향을 알아보았다. 몇 개의 암세포들은 세포사멸을 유도하기에는 너무 낮은 수준의 세포사멸 유도 물질을 발현하거나 너무 높은 수준의 세포사멸 저해 물질을 발현하였다. 이 물질들 중에서 procaspase-8은 PKCK2에 의해서 인산화 되었다. PKCK2는 procaspase-8의 threonine-373을 인산화

시키며 그 인산화는 procaspase-8이 활성형 caspase-2에 의해 processing되는 것을 저해하였다.

종합하여, 세포내 PKCK2 활성도는 여러 암세포주의 TRAIL 민감도를 결정하며, PKCK2는 procaspase-8을 인산화 시킴으로써 암세포의 TRAIL 민감도를 조절한다.

핵심되는 말 : TRAIL, 프로틴 카이네이즈 CK2, procaspase-2, procaspase-8, apoptosis, cancer cell lines.