Sensitization of Cancer Cells to TRAIL by Inhibiting Protein Kinase CK2

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Sensitization of Cancer Cell to TRAIL by Inhibiting Protein Kinase CK2

Directed by Professor **Kunhong Kim**

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This certifies that the Master's Thesis
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Hye-Yeon Choi

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ABSTRACT

Sensitization of Cancer Cells to TRAIL by Inhibiting Protein Kinase CK2

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Protein Kinase Casein Kinase 2 (PKCK2) is a serine/threonine kinase that is known to play an important role in cell cycle control, cellular differentiation, and proliferation. We revealed a mechanism by which cancer cells acquire a resistance against TRAIL-mediated apoptosis using six human cancer cell lines. We found that PKCK2 phosphorylates procaspase-2 at serine-157, thereby preventing its dimerization and activation. When PKCK2 activity is down-regulated 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 TRAIL-mediated apoptosis. To confirm whether this newly

discovered mechanism for regulating TRAIL-mediated apoptosis is generally applicable to other cancer cells lines or primary human tumors, we evaluated intracellular PKCK2 activity, procaspase-2 activity and correlated them with TRAIL sensitivity using 31 human cancer cell lines originated from colon, breast, liver, stomach, uterine cervix, brain, lung, esophagus, melanocyte, and blood. Among 31 cancer cell lines, only 6 cancer cell lines were TRAIL-sensitive (19.4%), and all the other TRAIL-resistant cancer cell lines were sensitized to TRAIL by PKCK2 inhibition. PKCK2 activity was high in TRAIL-resistant but low in TRAIL-sensitive cancer cell lines (P=0.0002). Contrary to the PKCK2 activity, caspase2 activity was high in TRAIL-sensitive but low in TRAIL-resistant cancer cell lines (P=0.0002). When we examined TRAIL sensitivity and PKCK2 activity in primary human hematologic malignancy, the results were the same as the results obtained from the cancer cell lines.

Taken together, we confirmed here that intracellular PKCK2 activity is a major determinant for TRAIL sensitivity in cancer cells.

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

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I. Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent inducer of apoptosis of transformed and cancer cells but not of most normal cells or tissues [1,2]. However, one potential obstacle that may limit the clinical efficacy of TRAIL lies in the fact that many cancer cells develop a TRAIL-resistance phenotype [3,4]. Post-translational modifications of pro- or anti-apoptotic molecules like Bid[5], BAD[6,7], or Bcl-2[8], such as their phosphorylation, can change the susceptibility of cancer cells to

TRAIL. Caspases play important roles in the initiation and execution of apoptosis and their activation can also be regulated by phosphorylation. For example, caspase-9 cannot be activated when it is phosphorylated by PKB/Akt at serine-196[9] or by ERK at threonine-125[10], and when caspase-8 and caspase-3 are phosphorylated by p38-mitogen-activated-protein-kinase (MAPK) they become inactivated[11]. Protein kinase casein kinase 2 (CK2) has traditionally been classified 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 [12-14]. It plays a key role in cell cycle control, cellular differentiation, and proliferation [12,15]. Since not much attention has been paid to caspase-2 because caspase-2-deficient mice showed only subtle phenotypes[16,17], little is known about the mechanism by which caspase-2 becomes activated or the role caspase-2 plays in particular apoptotic pathways. Recently, we found that PKCK2 phosphorylates procaspase-2 at serine-157 residue, thereby preventing its dimerization and activation. When PKCK2 is blocked by a specific inhibitor, 5,6–dichloro–1–beta–D-ribofuranosyl benzimidazole (DRB), procaspase-2 is dephosphorylated, dimerized, and activated, and then procaspase-8 is cleaved by 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 became fully activated followed by apoptosis (Fig. 1).

To confirm whether this newly discovered mechanism for regulating TRAIL-mediated apoptosis is generally applicable to other cancer cells lines or primary human

tumors, we evaluated intracellular PKCK2 activity, procaspase-2 activity and correlated them with TRAIL sensitivity.

II. Materials and methods

1. Cell lines and culture conditions.

The human leukemia cell lines (K562, U937, Jurkat, THP-1 and NB4), human colon cancer cell line (DLD-1), human hepatoma cell line (Hep3B), human gastric cancer cell lines (SNU638, SNU668), and human glioblastoma (A172) were cultured at 37°C in RPMI1640. The human esophageal cancer cell lines (TE2, HCE4) and human colon cancer cell line (SW480), human cervical carcinoma cell lines (HeLa, Caski) and human glioblastoma (U87MG) were grown in DMEM. The human breast cancer cell lines (MDAMB231, T47D, MDAMB435), human colon cancer cell line (HT29), human hepatoma cell lines (HepG2 and H4Ⅱ2), human melanoma cell lines (MALME3M, SkMel24, SkMel28), human gastric cancer cell line (AGS), human cervical carcinoma cell line (C33A), human glioblastoma (T98G), and human lung cancer cell lines (A549, NCI-H1299, NCI-H69) were cultured at 37°C in MEM. All the media contains 10% fetal bovine serum, 100 U/ml penicillin and 100 ug/ml streptomycin. The cells were treated for 2 h with 100 ng/ml TRAIL[18] and inhibition of PKCK2 was achieved by incubating the cells for 24 h with 5,6–dichloro–1–beta–D-ribofuranosyl benzimidazole (DRB, 40 μM, Calbiochem, Darmstadt, Germany) [19].

2. Isolation of leukemic cells.

Fifteen adults with *de novo* AML were enrolled in this study. In conjunction

with the institutional review board-approved treatment protocol, bone marrow aspirates were prepared prospectively from the patients before the initiation of chemotherapy. Marrows were sedimented on a Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient. After washing the mononuclear cells collected from the upper interface, T-cell depletion was performed using a high-gradient magnetic cell separation system/anti-CD3 monoclonal antibody (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instruction. A morphological evaluation indicated that >95% of the isolated cells were leukemic blasts [20].

3. Cell viability.

Cells (7×10^3 cells/well) were seeded in the 96-well plate and incubated for 24 h. The cells were treated with the 40 μM DRB for 24 h followed by subsequent incubation with TRAIL (100 ng/ml) for 2 h. For the MTT assay, cells were incubated with 2 mg/ml MTT (Sigma Chemical Co., St. Louis, MO, USA). The cells were further incubated at 37℃ for 3 h. The supernatants were then removed and 100 μl dimethylsulfoxide (DMSO) (Biobasic INC., Toronto, Canada) was added plates were gently shaken and incubated at 37℃ for 10 min. Absorbance (A) was then recorded at 570 nm using SpectraMax ELISA reader (Sunnyvale, CA, USA)[21]. For suspension cells, MTS assay was performed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Midison, WI, USA) according to the manufacturer's

instruction [22].

4. Caspase-2 activity assays.

Caspase-2 activity was evaluated by using a colorimetric assay kit (R & D systems, Minneapolis, MN, USA) [23].

5. PKCK2 kinase activity assay.

The phosphotransferase activity of PKCK2 was measured by using a Casein Kinase-2 Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA). Recombinant full-length human PKCK2 protein that contains an α-subunit N-terminal 6xHis tag and a β-subunit N-terminal GST tag (Upstate Biotechnology, Lake Placid, NY, USA) was used as a positive control [24].

III. Results

1. TRAIL cytotoxicity toward various cancer cell lines.

To generalize our previous observation that PKCK2 activity determines TRAIL sensitivity, we randomly chose 31 cancer cell lines originated from colon, breast, liver, stomach, uterine cervix, brain, lung, esophagus, melanocyte, and blood (Table 1). To examine TRAIL cytotoxicity toward cancer cell lines, cell viability was measured by using MTT or MTS assay. Among 31 cancer cell lines, only 6 cancer cell lines (TE2, SW480, A172, A549, SNU638, and SNU668) were TRAIL-sensitive (19.4 %, Fig. 2). All the TRAIL-resistant cancer cell lines were sensitized to TRAIL by a PKCK2 inhibitor, DRB, pre-treatment (Fig. 2).

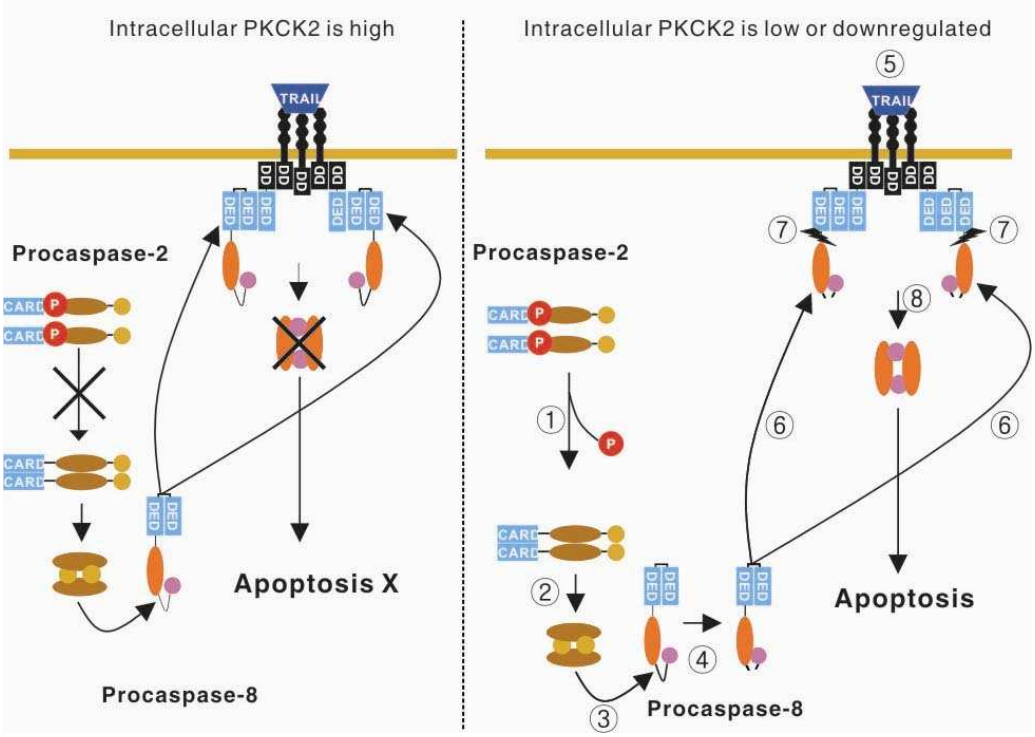


Figure 1. Proposed mechanism of PKCK2 involvement in TRAIL-mediated apoptosis. When PKCK2 is high, TRAIL-mediated apoptosis can not occur (Left). When PKCK2 is low or inhibited, procaspase-2 and -8 is dephosphorylated (①), dephosphorylated procaspase-2 is dimerized and activated (②), activated caspase-2 binds to dephosphorylated procaspase-8 (③), and cleaves the procaspase-8 between the large and small subunits (④). When TRAIL is engaged (⑤), the cleaved procaspase-8 is recruited into DISC (⑥). Once cleaved, further cleavage between the prodomain and large subunit can occur by induced oligomerization (⑦), and procaspase-8 can be fully activated (⑧) , and finally, TRAIL-mediated apoptosis can occur (Right).

Table 1. Cancer cell lines used in the experiment and their TRAIL sensitivity

Origin	Cell line	TRAIL sensitivity*
Colon cancer	SW480	S
	HT29	R
	DLD-1	R
Breast cancer	MDA-MB231	R
	T47D	R
	MDA-MB435	R
Hepatoma	HepG2	R
	H4 II 2	R
	Hep3B	R
Melanoma	MalMe3M	R
	SK-Mel24	R
	SK-Mel28	R
Gastric cancer	AGS	R
	SNU638	S
	SNU668	S
Cervical cancer	HeLa	R
	Caski	R
	C33A	R
Glioblastoma	T98G	R
	U87MG	R
	A172	S
Lung cencer	NCI-H69	R
	A549	S
	NCI-H1299	R
Leukemia	U937	R
	K562	R
	Jurkat	R
	THP-1	R
	NB4	R
Esophageal cancer	TE2	S
	HCE4	R

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

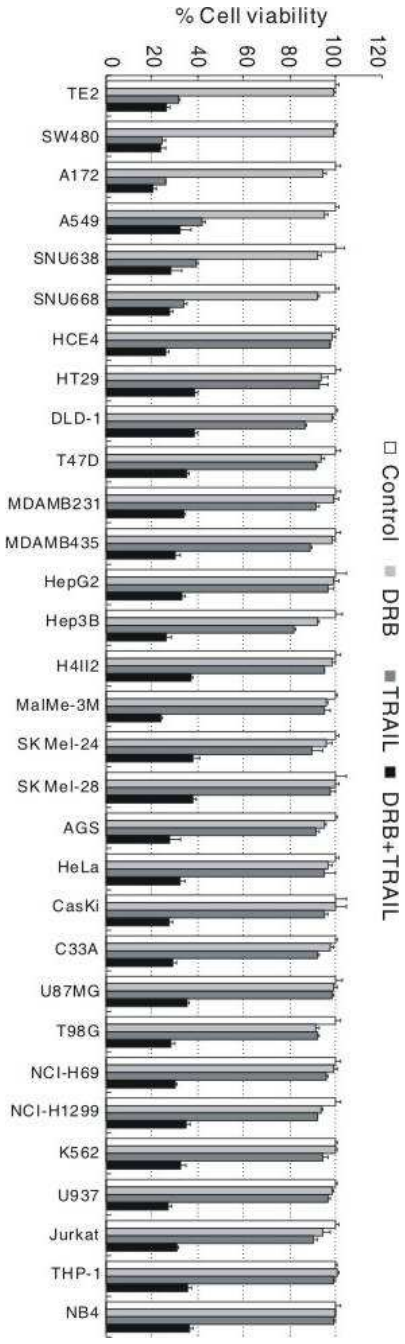
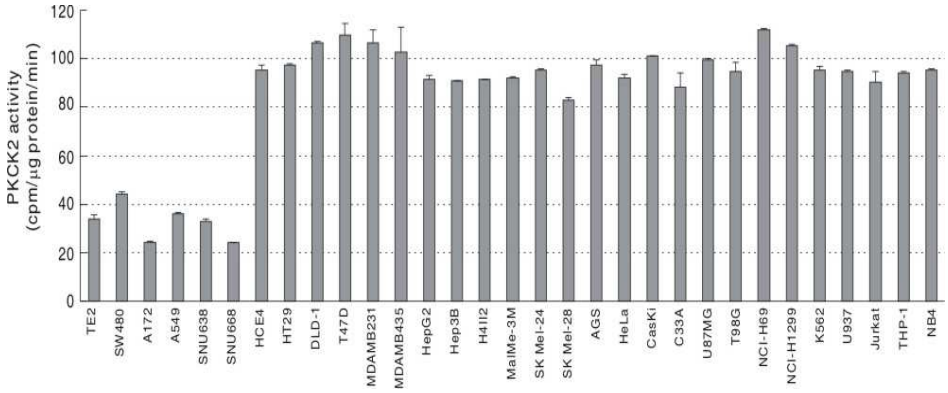

% Cell viability
0
20
40
60
80
100
120
TE2
SW480
A172
A549
SNU638
SNU668
HCE4
HT29
DLD-1
T47D
MDAMB231
MDAMB435
HepG2
Hep3B
H4II2
MalMe-3M
SK Mel-24
SK Mel-28
AGS
HeLa
CasKi
C33A
U87MG
T98G
NCI-H69
NCI-H1299
K562
U937
Jurkat
THP-1
NB4
Control
DRB
TRAIL
DRB+TRAIL

Figure 2. TRAIL cytotoxicity toward various cancer cell lines. Cell viability was measured by the MTT or MTS assay using cancer cells incubated with or without DRB (40 μM) for 24 h, and then subsequently treated with TRAIL for 2 h. The data are expressed as mean ± SD for triplicate, and similar results were obtained from two independent experiments.

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

Then, we checked intracellular PKCK2 activity of cancer cell lines. TRAIL-sensitive cancer cell lines showed low intracellular PKCK2 activity but TRAIL-resistant cancer cell lines showed high PKCK2 activity (Fig. 3, A and B).

A.



B.

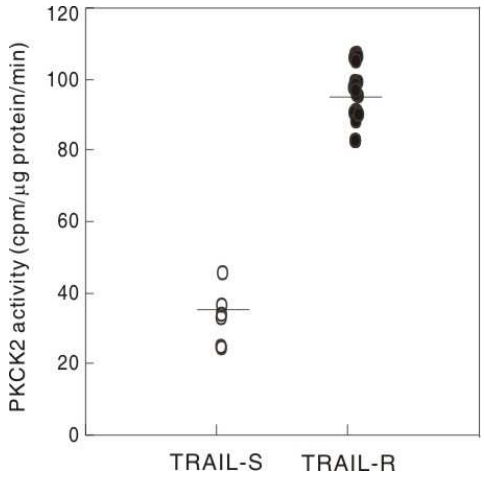


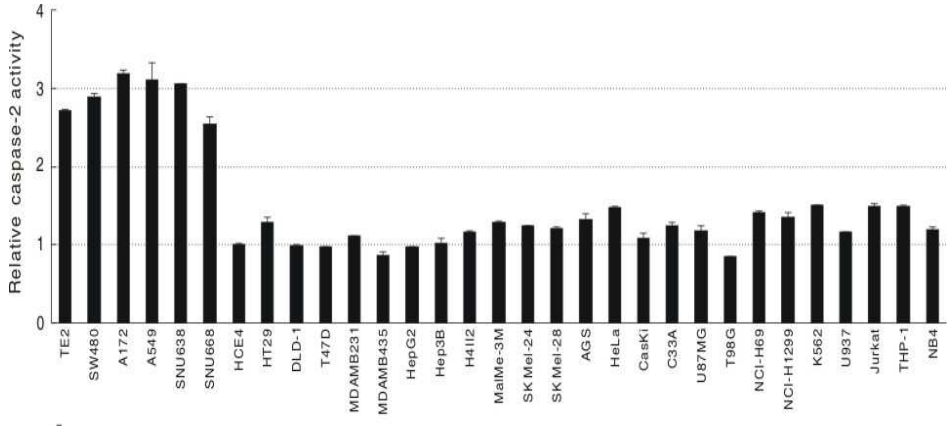
Figure 3. Correlation between TRAIL sensitivity and their intracellular PKCK2 activity. A. PKCK2 activity in various cancer cell lines. The phosphotransferase activity of PKCK2 was measured by using a Casein Kinase-2 Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA). The data are expressed mean ± SD for duplicate, and similar results were obtained from two independent experiments.

B. PKCK2 activity and TRAIL sensitivity. TRAIL-sensitive cancer cell lines (n=6) have low PKCK2 activity and TRAIL-resistant cancer cell lines (n=25) have high PKCK2 activity ($p=0.0002$, Wilcoxon rank sum test). The line represents the mean value.

3. Correlation between intracellular PKCK2 activity and caspase-2 activity.

As we previously demonstrated that PKCK2 activity regulates procaspase-2 activity by phosphorylating at serine-157, we then examined caspase-2 activity of cancer cell lines. As we expected, endogenous caspase-2 activity was high in TRAIL-sensitive cancer cell lines that have low PKCK2 activity, but low in TRAIL-resistant cancer cell lines that have high PKCK2 activity (Fig. 4, A and B).

A.



B.

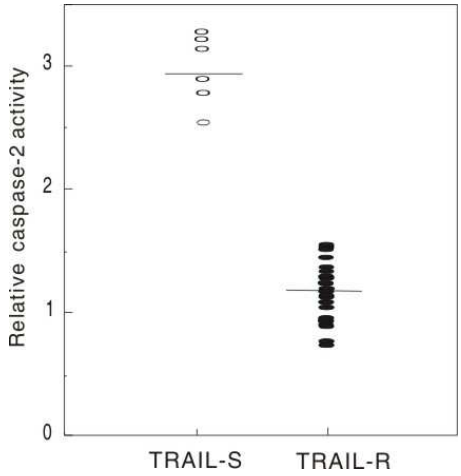


Figure 4. Correlation between intracellular PKCK2 activity and caspase-2 activity.

A. Caspase-2 activity. Caspase-2 activity was measured using colorimetric assay kits (R & D systems, Minneapolis, MN, USA). The data are expressed mean ± SD for duplicate, and similar results were obtained from two independent experiments. B.

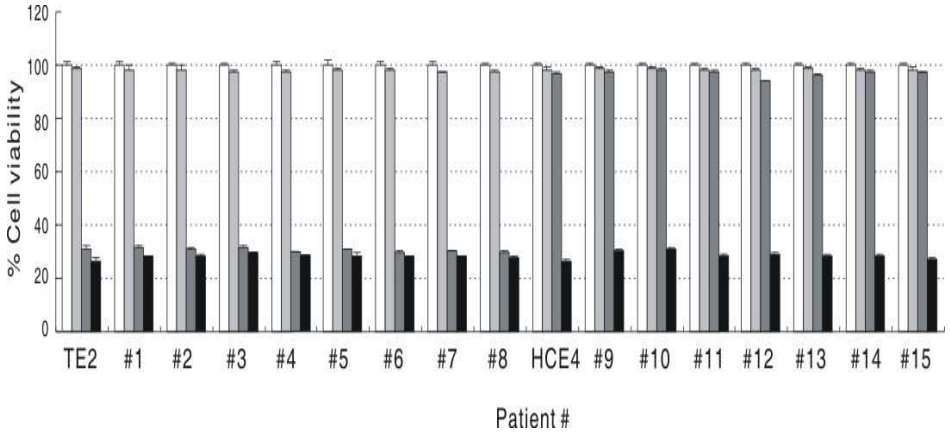
Caspase-2 activity and TRAIL sensitivity. TRAIL-sensitive cancer cell lines (n=6) have high caspase-2 activity and TRAIL-resistant cancer cell lines (n=25) have low caspase-2 activity ($p=0.0002$, Wilcoxon rank sum test). The line represents the mean value.

4. Correlation between TRAIL sensitivity of primary hematologic malignancy and their intracellular PKCK2 activity.

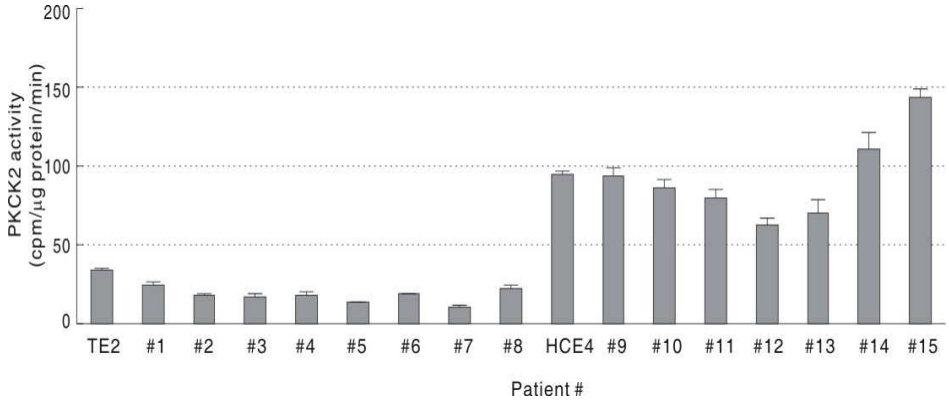
To examine whether PKCK2 also determines TRAIL sensitivity in primary human hematologic malignancy, leukemia blasts were isolated from 15 patients diagnosed as acute myelogenous leukemia (AML). Among 15 cases, 8 cases were TRAIL-sensitive (53.3%). All the TRAIL-resistant cases became sensitized to TRAIL by DRB pre-treatment (Fig. 5A). Then, we evaluated intracellular PKCK2 activity of 15 cases to see whether there is a correlation between TRAIL sensitivity and PKCK2 activity. As expected, all the TRAIL-sensitive cases had low PKCK2 activity but all the TRAIL-resistant cases had high PKCK2 activity (Fig. 5B)

A

.


120
100
80
60
40
20
0
% Cell viability
TE2
#1
#2
#3
#4
#5
#6
#7
#8
HCE4
#9
#10
#11
#12
#13
#14
#15
Patient #

B.


200
150
100
50
0
PKCK2 activity
(cpm/μg protein/min)
TE2
#1
#2
#3
#4
#5
#6
#7
#8
HCE4
#9
#10
#11
#12
#13
#14
#15
Patient #

C.

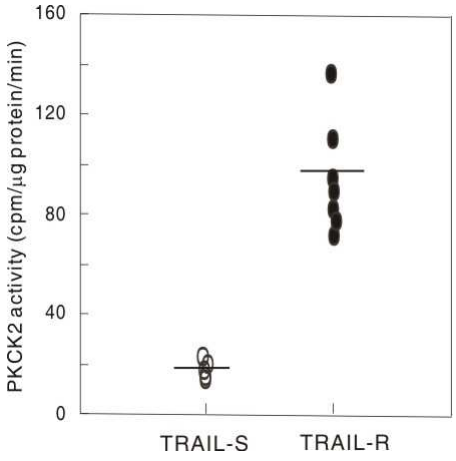


Figure 5. Correlation between TRAIL sensitivity of primary hematologic malignancy and their intracellular PKCK2 activity. A. TRAIL cytotoxicity toward primary hematologic malignancy. Cell viability was measured by the MTS assay using cancer cells isolated from 15 AML patients incubated with or without DRB (40 μM) for 24 h and then subsequently treated with TRAIL for 2 h. The data are expressed as mean ± SD for triplicate, and similar results were obtained from two independent experiments. B. PKCK2 activity in hematologic malignancy. TE2 and HCE4 were used as control for TRAIL-sensitive and TRAIL-resistant cancer cell lines, respectively. C. PKCK2 activity and TRAIL sensitivity. TRAIL-sensitive leukemia (n=8) have low PKCK2 activity and TRAIL-resistant leukemia (n=7) has high PKCK2 activity (p=0.0001, Wilcoxon rank sum test). The line represents the mean value.

VI. Discussion

Recently, we revealed a mechanism by which cancer cells acquire a resistance against TRAIL-mediated apoptosis. We found that PKCK2 phosphorylates procaspase-2 at serine-157, thereby preventing its dimerization and activation. When PKCK2 is inhibited by a specific inhibitor (DRB), 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 confirm whether this newly discovered mechanism for regulating TRAIL-mediated apoptosis is generally applicable to cancer cells, we examined intracellular PKCK2 activity, procaspase-2 activity and correlated them with TRAIL sensitivity of each cancer cell line. We tested 31 human cancer cell lines originated from colon, breast, liver, stomach, uterine cervix, brain, lung, esophagus, melanocyte, blood and tested 15 primary human hematologic tumors. Among 31 cancer cell lines, only 6 cancer cell lines were TRAIL-sensitive (19.4 %), and among 15 cases of primary tumors, 8 cases were TRAIL-sensitive (53.3%). All the TRAIL-resistant cancer cell lines and primary hematologic tumors were sensitized to TRAIL by PKCK2 inhibition. PKCK2 activity was high in TRAIL-resistant but low in TRAIL-sensitive cancer cell lines and primary tumors. These results confirm that intracellular PKCK2 activity is a major determinant for TRAIL sensitivity in cancer cells. However, this mechanism may not be applicable to 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 [25, 26], a homozygous deletion [27], mutations [28], or polymorphism [3, 28] found in the death domain region of DR4, inactivation of caspase-8 by DNA methylation or gene deletion [29-31].

Here we show that PKCK2 activity determines TRAIL sensitivity of cancer cells. Targeting PKCK2 for inhibiting its activity may widen the therapeutic window of TRAIL in cancer therapy.

V. Conclusion

1. PKCK2 inhibition sensitized TRAIL-resistant cancer cell lines to TRAIL.
2. PKCK2 activity was low in the TRAIL-sensitive cancer cells but high in the TRAIL-resistant cancer cells.
3. In contrast, caspase-2 activity was high in the TRAIL-sensitive but low in the TRAIL-resistant cancer cells.
4. The TRAIL-resistance phenotype of primary hematologic malignancy was also attributed from high intracellular PKCK2 activity.

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ABSTRACT (in Korean)

프로테인 카이네이즈 CK2활성 억제를 통한 암세포의 TRAIL에 대한 감작

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프로테인 카이네이즈 CK2 는 ser/thr 프로테인 카이네이즈로 세포주기 조절, 세포 분화 및 성장에 중요한 역할을 하는 효소이다. 본 실험실에서는 6 개의 암세포를 이용하여 암세포의 TRAIL 저항성에 기전을 밝혔다. 암세포 내 PKCK2 의 활성은 procaspase-2 의 serine 157 부위를 인산화하여 이분체 형성을 억제함으로써 활성형의 caspase-2 생성을 억제함을 확인하였고, caspase-2 가 활성화 되었을 때 procaspase-8 의 large subunit 과 small subunit 사이를 절단됨을 확인하였으며, 이러한 상태의 암세포는 TRAIL 매개 세포 사멸 과정이 일어날수 있도록 준비된 상태로 존재하다가 세포 사멸 수용체에 TRAIL 이 결합하였을 때 절단된 procaspase-8 이 수용체에 모집되면 prodomain 과 large subunit 사이가 또다시 절단됨으로써 활성형의 caspase-8 이 형성되고 따라서 세포 사멸이 유발된다는 것을 밝혔다. 본 연구에서는 대장, 유방, 간, 위, 자궁, 뇌, 폐,

식도, 멜라닌세포, 혈액으로부터 기원한 31 종의 암세포주와 급성 백혈구성 백혈병으로 진단된 환자로부터 채취한 암세포를 이용하여 이전 연구의 결과가 재현성을 가지는 지를 확인하고 TRAIL 저항성의 기전으로서 일반화할 수 있는 지 여부를 확인하고자 하였다. 31 개의 암세포주들의 TRAIL 내성을 확인한 결과 6 개의 암세포만이 TRAIL 에 민감성을 나타내었으며 (19.4%) TRAIL 내성을 보이는 모든 암세포주들은 PKCK2 의 억제제와 TRAIL 병합처리 시 감작되어 세포사멸이 유발됨을 확인할 수 있었다. 각 암세포주들의 세포 내 PKCK2 활성을 측정한 결과 TRAIL 내성 암세포주들은 PKCK2 활성이 높았고 TRAIL 민감성 암세포주들은 낮은 PKCK2 활성을 가짐을 확인할 수 있었다 (P=0.0002). PKCK2 의 활성과는 반대로 TRAIL 내성 암세포 주들은 caspase-2 활성이 높았으며, TRAIL 민감성 암세포주들은 caspase-2 활성이 낮다는 것을 확인하였다 (P=0.0002). 또한 일차 혈액암세포를 이용한 실험에서도 TRAIL 민감도가 암세포 내 PKCK2 의 활성에 따라 결정된다는 것을 확인할 수 있었다. 본 연구 결과는 암세포 내 세포 사멸 관련 물질의 유전적 또는 에피제네틱한 변화가 유발되지 않은 경우 암세포의 TRAIL 내성은 암세포 내 PKCK2 활성을 억제함으로써 극복될 수 있으며, 따라서 암세포 내 PKCK2 활성을 조절할 수 있는 약물의 발굴을 통해서 TRAIL 내성 암세포의 세포 사멸 유발을 통해 암 치료 효과를 획기적으로 증대시킬 수 있을 것이라고 판단된다.

핵심되는 말: TRAIL, 프로테인 카이네이즈 CK2 (PKCK2), procaspase-2, 세포 사멸, 암세포