Role of parkin as a tumor suppressor protein in cervical cancer cells resistant to tumor necrosis factor-alpha

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ABBREVIATIONS

APC **: Adenomatous polyposis coli**

TNF-α : Tumor necrosis factor-alpha

M.O.I. : Multiplicity of infection

PBS : Phosphate-buffered saline

SDS : Sodium dodecyl sulfate

DAPI : 4',6-diamidino-2-phenylindole

BAK1 : Bcl-2 homologous antagonist killer

Bcl-xL : B-cell lymphoma-extra large

Bax : Bcl-2-associated X protein

MCL1 : Myeloid leukemia cell differentiation protein

GAPDH : Glyceraldehyde 3-phosphate dehydrogenase

PARP : Poly ADP-ribose polymerase

PKC : Protein kinase C

MAPK : Mitogen-activated protein (MAP) kinases

NF-κB : Nuclear factor kappa-light-chain-enhancer of activated B cells

PI3-K : Phosphatidylinositol 3-kinases

p38 MAPK : p38 mitogen-activated protein kinases

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ABSTRACT

Parkin is a putative tumor suppressor protein. Various types of cancers exhibit reduced expression of parkin or mutation in the parkin gene (*PARK2*). Many malignant tumors become resistant to tumor necrosis factor-alpha (TNF-α) during carcinogenesis, thus can escape from TNF-α-induced cell death. In the present study, I examined whether parkin acts as a tumor suppressor in HeLa cells, a human cervical cancer cell line resistant to TNF-α-induced cell death. TNF-α-treatment alone did not affect HeLa cell viability. However, expression of parkin restored TNF-α-induced apoptosis in HeLa cells. Increased cell death was due to activation of the apoptotic pathway. Expression of parkin in TNF-α-treated HeLa cells stimulated cleavage of the pro-apoptotic proteins caspase-8, -9, -3, -7 and poly ADP ribose polymerase (PARP). In addition, parkin expression resulted in decreased expression of the caspase inhibitory protein, survivin and the positive regulator of survivin, β-catenin. Inhibition of the PKC pathway resulted in restoration of β-catenin and survivin levels. These results suggest that parkin acts as a tumor suppressor through the PKC─β-catenin─survivin─caspase pathway. I propose that this pathway is a novel molecular mechanism by which parkin functions as a tumor suppressor.

Key Words: Parkin, tumor suppressor, TNF-α, cervical cancer, apoptosis

Ⅰ. Introduction

Parkin protein (parkin) was originally implicated in Parkinson's disease.[1] Parkin gene (*PARK2*) is located on chromosome 6q25.2-6q27 and loss of heterozygosity (LOH) in 6q26 was reported in ovarian, breast, hepatocellular and squamous cell lung cancers.[2-4] Since these reports, association between cancer and genetic mutations in parkin gene (*PARK2*) has been an active area of research. Deletion in *PARK2* gene was reported in ovarian tumor cells.[5] In addition, in a variety of cancers including brain, liver, colorectal, ovarian, cervical, pancreas, kidney and breast cancers alternative transcripts were found due to gene deletion and duplication in *PARK2*.[6-10] In acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and some colorectal cancer cells, *PARK2* gene was abnormally methylated and its expression was decreased.[10, 11] Recent reports showed that decrease in parkin expression has an essential role in tumorigenesis suggesting that parkin is a putative tumor suppressor. Consistent with this notion, overexpression of parkin in hepatocarcinoma, glioblastoma, lung cancer, breast cancer and colon cancer cell lines repressed cell growth.[7-10, 12] These studies attracted interest in studying mechanisms by which parkin acts as a tumor suppressor. In breast cancer, parkin stabilizes microtubules and increases susceptibility to anti-cancer agents. In the breast cancer cell line MCF7, parkin reduces cell growth by inducing expression of cyclin-dependent kinase 6 (CDK6).[8] In glioblastoma and other cancers, parkin overexpression results in degradation of ubiquitin-mediated cyclin E and subsequent cell cycle arrest.[9] Simultaneous mutation in both *PARK2* and *APC* gene dramatically accelerates colorectal carcinogenesis.[10] The mechanisms by which parkin suppresses tumorigenesis

are being elucidated but many questions remain unanswered. Moreover, parkin seems to exert different effects in different types of cancers.

Immune cells secrete various cytokines in response to cancers.[13] As the cancer develops, they become resistant to anti-tumor cytokines such as tumor necrosis factor-alpha (TNF-α).[14] TNF-α is a pro-inflammatory cytokine secreted by macrophages which has a suppressive effect on tumors.[15] However, the tumor can become TNF-α resistant as the tumor develops.[16, 17] Almost all malignant tumors are TNF-α resistant.[18] In some reports, TNF-α enhanced tumor proliferation and metastasis.[18, 19] For these reasons, many malignant tumors can escape from TNF-α-induced cell death.[16]

Cervical cancer is one of the most common cancers among women worldwide, accounting for 9% (529,800) of total new cancer cases and the fourth leading cause of cancer death in females accounting for 8% (275,100) of the total cancer deaths among females in 2008.[20] LOH in *PARK2* gene was reported in primary tumor samples from patients with cervical cancer.[21] The HeLa cell, a human cervical cancer cell line, also harbors a *PARK2* gene deletion[6] and is resistant to TNF-α induced cell death.[22]

Apoptosis, the process of programmed cell death, is one of the regressions induced by TNF-α.[17] Insufficient apoptosis causes uncontrolled cell proliferation resulting in cancer development and progression. The binding of TNF-α to tumor necrosis factor receptor 1 (TNFR1) initiates the apoptosis pathway that leads to caspase activation via the intermediate membrane proteins TNFR-associated death domain and Fas-associated death domain protein.[23] Various molecules are related to this pathway. In particular, caspases play a central role in the transduction of apoptotic signals. Initiator caspases (caspase-8 and -9) and effector caspases (caspase-3 and -7) are prominent apoptotic signaling molecules involved in this signal transduction.[24] Effector

caspases are cleaved and activated by the activated initiator caspases. Cleaved effector caspases inactivate poly ADP ribose polymerase (PARP).[25] PARP is involved in DNA repair[26] and programmed cell death.[27] PARP is inactivated by cleaved caspases during programmed cell death.[28]

Moreover, there are several molecules involved in the regulation of apoptosis such as the pro-apoptotic molecules (BAK1, Bax, etc) and the anti-apoptotic proteins (Bcl-xL, MCL1, Bcl-2, survivin, etc).[29] In particular, survivin is a member of the inhibitors of apoptosis (IAP) family. Survivin protein inhibits caspases activation thereby leading to negative regulation of apoptosis.[30] Survivin binds to only active caspase-3 and -7 and it is likely here that survivin inhibits the active forms of the caspases. Thus, survivin acts as an anti-apoptotic protein possibly by preventing cleavage of caspases and resulting in decreased apoptosis.[31] Recent studies have shown that disruption of the survivin induction pathways leads to increase in apoptosis and decreased tumor growth. In recent years survivin has been a target of attention for cancer immunotherapy.[32]

Survivin is a direct target gene of the Wnt pathway and is upregulated by β-catenin.[33] Wnt/β-catenin signaling is associated with the development and progression of multiple cancers.[34] β-catenin is a subunit of the cadherin protein complex and has been implicated as an integral component in the Wnt signaling pathway.[35] Activation of this pathway is linked to accumulation of β-catenin in the cell followed by altered expression of genes which are the known to contribute to cancer progression including survivin.[36] In addition, a recent report demonstrated that expression of parkin reduces steady state levels of β-catenin protein.[37]

I hypothesize that expression of parkin affects β-catenin expression and subsequent disruption of Wnt/β-catenin signaling causes downregulation of the anti-apoptotic protein survivin resulting in apoptotic death of cancer cells.

Accordingly, in this study, I investigated the role of parkin expression in TNF-α-induced-apoptosis in HeLa cells which are naturally resistant to TNF-α. I found that parkin expression induces downregulation of β-catenin and survivin. This downregulation is mediated by the PKC signaling pathway. Parkin expression restores susceptibility to TNF-α-induced cell death and that this process is mediated by decreased expression of survivin and activation of caspase-8, -9, -3, -7, and PARP.

Ⅱ. MATERIALS AND METHODS

1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin- streptomycin, trypsin-EDTA, and trypan blue stain solution were obtained from Gibco BRL (Grand Island, NY, USA). Recombinant adenoviral vector including parkin gene (Parkin) was produced as previously described.[38] Recombinant human TNF-α was purchased from R&D System (Minneapolis, MN, USA). Propidium iodide was purchased from BD Biosciences (San Jose, CA, USA). Trizol reagent, random hexamer, and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) were purchased from Invitrogen (Grand Island, NY, USA). Ro 31-7549, PD 98059, Ly 294002, SB 203580, and GW 5074 were purchased from Calbiochem (Darmstadt, Germany). BAY 11-7085 was purchased from Enzo life science (New York, NY, USA). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies specific for PARP, cleaved forms of caspase-3, -7 -8, and -9 and β-catenin were purchased from Cell Signaling technology (Danvers, MA, USA). Antibodies specific for parkin and β-actin were purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA).

2. Cell culture and viral infection with parkin-expressing adenovirus

HeLa (human cervical adenocarcinoma cells, ATCC) cells were grown in DMEM supplemented with 10% FBS and penicillin-streptomycin and maintained at 37℃ in humidified atmosphere with 5% CO_2. Cells were seeded in 6-well plates at a density of 2×10^5/well. After 24 h, Cells were infected with 150 multiplicity of infection (M.O.I.) of parkin-expressing adenovirus (Parkin) or mock adenovirus (Mock) for 24 h. For dose- dependent experiments, cells were infected with 0, 19, 38, 75, and 150 M.O.I. of Parkin. Various concentrations of Parkin were mixed with Mock and normalized for a total of 150 M.O.I. per infection.

3. Trypan blue exclusion assay

Cells (2×10^5/well) were seeded in 6-well culture plates, cultured for 24 h and then infected with Parkin or Mock for an additional 24 h. The cells were then treated with TNF-α (5 ng/ml) for 24 h and the cells trypsinized and stained with trypan blue dye solution. Viable cells (i.e., trypan blue dye negative) were counted on a hemocytometer (Marienfeld, Lauda-Königshofen, Germany) under a microscope.

4. RNA extraction and reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from cultured cells using Trizol reagent according to the manufacturer's instructions. cDNA was synthesized by reverse transcription with 2 μg of total RNA, 0.25 μg of random hexamer, and 200 unit of MMLV-RT for 50 min at 37℃ and 15 min at 70℃. Subsequent PCR amplification using 0.2 units of Taq polymerase (Cosmogenetech, Seoul, Korea) was performed in a thermocycler using specific primers. The primers used and the PCR conditions are listed in Table 1. GAPDH was amplified as an internal control. PCR products were electrophoresed on 1.5% (w/v) agarose gels containing 0.4% μg/ml ethidium bromide and the size of the PCR products compared with a 100 bp DNA ladder (Bioprince, Atlanta, GA, USA). Gel images were taken using Gel Doc (Bio-Rad, Hercules, CA, USA).

Table 1. Summary of PCR primers used for analyses of mRNA expression

Primers	Sequences (5' - 3')		Product length (bp)	Annealing temperature (℃)	Cycles
	Forward	Reverse			
BAK1	GCCCAGGACACAGAGGAGGTT	AAACTGGCCCAACAGAACCACACC	527	60	40
Bcl-xL	TTGGACAATGGACGGTTGA	GTAGAGTGGATGGTCATGG	575	60	30
Bax	AAGAAGCTGAGCGAGTGTC	CGGCCCCAGTTGAAGTTGC	158	60	35
MCL1	TGGGTTTGTGGAGTTCTTCCA	CTCCTCCATAGCTTCCCAAAC	474	55	30
Bcl-2	CATTTCCACGTCAACAGAATTG	AGCACAGGATTGGATATTCCAT	505	55	30
Survivin	AGCCCTTTCTCAAGGACCAC	GCACTTTCTTCGCAGTTTCC	363	60	24
β-catenin	TTGAAGTATACCATACAACTG	GCAGCATCAAACTGTGTAGAT	393	55	30
GAPDH	CGGGAAGCTTGTCATCAATGG	GGCAGTGATGGCATGGACTG	357	55	24

5. DAPI staining

Cells (2×10^5/well) were seeded onto cover glass slides (24 mm × 24 mm) inside 6-well culture plates and cultured for 24 h. The cells were infected with Parkin or Mock for an additional 24 h and then treated with TNF-α (5 ng/ml) for 24 h. The culture medium was aspirated and the cover glass containing adherent cells was washed with PBS twice. Cells were then fixed with 2% paraformaldehyde for 5 min. After fixation, the cover glass was washed with PBS twice and then permeabilized with 0.1% Triton X-100 for 5 min. Slides were washed with PBS and incubated with DAPI stain solution (Vector lab, Burlingame, CA, USA) in the dark for 10 min. The cover slide was placed on a microscope slide and the DAPI stained cells observed under a fluorescence microscope (Olympus, Tokyo, Japan) at 430 nm.

6. Cell cycle analysis by flow cytometry

HeLa cells seeded in 6-well plates (2×10^5 cells/well) and cultured in 10% FBS DMEM for 24 h. Cells were then infected with Parkin or Mock for 24 h and then treated with TNF-α (5 ng/ml) for an additional 24 h. Thereafter, the cells were trypsinized into single cell suspension and then fixed with 70% ethanol in PBS at 4℃ for 2 h. The fixed cells were stained with PBS solution containing propidium iodide (2.5 mg/ml) and RNase (0.1 mg/ml) (ELPIS biotech, Taejeon, Korea) at 37℃ for 40 min. The cells were washed to remove excess propidium iodide and the cell suspension analyzed for DNA content using FACS Calibur (BD Biosciences, San Jose, CA, USA).

7. Western blot analysis

Cells were lysed with a PBS buffer containing 1% Triton X-100 and protease inhibitor cocktail and then centrifuged at 19,000*g* for 10 min at 4℃. The supernatant was collected and the total protein concentration quantified using the Lowry protein assay (Bio-Rad, Hercules, CA, USA). Protein samples (15 μg per lane for β-actin, parkin, and PARP; 50 μg per lane for cleaved caspase-3, -7, -8, and -9) were mixed with loading buffer and the proteins separated by SDS-polyacrylamide gel electrophoresis (10% SDS-PAGE for detection of β-actin, parkin, β-catenin and PARP; 12% SDS-PAGE for detection of cleaved caspases). The proteins were then transferred to nitrocellulose membrane and blocked with TBST containing 5% (w/v) skim milk for 30 min. The nitrocellulose membrane was then incubated with primary antibody (see Table 2) at 4℃ overnight and then with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit). The immunolabeled proteins were visualized using ECL (Thermo, Waltham, MA, USA) and exposed onto X-ray film (Agpa, Mortsel, Belgium). β-actin was used as an internal control.

Table 2. Summary of primary antibodies used for analyses of western blot

Primary Ab	Detected size (kDa)	Primary Ab	Detected size (kDa)
Parkin	50	Cleaved caspase-3	17, 19
PARP	Full length : 116 Cleaved form : 89	Cleaved caspase-7	20
Beta-catenin	92	Cleaved caspase-8	18
Beta-actin	43	Cleaved caspase-9	37

Ⅲ. RESULTS

1. Expression of parkin restores susceptibility to TNF-α-induced HeLa cell death.

The human cervical cancer cell line, HeLa, is resistant to TNF-α-induced cell death.[22] To examine whether parkin influences susceptibility of HeLa cells to TNF-α, HeLa cells were infected with mock adenovirus (Mock) or parkin-expressing adenovirus (Parkin) and then treated with TNF-α. TNF-α treatment alone did not affect HeLa cell viability (Figure 1A left image and Figure 1B white bar). However, parkin expression reduced viability of TNF-α-treated HeLa cells (Figure 1A right image and Figure 1B black bar) and reduction of cell viability by parkin expression was also TNF-α-dose-dependent (Figure 1B black bar). Expression of parkin resulted in decreased viability of TNF-α-treated HeLa cells in a parkin-dose-dependent manner (Figure 1C). These results show that expression of parkin in HeLa cells results in TNF-α-induced reduction of viability in HeLa cells.

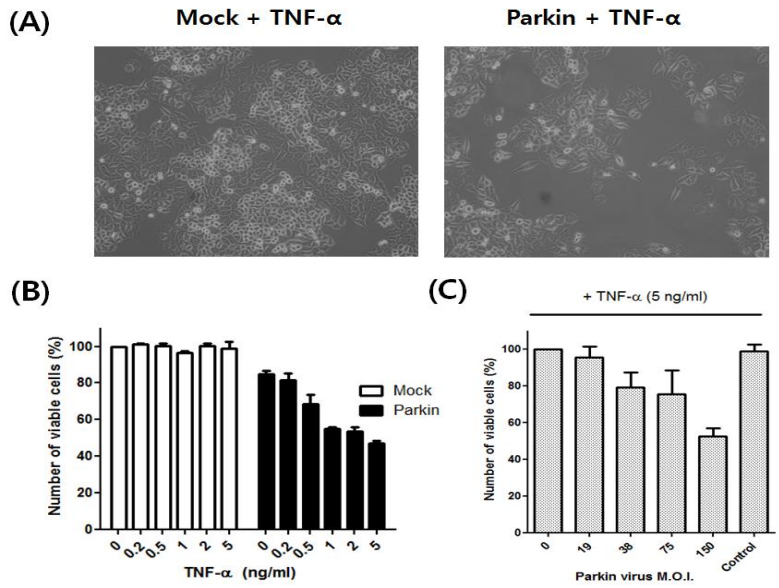

Figure 1. Expression of parkin restores susceptibility to TNF-α-induced HeLa cell death. **(A)** Cells were infected with either parkin-expressing adenovirus (Parkin) or mock adenovirus (Mock) (150 M.O.I. respectively) for 24 h and treated with 5 ng/ml TNF-α for 24 h. Images were captured using an inverted microscope (200X). **(B)** Cells were infected with either Mock or Parkin (150 M.O.I. respectively) for 24 h and then treated with TNF-α (0, 0.2, 0.5, 1, 2, 5 ng/ml) for 24 h. Viable cells were counted by trypan blue exclusion assay (3 independent experiments). Number of viable cells in 150 M.O.I. Mock-infected group without TNF-α treatment was set as 100%. **(C)** Cells were infected with indicated concentrations (0, 19, 38, 75, 150 M.O.I.) of Parkin for 24 h and then treated with 5 ng/ml TNF-α for 24 h. Viable cells were counted by trypan blue exclusion assay (3 independent experiments). Number of viable cells in 0 M.O.I. Parkin-infected group was set as 100%.

2. Parkin expression results in TNF-α-induced apoptotic cell death.

To examine whether the mechanism by which parkin increases TNF-α-induced cell death is due to apoptosis, cells were infected with either Parkin or Mock and cultured in the absence or presence of TNF-α. Then, cell cycle analysis was performed by flow cytometry. The percentage of cells in the sub-G1 phase increased when parkin was expressed in TNF-α-treated HeLa cells (Figure 2). In addition, apoptotic bodies were observed when cells were infected with Parkin and then treated with TNF-α (Figure 3). Increase of cells in the sub-G1 phase and the presence of apoptotic bodies imply that parkin induces cell death in TNF-α-treated HeLa cells via the apoptotic pathway.

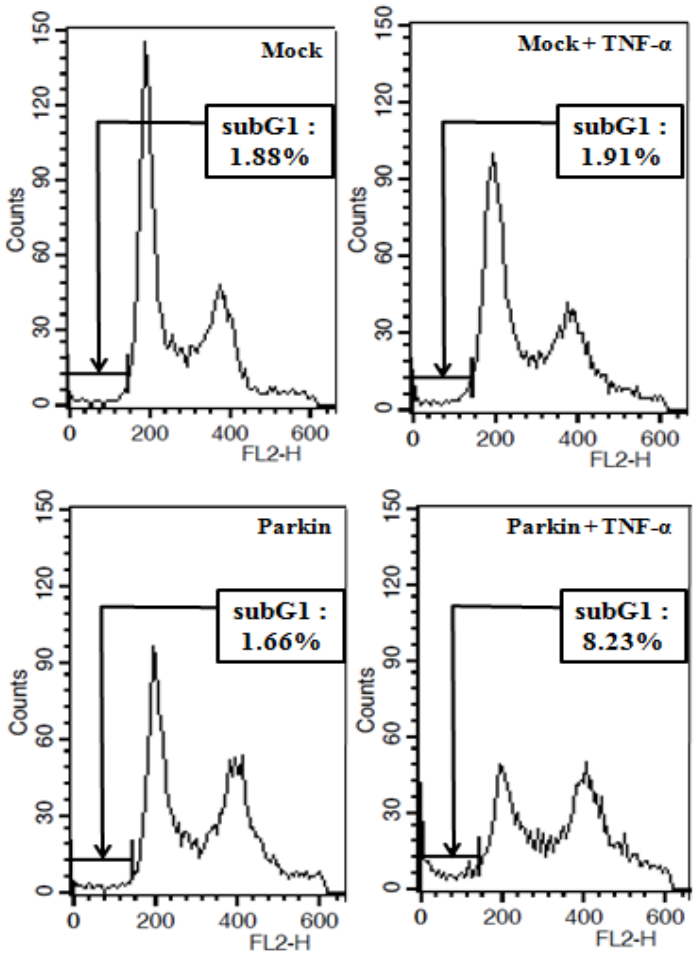

Figure 2. Combination of parkin expression and TNF-α treatment induces increase in cells at the subG1 phase. HeLa cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h and then cultured in the absence or presence of TNF-α (5 ng/ml) for 24 h. Cell cycle analysis was performed using propidium iodide staining and flow cytometry.

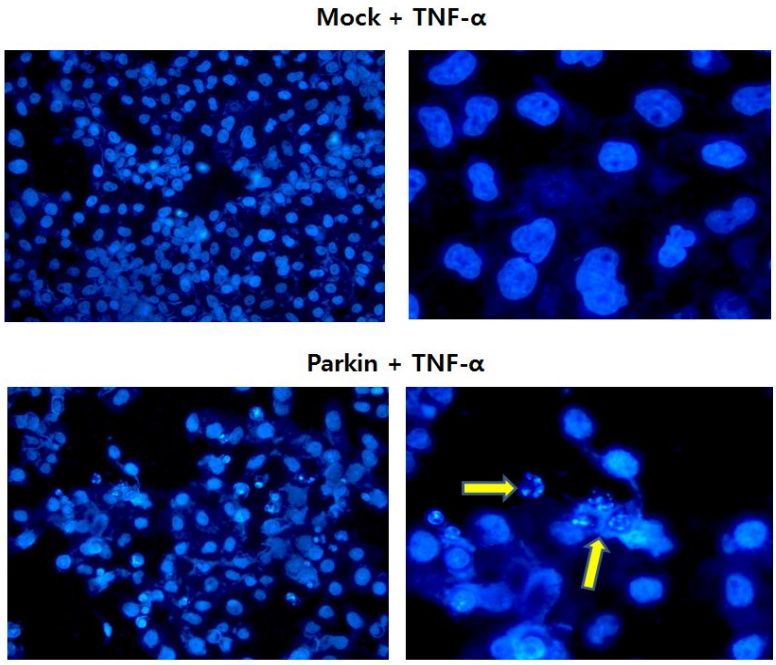

Figure 3. Apoptotic bodies are observed in parkin expressed and TNF-α treated HeLa cells. HeLa cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h and treated with TNF-α (5 ng/ml) for 24 h. Cells were stained with DAPI and images captured using fluorescence microscopy (430 nm). Apoptotic bodies (arrow).

3. Parkin expression in TNF-α-treated HeLa cells stimulates cleavage of caspases and PARP.

Parkin induces cell death in TNF-α-treated HeLa cells via the apoptotic pathway. Therefore, I investigated which apoptotic molecules were affected by parkin expression in TNF-α-induced HeLa cells. First, we examined whether cleavage of PARP, a hallmark of apoptosis, was stimulated by parkin expression in TNF-α-treated HeLa cells. I found that expression of parkin induced TNF-α-mediated cleavage of PARP in a TNF-α-dose-dependent manner (Figure 4A). In addition, parkin expression enhanced cleavage of PARP in a parkin-dose-dependent manner (Figure 4B). Next, we determined whether caspase-3 and/or caspase-7, upstream effector molecules of PARP, are activated by parkin in TNF-α-treated HeLa cells. Parkin expression resulted in activation of both caspase-3 and caspase-7 in a TNF-α-dose-dependent manner (Figure 5A). In addition, parkin expression resulted in TNF-α-mediated activation of caspase-3 and -7 in a parkin-dose-dependent manner (Figure 5B). Caspase-8 and -9 are upstream molecules of caspase-3 and -7. Therefore, I examined the activation of caspase-8 and caspase-9 and found that parkin expression induced cleavage of both caspase-8 and -9 in TNF-α-treated HeLa cells (Figure 6). Caspase-8 and -9 are known to be involved in the mitochondria-independent and mitochondria-dependent apoptotic pathway, respectively. Therefore, these results show that parkin induces apoptotic cell death in TNF-α-treated HeLa cells via activation of both the mitochondria-dependent and mitochondria–independent pathway.

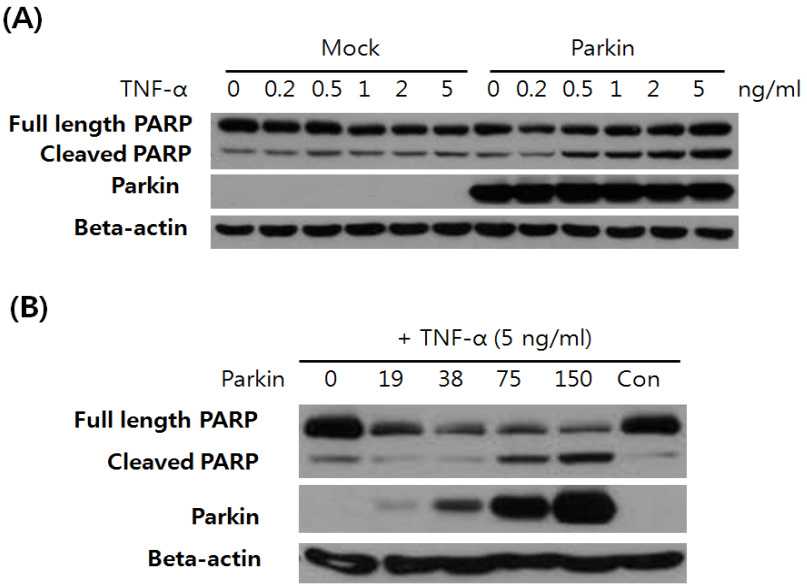

Figure 4. Parkin expression in TNF-α-treated HeLa cells stimulates cleavage of PARP. **(A)** Cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h and then treated with TNF-α (0, 0.2, 0.5, 1, 2, 5 ng/ml) for 24 h. **(B)** Cells were infected with indicated concentrations (0, 19, 38, 75, 150 M.O.I.) of Parkin for 24 h and then treated with TNF-α (5 ng/ml) for 24 h. Cell extracts were separated on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Cleavage of PARP was detected by Western blotting. Expression of parkin was determined by Western blot using anti-parkin antibodies. Levels of β-actin were analyzed as internal controls.

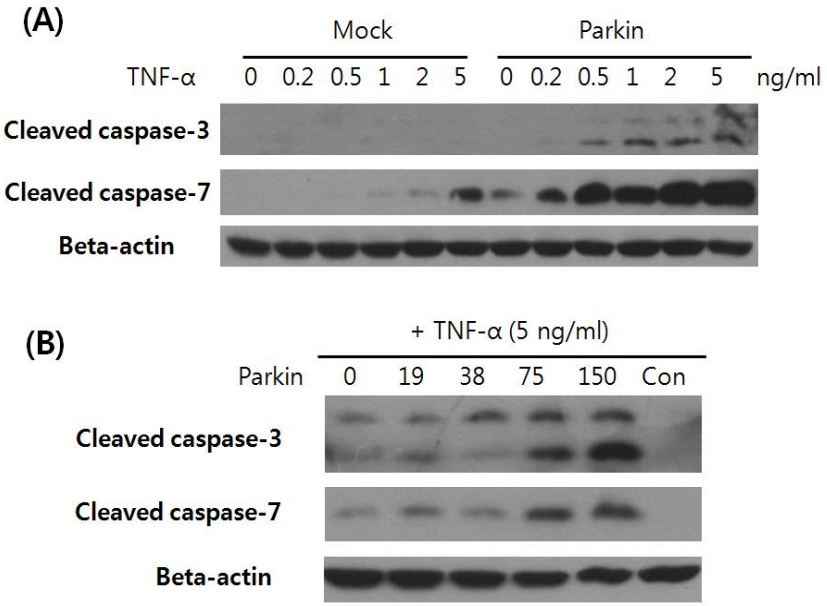

Figure 5. Parkin expression in TNF-α-treated HeLa cells stimulates cleavage of caspase-3 and caspase-7. **(A)** Cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h and then treated with TNF-α (0, 0.2, 0.5, 1, 2, 5 ng/ml) for 24 h. **(B)** Cells were infected with indicated concentrations (0, 19, 38, 75, 150 M.O.I.) of Parkin for 24 h and then treated with TNF-α (5 ng/ml) for 24 h. Cell extracts were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Cleaved forms of caspase-3 and caspase-7 were detected by Western blot using antibodies specific for cleaved fragment(s) derived from activated caspase-3 and -7 respectively. Levels of β-actin were analyzed as internal controls.

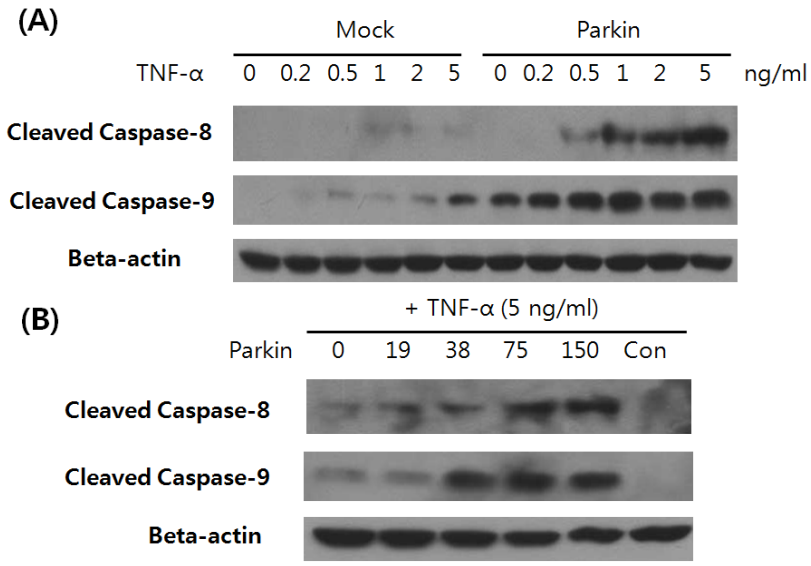

Figure 6. Parkin expression in TNF-α-treated HeLa cells stimulates cleavage of caspase-8 and caspase-9. **(A)** Cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h and then treated with TNF-α (0, 0.2, 0.5, 1, 2, 5 ng/ml) for 24 h. **(B)** Cells were infected with indicated concentrations (0, 19, 38, 75, 150 M.O.I.) of Parkin for 24 h and then treated with TNF-α (5 ng/ml) for 24 h. Cell extracts were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Cleaved forms of caspase-8 and caspase-9 were detected by Western blot using antibodies specific for cleaved fragment(s) derived from activated caspase-8 and -9 respectively. Levels of β-actin were analyzed as internal controls.

4. Parkin expression results in reduced expression of survivin.

A variety of molecules other than caspases are known to be involved in the regulation of apoptosis.[29] To identify other potential molecules involved in parkin-induced apoptosis in TNF-α-treated HeLa cells, I determined the expression level of apoptosis regulatory molecules. Cells were infected with Parkin and then treated with TNF-α. First, cDNA was subjected to PCR to detect mRNA expression level of the pro-apoptotic regulators BAK1 (Bcl-2 homologous antagonist killer) and Bax (Bcl-2-associated X protein) and the anti-apoptotic regulators Bcl-xL (B-cell lymphoma-extra large), MCL1 (myeloid leukemia cell differentiation protein), and Bcl-2 (B-cell lymphoma 2).[29] Parkin expression did not influence the mRNA level of these apoptosis regulatory molecules (Figure 7A). However, the mRNA expression level of another anti-apoptotic molecule, survivin, was decreased in a dose-dependent manner (Figure 7B). Since survivin is known to bind and inhibit activated caspase-3 and -7,[39] it is possible that decrease of survivin by parkin expression attenuates inhibition of activated caspase-3 and -7, leading to increased apoptosis.

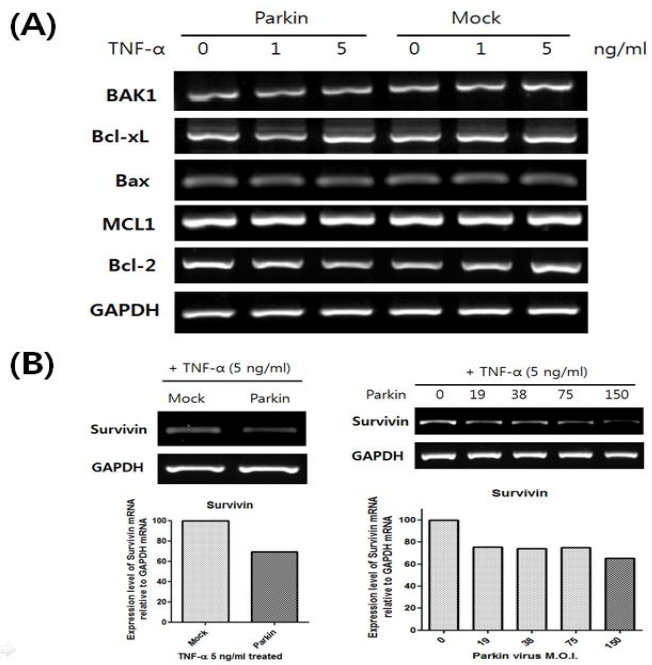

Figure 7. Parkin expression results in reduced expression of survivin. **(A)** HeLa cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h and then treated with indicated concentrations (0, 1, 5 ng/ml) of TNF-α for 24 h. cDNA was subjected to PCR to amplify apoptosis regulatory molecules (BAK1, Bcl-xL, Bax, MCL1, and Bcl-2). The PCR products were resolved on a 1.5% agarose gel. GAPDH was used as an internal control. **(B)** HeLa cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h and then treated with 5 ng/ml of TNF-α for 24 h (left panel). HeLa cells were infected with indicated concentrations (0, 19, 38, 75, 150 M.O.I.) of Parkin for 24 h and then treated with TNF-α (5 ng/ml) for 24 h (right panel). cDNA was subjected to PCR to amplify survivin. The PCR products were resolved on a 1.5% agarose gel. GAPDH was used as an internal control.

5. Parkin expression reduces β-catenin in TNF-α treated HeLa cells via the PKC pathway.

β-catenin is a known anti-apoptotic molecule and is a transcription factor responsible for expression of survivin.[40, 41] I found that expression of parkin reduces protein level of β-catenin in a parkin dose-dependent manner (Figure 8A and B). However, the mRNA level of β-catenin remains unchanged by parkin expression (Figure 8C). This implies that parkin-induced reduction of β-catenin is resulted from decrease of β-catenin protein translation or increase of β-catenin protein degradation. Next, to determine which signaling molecules are involved in the parkin-induced reduction of β-catenin, protein level of β-catenin was detected after treatment of parkin-expressing cells with inhibitors specific for various signaling molecules. Expression level of β-catenin was restored by treatment with Ro 31-7549, an inhibitor specific for protein kinase C (PKC) (Figure 9). In addition, inhibition of PKC-related signaling pathway resulted in restoration of survivin expression (Figure 10A) and attenuated parkin-induced cell death in TNF-α-treated HeLa cells (Figure 10B). However, restoration was not complete implying that other signaling pathway(s) exist which are responsible for restoration of β-catenin and survivin and attenuation of parkin-induced cell death. These results suggest that parkin expression results in the decrease of β-catenin protein level via the PKC signaling pathway and corresponding decrease of survivin followed by increased activation of caspase leading to apoptosis of cancer cells.

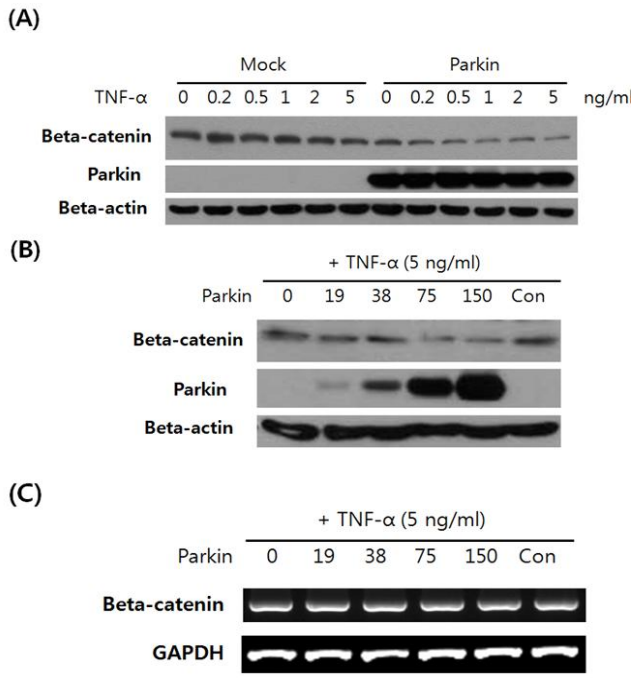

Figure 8. Parkin expression decreases protein level of the β-catenin. **(A)** Cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h and then treated with TNF-α (0, 0.2, 0.5, 1, 2, 5 ng/ml) for 24 h. **(B)** Cells were infected with indicated concentrations (0, 19, 38, 75, 150 M.O.I.) of Parkin for 24 h and then treated with TNF-α (5 ng/ml) for 24 h. Cell extracts were separated on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Protein level of β-catenin was detected by Western blot. Levels of β-actin were analyzed as internal controls. **(C)** HeLa cells were infected with indicated concentrations (0, 19, 38, 75, 150 M.O.I.) of Parkin for 24 h and then treated with TNF-α (5 ng/ml) for 24 h. cDNA was subjected to PCR to amplify β-catenin. The PCR products were resolved on a 1.5% agarose gel. GAPDH was used as an internal control.

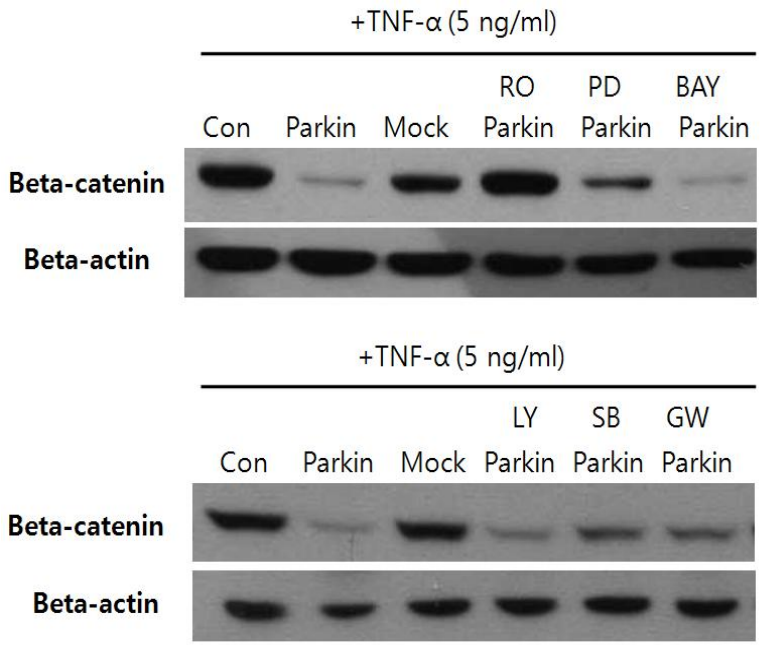

Figure 9. Parkin expression-induced reduction of β-catenin was restored by inhibitors specific for protein kinase C (PKC).

Cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h. Parkin-expressing cells were treated with 0.5 μM Ro 31-7549 (PKC inhibitor), 2.5 μM PD 98059 (MAPK inhibitor), 10 μM BAY 11-7085 (NF-κB inhibitor), 2.5 μM Ly 294002 (PI3-K inhibitor), 2.5 μM SB 203580 (p38 MAPK inhibitor), and 0.5 μM GW 5074 (cRaf1 inhibitor). After 1 h, cells were treated with TNF-α (5 ng/ml) for 24 h. Cell extracts were separated on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Expression of β-catenin was detected by Western blot. Levels of β-actin were analyzed as internal controls.

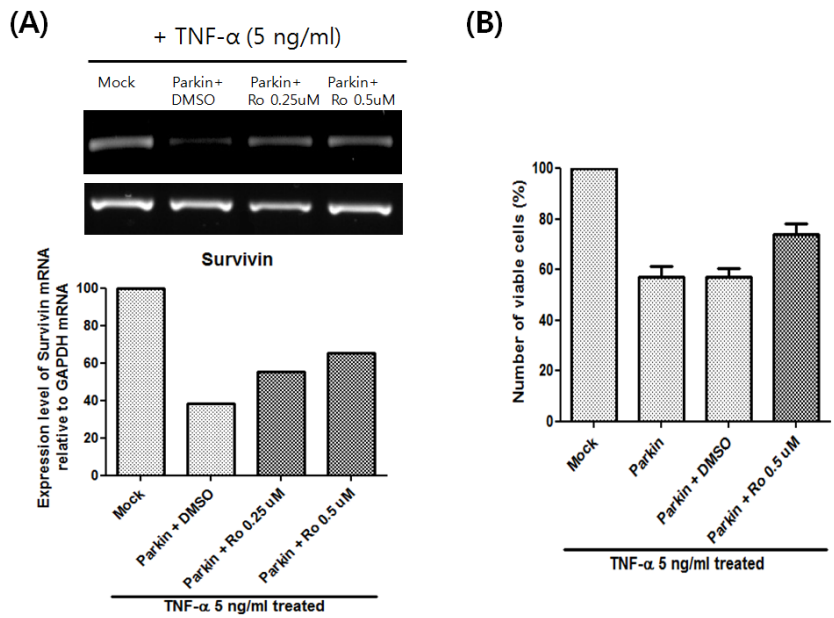

Figure 10. PKC inhibition restores expression of survivin and cell viability in parkin-expressed TNF-α-treated HeLa cells. **(A)** Cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h. Parkin-expressing cells were treated with indicated concentrations either vehicle control (DMSO) alone or 0.25 and 0.5 μM of Ro 31-7549 (PKC inhibitor) for 1 h, then cells were treated with TNF-α (5 ng/ml) for 24 h. cDNA was subjected to PCR to amplify survivin. The PCR products were resolved on a 1.5% agarose gel. GAPDH was used as an internal control. **(B)** Cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h. Parkin-expressing cells were treated with DMSO or 0.5 μM Ro 31-7549 for 1 h, then cells were treated with TNF-α (5 ng/ml) for 24 h. Viable cells were counted by trypan blue exclusion assay (3 independent experiments). Number of viable cells in Mock-infected group was set as 100%.

6. Effects of parkin expression in HeLa cell (TNF-α untreated) and other cancers

I performed experiments to examine whether HeLa cell death was affected by expression of parkin in the absence of TNF-α treatment. Parkin expression without TNF-α also resulted in a minor decrease of cell viability (Figure. 11A). Cleavage of PARP was also detected in cells infected with high dose (150 M.O.I) of Parkin (Figure. 11B). Next, we examined whether parkin expression affects cell viability in different types of cancer cells. MCF7 (breast cancer cell line) and A549 (lung cancer cell line) were infected with Parkin both in a dose-dependent and time-dependent manner then viable cells were counted. MCF7 showed parkin-dose-dependent decrease of cell viability. However, in A549 cells infected with Parkin, cell viability did not decrease (Figure 12). These results suggest that parkin also can be implicated in TNF-α-independent apoptotic pathway and parkin exerts different effects in different types of cancers.

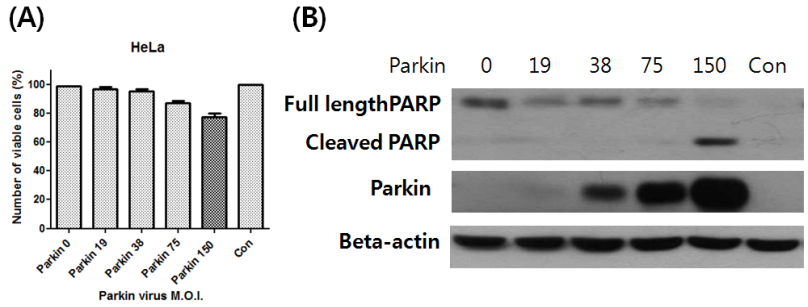

Figure 11. Parkin expression in the absence of TNF-α treatment also leads to a minor increase in cell death. **(A)** Cells were infected with indicated concentrations (0, 19, 38, 75, 150 M.O.I.) of Parkin for 24 h. Viable cells were counted by trypan blue exclusion assay (3 independent experiments). Number of viable cells in 0 M.O.I. Parkin-infected group was set as 100%. **(B)** Cells were infected with indicated concentrations (0, 19, 38, 75, 150 M.O.I.) of Parkin for 24 h. Cell extracts were separated on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Cleavage of PARP was detected by Western blot. Parkin expression was determined using anti-parkin antibodies. Levels of β-actin were analyzed as internal controls.

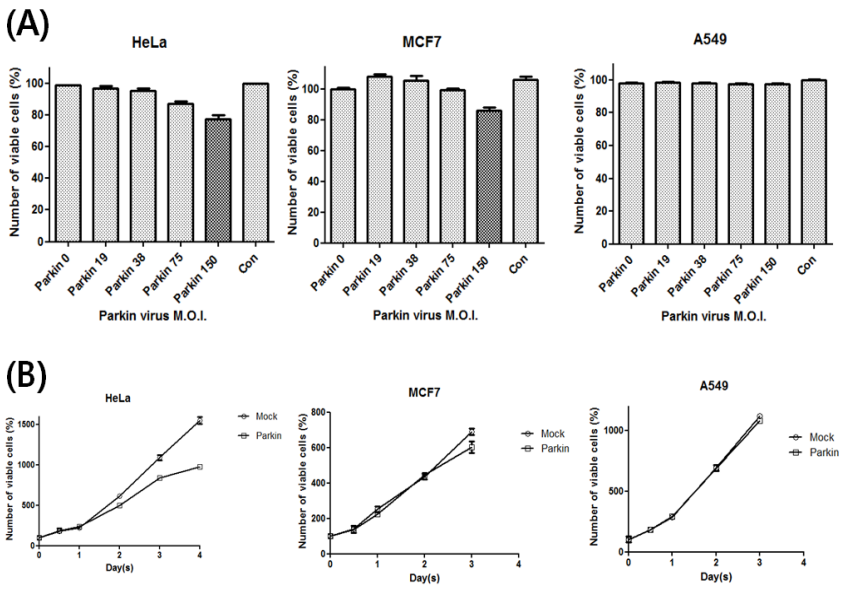

Figure 12. Parkin exerts different effects in different types of cancers. **(A)** HeLa, MCF7, and A549 cells were infected with indicated concentrations (0, 19, 38, 75, 150 M.O.I.) of Parkin for 24 h. Viable cells were counted by trypan blue exclusion assay (3 independent experiments). Number of viable cells in 0 M.O.I. Parkin-infected group was set as 100%. **(B)** HeLa, MCF7, and A549 cells were infected with Parkin or Mock virus (150 M.O.I. respectively) for indicated periods of time (0, 0.5, 1, 2, 3 days; for HeLa cells, until 4 days). Viable cells were counted by trypan blue exclusion assay (3 independent experiments). Number of viable cells in 0 day group was set as 100%.

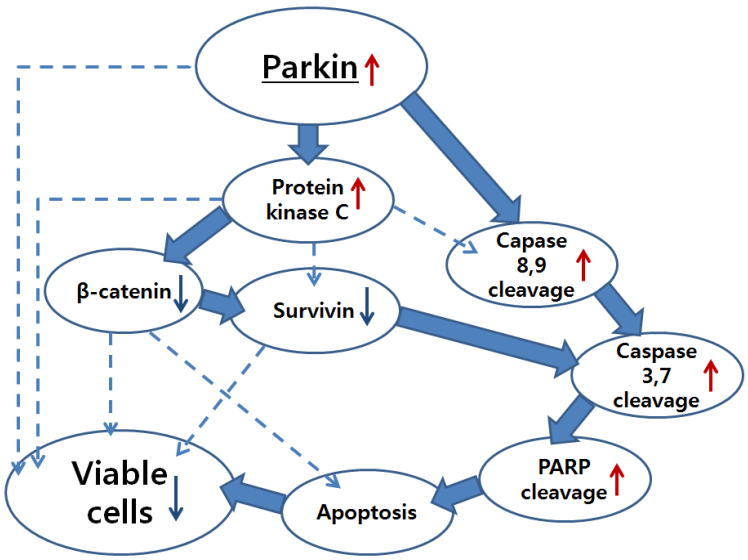

Figure 13. Schematic diagram showing putative points of parkin involvement in TNF-α-induced apoptotic death of HeLa cells. Thick arrows show putative pathway by which parkin restores TNF-α-stimulated HeLa cell death. Parkin expression reduces β-catenin and survivin via PKC signaling in TNF-α treated HeLa cells and consequent increased activation of caspase-3 and -7. Also, parkin expression stimulates activation of caspase-8 and -9, upstream molecules of caspase-3 and -7. These processes consequently can induce activation of apoptosis. Dotted arrows show other possible pathways.

Ⅳ. Discussion

Parkin is implicated in Parkinson's disease but more recently has been proposed to be a tumor suppressor. However, the exact inhibitory role of parkin in carcinogenesis and the mechanisms by which parkin functions as a tumor suppressor are unknown. In the current study, I investigated the role of parkin in TNF-α-induced apoptosis of HeLa cells which are inherently resistant to TNF-α-induced cell death and also lack parkin expression. I found that (1) parkin expression restored TNF-α-induced cell death in HeLa cells, (2) TNF-α-induced cell death was mediated via the apoptotic pathway involving survivin, caspase-8, -9, -3, -7, and PARP, (3) parkin expression reduces β-catenin, positive regulator of survivin, in TNF-α treated HeLa cells and (4) parkin expression induced reduction of β-catenin via the PKC signal pathway.

Apoptosis can be induced by a variety of stimuli and one potent activator of this pathway is TNF-α.[17] Lack of apoptosis causes uncontrolled cell proliferation leading to development of cancers. The binding of TNF-α to tumor necrosis factor receptor 1 (TNFR1) has been shown to initiate the apoptosis pathway eventually leading to caspase activation.[23] In the current study, I showed that parkin expression dramatically enhances TNF-α-induced apoptosis of HeLa cells in a TNF-α-dose-dependent manner (Figure 1B). These results strongly suggest that parkin expression in HeLa cells restores the TNF-α-dependent apoptotic pathway. However, a role of parkin in the TNF-α-independent apoptosis pathway cannot be ruled out altogether because parkin expression in the absence of TNF-α treatment can also lead to a minor (~15% reduction of viable cells) increase in cell death (Figure 11). Based on these results, I suggest that decreased expression of parkin can aid cancerous cells to

escape from TNF-α-stimulated apoptosis ultimately leading to development of cancer.

Caspases play a central role in transduction of apoptotic signals.[42] There are two major apoptotic pathways; mitochondria-independent and mitochondria-dependent. Both pathways can be activated by treatment with TNF-α. Caspase-8 is mainly involved in the mitochondria-independent pathway and caspase-9 is known to play an important role in the mitochondria-dependent pathway.[43, 44] The results from this study show that both initiator caspase-8 and -9 are activated by expression of parkin (Figure 6). Therefore, parkin expression may exert an effect between the TNF-α receptor and the initiator caspases (Figure 13). A simpler explanation for the restoration of TNF-α induced cell death in HeLa cells could be that parkin expression restored expression of the TNFR1 and therefore restored susceptibility to TNF-α. However, I found that HeLa cells express a stable level of TNFR1 mRNA irrespective of parkin expression.

There are numerous pro-apoptotic molecules and anti-apoptotic proteins involved in the regulation of apoptosis.[29] Among them, survivin is a member of the inhibitors of apoptosis (IAP) family. Survivin inhibits activation of caspases leading to negative regulation of apoptosis.[30] Survivin binds to and inhibits the active forms of caspase-3 and -7.[39] In the present study, expression of parkin in HeLa cells reduced the expression of survivin (Figure 7B). Therefore, reduction of parkin in cancer cells may lead to increased levels of survivin and finally to inhibition of active caspases and ultimately to decreased apoptosis. Recent studies have shown that disruption of the survivin induction pathways leads to increase in apoptosis and decreased tumor growth.[32] Decrease of survivin by parkin may contribute to the parkin-induced restoration of TNF-α-induced apoptosis of HeLa cells.

Parkin is known to be an E3 ubiquitin ligase which initiates the proteosome mediated degradation of proteins.[45] Parkin can be involved in signaling pathways by influencing ubiquitin mediated degradation of signaling molecules. β-catenin is a transcription factor involved in the Wnt/β-catenin signaling pathway and *APC* gene-mediated colorectal tumorigenesis.[46] In this study, I found that parkin induces downregulation of β-catenin. Parkin expression had no influence β-catenin transcription; instead it decreased the protein level of β-catenin in the cytosol fraction (Figure 8). I surmise that the β-catenin protein is degraded or translocated to the nucleus in TNF-α-treated HeLa cells in response to parkin expression. Reports suggest that β-catenin regulates cell cycle-related molecules.[47] In Figure 2, although percentage of cells in the subG1 phase increased implying activation of apoptotic pathway, cells in G2/M phase also increased which also implies G2/M arrest. Decrease of β-catenin can be associated with this change in cell cycle. Therefore, it is necessary to examine whether parkin expression affects expression of molecules regulated by β-catenin such as E-cadherin, c-Myc, cyclin D1, CD44 and VEGF.[48]

It has been reported that β-catenin degradation can be induced by PKC-α.[49] Inhibitor studies suggest that parkin down-regulates β-catenin via the PKC pathway. PKC is an important enzyme in the signal transduction cascade and is influenced by Ca^{2+}, diacylglycerol (DAG) and phospholipid.[50] Various isoforms exist and the function of isoform differ.[51] In this study, I used the broad-spectrum PKC inhibitor. Therefore, further experiments are required to determine which PKC isoform is involved in parkin-induced reduction of β-catenin. In addition, further studies are needed to elucidate whether parkin expression affects expression or activity of β-catenin regulating proteins such

as APC, GSK3-β, Wnt and Axin[46] to further understand the mechanism of parkin-induced downregulation of β-catenin.

Recently it has been reported that parkin is involved in glucose metabolism.[52] Therefore, it is possible that expression of parkin reduces proliferation or induces apoptosis of cells by influencing energy metabolism in cancer cells. In the present study, I showed that expression of parkin exerts different effects on different types of cancer cells (Figure 12). For example, in one study, parkin gene mutation in neuronal cells induced cell death but in other type of cells contributed to tumorigenesis.[53] Further studies for elucidating the various roles of parkin in diverse types of cancer cells seems warranted to understand mechanisms by which parkin act as a tumor suppressor.

In conclusion, I report herein that parkin expression restored TNF-α-induced cell death in HeLa. Although further studies are needed to determine the exact mechanism(s) by which parkin expression restores TNF-α-stimulated HeLa cell death, I have identified several potential molecules and signaling pathways that will provide a foundation in understanding how parkin acts as a tumor suppressor.

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ABSTRACT IN KOREAN

종양괴사인자-알파(Tumor necrosis factor-alpha)내성 자궁경부암 세포에서 파킨단백질의 종양억제능 연구

연세대학교 대학원

임상병리학과

이 경 홍

파킨단백질은 종양억제인자로 기능할 것으로 추정되는 단백질로, 다양한 암세포에서 파킨유전자(*PARK2*)의 돌연변이와 파킨단백질의 발현감소가 보고되고 있다. 많은 암세포가 암 발달 과정 중에 종양괴사인자-알파에 대한 저항성을 가지게 되며 그 결과 대부분의 악성종양세포에서는 종양괴사인자-알파에 의한 세포사가 일어나지 않는다. 본 연구에서는, 종양괴사인자-알파에 저항성이 있는 자궁경부암 HeLa 세포에서 파킨단백질의 종양억제기능에 대해 연구하였다. 파킨단백질 발현 후 종양괴사인자-알파 처리 시, HeLa

세포의 생존세포수가 감소하였으며 이는 세포자멸사(apoptosis)에 의해 야기됨을 확인하였다. 파킨단백질에 의한 세포사 과정에서 caspase-8. -9, -3, -7 이 활성화되며 결과적으로 poly ADP ribose polymerase(PARP)가 불활성화 되는 것을 확인하였다. 또한, 파킨단백질이 항세포자멸사 단백질인 survivin 의 발현을 감소시키고 survivin 의 전사조절인자인 베타-카테닌(β-catenin)의 단백질 양을 감소시킨다는 사실을 확인하였다. 이러한 과정이 protein kinase C (PKC)를 포함한 신호전달체계에 의해 일어남을 확인하였다. 이 같은 결과는 파킨단백질이 PKC – 베타-카테닌 – survivin – caspase 경로에 영향을 주어 종양괴사인자-알파에 의한 HeLa 세포사멸에 관여함을 보여준다. 본 연구에서 제시한 새로운 기전이 파킨단백질의 종양억제능을 이해하는데 도움이 될 것이라 사료된다.

핵심되는 말 : 파킨단백질, 종양억제, 종양괴사인자-알파, 자궁경부암, 세포자멸사