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■ Original Article ■

Parkin-induced Decrease of β-catenin is Mediated by Protein Kinase C in TNF-α-treated HeLa Cells

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Parkin is a protein known to have tumor suppressive functions. In a previous study, we determined that Parkin expression restores susceptibility to TNF-α-induced death in HeLa cells. β-catenin is a key protein in the Wnt signaling pathway and excessive activation of the β-catenin pathway can promote cancer development. In this study, we found that β-catenin levels decreased dramatically in Parkin over-expressing HeLa cells treated with TNF-α. We used chemical inhibitors of cell signaling pathways to identify the signaling molecules involved in β-catenin down-regulation. Our results indicate that the PKC inhibitor (RO-31-7549) blocked parkin-induced down-regulation of β-catenin. We also show that Parkin-induced decrease in cell viability in TNF-α-treated HeLa cells is alleviated upon treatment with a PKC inhibitor. Taken together, these results suggest the possibility that β-catenin reduction may be associated with Parkin-induced decrease of cell viability in TNF-α treated HeLa cells.

Key Words: Parkin, TNF-α, β-catenin, Cervical cancer, Tumor suppressor

INTRODUCTION

The Parkin gene (*PARK2*) was first described by Kitada *et al*. to cause autosomal recessive juvenile parkinsonism (Kitada et al., 1998). PARK2 is located on chromosome 6q25.2-6q27 which is implicated as a common fragile site (Denison et al., 2003b). In many cancers including hepatocellular carcinoma, ovarian, breast, lung, and squamous cell lung cancers, loss of heterozygosity (LOH) on chromosome 6q26 can be found (Oates et al., 1998, Shridhar et al., 1999; Kong et al., 2000) In addition, alternative transcripts of Parkin gene were found in various cancers resulted from deletion or duplication of the gene (Denison et al., 2003a; Denison et al., 2003b; Wang et al., 2004; Poulogiannis et al., 2010; Tay et al., 2010; Veeriah et al., 2010). In acute lymphoblastic leukemia, chronic myeloid leukemia, and colorectal cancers, hypermethylation of the *Parkin* gene promoter region diminished the expression of Parkin (Agirre et al., 2006; Poulogiannis et al., 2010). Moreover, it was reported that Parkin over-expression resulted in growth inhibition of cancer cells (Picchio et al., 2004; Wang et al., 2004; Poulogiannis et al., 2010; Tay et al., 2010; Veeriah et al., 2010). In MCF7, a breast cancer

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cell line, Parkin stabilized microtubules, increased sensitivity to anti-cancer agents, and induced growth arrest (Tay et al., 2010). Ubiquitination of cyclin E by Parkin resulted in cyclin E degradation and subsequent cell cycle arrest in a glioblastoma cell line (Veeriah et al., 2010). Simultaneous mutation in both Parkin and APC gene accelerated development of colorectal carcinoma (Poulogiannis et al., 2010). Collectively, these reports strongly implicate Parkin as a tumor suppressor. However, the mechanism by which Parkin acts as a tumor suppressor remains to be fully elucidated.

The β-catenin signaling pathway plays an important role in regulating gene transcription involved in embryonic development and cellular proliferation. Moreover, activation of β-catenin signaling is involved in the development and progression of many cancers (Beachy et al., 2004). The β-catenin pathway is promoted by Wnt signals in developmental and physiological processes or by mutations in degradation complex during tumor formation. In the absence of Wnt signal, a complex which includes the adenomatous polyposis coli (APC) phosphorylates and degrades β-catenin. In the presence of a Wnt signal, β-catenin is hypophosphorylated and accumulated by activation of the pathway and it is followed by increased target gene expression (Heeg- Truesdell and LaBonne, 2006; Kimelman and Xu, 2006). Increased cytosolic β-catenin level and subsequent nuclear import permits its interaction with Tcf/Lef family that controls the expression of genes which contribute to cancer progression such as cyclin D1, VEGF, and survivin (Altieri, 2004; Nagy et al., 2007).

In this study, we investigated the implication of β-catenin during Parkin mediated suppression of cancer progression. We report that Parkin reduces β-catenin via protein kinase C (PKC)-dependent signaling pathway.

MATERIALS AND METHODS

Materials

Recombinant adenoviral vector including parkin gene (Parkin virus) was produced as previously described by Kim et al. (Kim et al., 2006). Recombinant human TNF-α was purchased from R&D System (Minneapolis, MN, USA). RO-31-7549, PD 98059, Ly 294002, SB 203580, GW 5074 were purchased from Calbiochem (Darmstadt, Germany). BAY 11-7085 was purchased from Enzo Life Science (New York, NY, USA).

Cell lines and cell culture

HeLa (Human cervical adenocarcinoma cells) (ATCC, Manassa, CO, USA) was grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) and streptomycin-penicillin (Gibco BRL). The cells were maintained at 37℃ in humidified atmosphere with 5% CO_2.

Parkin gene expression

HeLa cells (2×10^5) were seeded into each well of a 6-well plate and 24 h later infected with different concentrations of Parkin virus and Mock virus in serum-free DMEM. After an additional 90 min 10% FBS-DMEM was added to each well. In Parkin virus dose-dependent experiments, cells were infected with Parkin virus at different multiplicity of infection (M.O.I.) (0, 19, 38, 75, 150). To compensate for the effect of the viral vector, Mock virus was added with the Parkin virus to maintain equal concentration of 150 M.O.I. A non-infected group was added as a negative control.

Trypan blue dye exclusion assay

The cell culture medium was removed and treated with trypsin-EDTA (Gibco BRL) for 2 min. Cells were dislodged and an equal volume of cell suspension mixed with 0.4% trypan blue stain solution (Gibco BRL). Viable cells were enumerated using a hematocytometer (Marienfeld, Lauda- Königshofen, Germany).

RT-PCR(Reverse transcriptase - polymerase chain reaction)

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription with 2 μg total RNA, 0.25 μg of random hexamer

(Invitrogen) and 200 U of Moloney Murine Leukemia Virus- Reverse Transcriptase (MMLV-RT; Invitrogen) for 50 min at 37℃ and 15 min at 70℃ Subsequent PCR amplification using 0.2 U of *Taq* polymerase (Cosmogenetech, Seoul, Korea) was performed in a thermocycler using specific primers.

The sequences of the PCR primers are as follows: 5′-TTGAAGTATACCATACAACTG-3′ (forward) and 5′-GCAGCATCAAACTGTGTAGAT-3′ (reverse) for β-catenin and 5′-CGGGAAGCTTGTCATCAATGG-3′ (forward) and 5′-GGCAGTGATGGCATGGACTG-3′ (reverse) for GAPDH. PCR products were electrophoresed on 1.5% agarose gels, stained for 10 min with ethidium bromide and destained for 20 min. Product size were determined by comparing to a 100 bp DNA ladder marker (Bioprince, Atlanta, GA, USA). The intensity of each band was quantitated using a Gel Doc (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Cells were lysed with a PBS buffer containing 1% Triton X-100 and protease inhibitor cocktail and then centrifuged at 19,000g for 10 min at 4℃. The supernatant was collected and the total protein concentration quantified using the Lowry protein assay (Bio-Rad). Protein samples (15 μg per lane for β-actin, parkin, and; 30 μg per lane for β-catenin) were mixed with loading buffer and the proteins separated by SDS-polyacrylamide gel electrophoresis (10% SDS- PAGE for detection of β-actin, Parkin, and β-catenin). The proteins were then transferred to a nitrocellulose membrane. The membrane was then incubated optimal concentrations of primary antibody at 4℃ overnight and then with the appropriate secondary antibody (anti-mouse or anti-rabbit) for 1 h at room temperature. The immunolabeled proteins were visualized using ECL (Thermo, Waltham, MA, USA). β-actin was used as an internal control.

RESULTS

Parkin induces cell death in TNF-α-treated HeLa cells

The human cervical cancer cell line, HeLa, is resistant to TNF-α-induced cell death (Franco et al., 2002). In a previous study, we found that expression of Parkin induces cell death in TNF-α-treated HeLa cells (Lee et al., 2012). To reconfirm 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 significantly affect HeLa cell viability (Fig. 1A, white bar). However, Parkin expression reduced viability of TNF-α-treated HeLa cells (Fig. 1A, black bar). These results show that expression of Parkin in HeLa cells results in decrease of cell viability in TNF-α-treated HeLa cells.

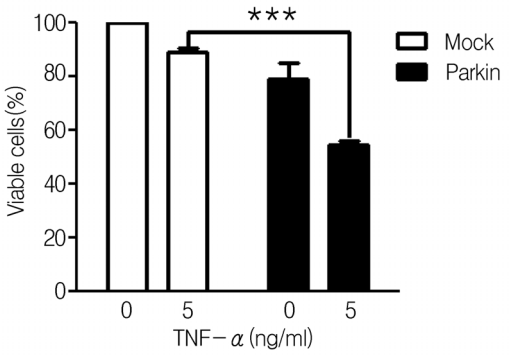

Fig. 1. Parkin expression reduces viable cells of TNF-α treated cervical cancer HeLa cells. Cells were infected with either Mock or Parkin (150 M.O.I. respectively) for 24h and then treated with TNF-α (0, 5 ng/ml) for 24 h. Viable cells were counted by trypan blue exclusion assay. Number of viable cells in 150 M.O.I. Mock-infected group without TNF-α treatment was set as 100%. Data are from three independent experiments. Values are shown as mean and standard error. P-values were determined by the Student's *t*-test. ***$P <$ 0.001

Parkin reduces protein level of β-catenin in TNF-α treated HeLa cells.

Activation of the β-catenin pathway results in expression of multiple genes that contributes to cancer progression. We found that expression of Parkin reduced β-catenin protein levels in TNF-α treated HeLa cells in a TNF-α dose- dependent manner (Figure 2A). Likewise, β-catenin protein levels were reduced in TNF-α treated HeLa cells in a Parkin dose-dependent manner (Figure

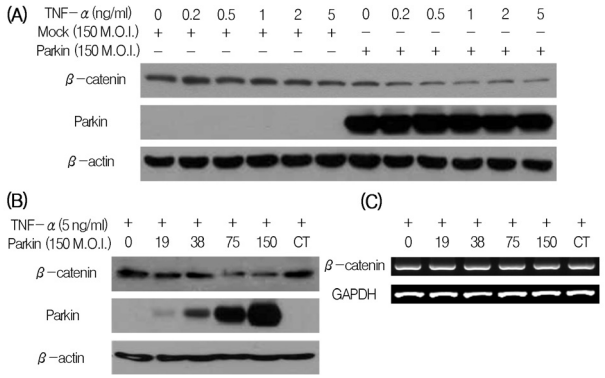

Fig. 2. Parkin expression decreases β-catenin protein levels. (A)Cells were infected with either Parkin or Mock (150 M.O.I. respectively) for 24 h and then treated with TNF-α (0, 0.2, 0.5, 1, 2, 5 ng/ml) for 24 h. (B) Cells were infected with indicated concentrations(0, 19, 38, 75, 150 M.O.I.) of Parkin for 24h and then treated with TNF-α (5ng/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.

2B). However, the mRNA level of β-catenin remained unchanged by Parkin expression suggesting that β-catenin transcription is similar (Figure 2C). These results suggest that Parkin-induced reduction of β-catenin is due to either a decrease in β-catenin protein translation or an increase in β-catenin protein degradation.

PKC mediates parkin-induced decrease of β-catenin

To identify the signaling pathways by which parkin reduces β-catenin, Parkin and TNF-α-treated HeLa cells were co-cultured with various chemical inhibitors of signaling pathways and β-catenin protein levels were assessed by Western blot analysis. We found that β -catenin level was recovered completely in Parkin and TNF-α-treated HeLa cells treated with RO-31-7549, a chemical inhibitor of the PKC pathway (Fig. 3). The chemical inhibitor PD 98059 (MEK inhibitor) and BAY 11-708598 (NF-κB inhibitor) did not show a significant effect. These results suggest that Parkin expression reduces β-catenin level via PKC signaling pathway.

Inhibition of PKC recovers parkin-induced cell death in TNF-α-treated HeLa cells

Thus far, Parkin expression on TNF-α-treated HeLa

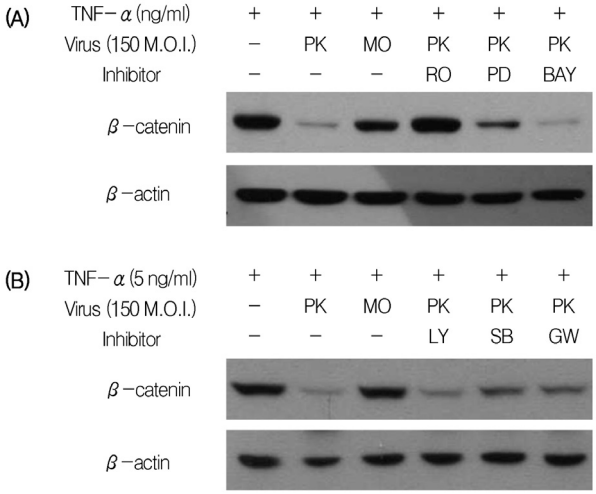

Fig. 3. Parkin expression-induced reduction of β-catenin was restored by protein kinase C (PKC) inhibitor. HeLa cells were infected with either Parkin virus or Mock virus (150 M.O.I. each) for 24 h. Parkin expressing cells were treated with 0.5 mM RO-31-7549 (PKC inhibitor), 2.5 mM PD 98059 (MEK1 inhibitor), 10 mM BAY 11-7085 (NF-κB inhibitor), 2.5 mM LY 294002 (PI3K inhibitor), 2.5 mM SB 203580 (p38 MAPK inhibitor), or 0.5 mM GW 5074 (c-Raf-1 kinase inhibitor). After 1 h, cells were treated with TNF-α(5ng/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. PK or MO indicates Parkin or Mock infected group respectively. RO, PD, BAY, LY, SB, or GW indicates the name of inhibitors that are RO-31-7549, PD 9805, BAY 11-7085, LY 294002, SB 203580, or GW 5074 respectively.

cells resulted in reduction of β-catenin which positively correlates with decreased cell viability. It is inferred from these data that reduction of β-catenin may contribute to Parkin-induced decreased cell viability of HeLa cells. If this hypothesis is true, then treatment with PKC inhibitor should induce recovery of cell viability in Parkin + TNF-α-treated HeLa cells, since PKC inhibitor induced recovery of β-catenin protein level. To test our hypothesis, Parkin + TNF-α-treated cells were pre-treated with the PKC inhibitor. We found that Parkin expression induced marked decrease in cell viability (57.1% viable cells) in TNF-α-treated HeLa cells but Parkin + TNF-α cells treated with PKC inhibitor showed an increase in cell viability to ~74.1% (Fig. 4). These results suggest a positive correlation between β-catenin levels and cell viability in Parkin + TNF-α-treated HeLa cells.

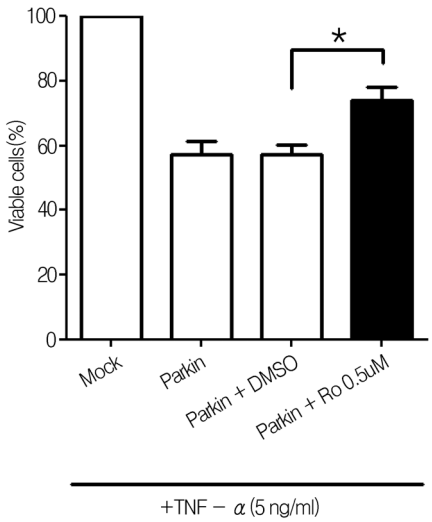

Fig. 4. PKC inhibition restores cell viability in parkin-expressed TNF-α-treated HeLa cells. HeLa cells were infected with either Parkin virus or Mock virus (150 M.O.I.) for 24 h. Parkin expressing cells were treated with DMSO alone or 0.5 mM of RO-31-7549 (PKC inhibitor). After 1 h, cells were treated with TNF-α (5 ng/ml) for 24 h. Viable cells were enumerated by trypan blue dye exclusion assay. Number of viable cells in mock-infected group was set as 100%. Data are from three independent experiments. Values are shown as mean and standard error. *P*-values were determined by the Student's *t*-test. $*P < 0.001$

DISCUSSION

Tumor suppressive role of Parkin has been frequently reported, but the mechanism by which it acts remains to be further investigated. In this study, we studied the implication of β-catenin in Parkin-induced decrease of cell viability in TNF-α-treated HeLa cells. We found that adenovirus- mediated expression of Parkin protein reduces β-catenin which is mediated through PKC signaling pathway and blockade of PKC signaling pathway alleviates decrease of cell viability in Parkin+TNF-α-treated HeLa cells. TNF-α is a potent activator of apoptosis, and a defect in this apoptotic pathway can lead to uncontrolled cell proliferation resulting in cancer progression (Balkwill, 2009). It has been reported that the HeLa cell line is resistant to TNF-α-induced cell death (Franco et al., 2002). We previously reported that Parkin induces apoptotic cell death in TNF-α-treated HeLa cells via activation of the apoptotic cascade (Lee et al., 2012). However, the mechanistic details by which Parkin regulates the apoptotic signaling remain to be investigated.

β-catenin is associated with many cancers and regulated by Wnt signaling. Increased cytosolic β-catenin results in translocation of β-catenin into the nucleus and forms a complex with the transcription factors of the Lef/Tcf family. β-catenin/Tcf-mediated transcription regulates many oncogenic genes, including c-myc, cyclin D1, and metalloproteinases (Chen et al., 2001). A previous study reported that stabilized β-catenin promotes hepatocyte proliferation and inhibits TNF-α-induced apoptosis (Shang et al., 2004). In the current study we investigated the expression of β-catenin during reduction of cell viability in Parkin + TNF-α-treated HeLa cells. In this study, we revealed that expression of parkin in HeLa cells induces decrease in β-catenin protein level which decreases further when treated with TNF-α (Fig. 2). This result suggests the correlation between decrease of β-catenin and cell viability both of which are induced by Parkin expression. A minor decrease in β-catenin by TNF-α was found consistent with reports that TNF-α inhibits epithelial cell proliferation through suppression of β-catenin/TCF signaling (Capaldo et al., 2012). To determine the mechanism by which Parkin decreases β-catenin protein levels, we investigated signaling pathways by using chemical inhibitors specific for PKC, MAPK, NF-κB, PI3K, p38 MAPK, c-Raf. Our data indicate that inhibition of PKC pathway restores β-catenin expression dramatically (Fig. 3A), thus the mechanism of β-catenin reduction by Parkin may be mediated by the PKC signaling pathway. Here, we used a pan-PKC inhibitor therefore it remains to be seen which PKC isoform is involved in decrease of β-catenin. Furthermore, we found that inhibition of PKC signaling pathway partially restores Parkin-induced decrease of cell viability in TNF-α-treated HeLa cells (Fig. 4). These results indirectly show that reduction of Parkin-induced reduction of β-cateninis implicated in cell death in TNF-α-treated HeLa cells. Nevertheless, how PKC pathway signaling affect β-catenin expression and decrease of cell viability in Parkin+TNF-α-treated HeLa cells remains to be

elucidated. In conclusion, we report that parkin expression reduces β-catenin protein levels via the PKC pathway.

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