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Impairment of autophagy induces p53 aggregation-mediated apoptosis in cadmium-exposed mouse kidney cells

Graduate School of Chosun University

Department of Biomedical Sciences

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카드뮴에 노출된 마우스 신장세포에서 자가포식 손상과 p53 응집에 의한 세포사멸 유도에 관한 연구

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스트레스에 의해 유전자의 손상을 받게 되면, 세포는 p53 의존성의 세포자가포식을 유도한다. 본 연구는 세포질 존재 p53이 카드뮴 독성에 의한 세포사멸 과정에서 중요한 역할을 한다는 것을 신장 사구체 세포 (MES13E)에서 밝혔다. MES13E 세포에 카드뮴 노출은 카스 페이스 의존성 PARP-1 절단과 소포체 스트레스 매개 CHOP의 유도를 통해 세포사멸을 유도했다. 카드뮴에 노출된 ME13E 세포에서 p53 단백질은 단량체 p53 및 폴리 유비퀴틴 화된 p53 (polyUb-p53)으로 발현되었다. 단량체 p53은 카드뮴의 농도와 노출 시간에 의존적으로 감소되었다. PolyUb-p53은 세포질에서 일시적인 축적 현상과 함께 응집 현상을 나타냈으며, 이 결과는 프로테아좀 활성에 비의존적인 것으로 확인 되었다. 카드뮴 처리 후 세포 자가포식 기질인 p62 / sequestosome-1

(SQSTM1)는 면역 침강 분석에 의해 유비퀴틴 (Ub) 과 상호 작용하는 것으로 확인되었다. p62 단백질은 단량체 및 폴리 유비퀴틴화 (polyUb) 된 형태로, 세포질에서 일시적으로 축적되었으며, 축적 패턴은 p53의 축적 패턴과 유사했다. 면역 침강분석에 의해 p53은 Ub 및 p62과 상호 작용하는 것으로 규명하였다. p62의 유전자 발현 억제는 polyUb-p53을 억제하였으며, 단량체 p53의 핵으로의 이동을 증가시켰다. 자가포식 관련 유전자 Atg5의 발현 억제는 카드뮴 유도 polyUb-p62 및 polyUb-p53을 억제하고 단량체 p53의 핵으로의 이동을 증가시켰다. LC3B의 면역 침강분석을 통해 LC3B는 p53 및 p62와 상호 작용하는 것으로 나타났다. 이러한 결과는 p53의 단백질 수준과 세포 내 분포가 p62 매개 세포 자가포식작용에 의해 조절됨을 나타낼 뿐 아니라 세포질 내의 축적이 세포 자가포식작용의 일시적인 손상에 의해 초래되었음을 나타낸다. 결론적으로, 본 연구 결과는 polyUb-p62에 의해 p53이 세포 자가포식 소포로 이동된 후 일시적인 축적을 통해 세포사멸을 유도함으로써, 이는 카드뮴에 의한 세포 및 신장의 조직 손상에 중요한 역할을 한다는 결과를 보여준다.

ABSTRACT

Impairment of autophagy induces p53 aggregation-mediated apoptosis in cadmium-exposed mouse kidney cells

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Upon genotoxic stress, cells can induce p53-dependent autophagy. Here we show that cytoplasmic p53 plays a critical role in cadmium (Cd) induced apoptosis in kidney messangial cells (MES13E), which was regulated by p62-mediated autophagy. Cd exposure to MES13E cells induced apoptosis through caspase-dependent PARP-1 cleavage and endoplasmic reticulum (ER) stress-mediated CHOP induction. In Cd-exposed ME13 cells, p53 protein detected as monomeric p53 and poly-ubiquitinated (PolyUb)-p53 forms.

Monomeric p53 decreased concentration- and exposure time-dependent manner, and PolyUb-p53 transiently accumulated and revealed aggregated in the cytosol compartment, which was independent of proteasome. After Cd exposure, p62/sequestosome-1 (SQSTM1), an autophagy substrate, interacted with ubiquitin (Ub) as demonstrated by immunoprecipitation analysis. p62 accumulated in monomeric and polyubiquitinated (polyUb) forms, and their accumulation patterns were similar as those of p53. Immunoprecipitation analysis revealed that p53 interacted with Ub and p62, respectively. Knockdown of p62 suppressed polyUb-p53, upregulating monomeric p53, and increasing nuclear localized p53. Autophagy inhibition by knockdown of *autophagy related 5* (*Atg5)* suppressed Cd-induced polyUb-p62 and polyUb-p53, upregulating monomeric p53, and increasing nuclear p53, indicating that p53 protein level and subcellular localization were regulated by p62-mediated autophagy. IP analysis for LC3B revealed that LC3B interacted with p53 as well as p62. Overall, our findings show that polyUb-p62 targeted p53 to autophagosomes, playing a crucial role in Cd-induced cell death and kidney damage.

ABBREVIATIONS

Cd, cadmium

MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

CHOP, C/EBP homologous protein

ER, endoplasmic reticulum

GFP, green fluorescent protein

LC3, microtubule-associated proteins 1A/1B light chain 3B

p62, sequestosome-1/SQSTM1

BaF1, bafilomycin A1

IF, immunofluorescence

IP, immunoprecipitation

PolyUb, poly-ubiquitnated

Ub, ubiquitin

siRNA, small interfering RNA

ROS, reactive oxygen species

NAC, *N*-acetyl-L-cysteine

1. INTRODUCTION

The kidneys are essential for maintaining homeostasis in the body by filtering blood and removing waste as urine (Guyton, 1991) These vital organs are damaged by various stressors that affect their function that can result in severe diseases. A number of acute and chronic insults, including those caused by drugs, toxins, metabolism, and inflammation, can damage the kidneys (Kimura *et al.*, 2017; Lee. 2018; Satarug *et al.*, 2004)

Cadmium (Cd) is a toxic environmental pollutant toxic heavy metal (ATSDR, 1999) and has known to be detrimental effects on human. In addition to a variety of acute toxicity to liver and lung, the kidney is also a major target organ for chronic Cd exposure (Jarup *et al.*, 1998). Due to its long biological half life (10~30 years) (IPCS 1992; Satarug *et al.*, 2004; Hartwig, 2013), chronic Cd exposure leads to accumulation in the human body and caused age-related diseases including nephrotoxicity, osteotoxicity, and immunotoxicity (Friberg *et al.*, 1984; ATSDR, 1999; Liu *et al.*, 2007). By IACR, Cd is also classified as a human carcinogen causing tumors of the lung and other tissues (Waalkes, 2003). Humans are continuously exposed to Cd, a toxic heavy metal, from contaminated foods, industrial sources, and cigarette smoke (Jarup, 2003). Cd accumulates in various tissues and organs; however, the kidney is very sensitive to Cd exposure, (Jarup *et la.*, 1998; Goering and Klaassen, 1983; Klaassen *et al.*, 2009). Cd enters the body through inhalation and ingestion contaminated air, water, and foods. Once absorbed, Cd transported to the liver and bound to metallothionein (Cd-MT), which transported to the kidney via blood (Klaassen *et al.*, 1999). Cd-MT is filtered through the glomeruli and reabsorbed by

proximal tubular cells, where it is degraded and released free Cd (Cd^{2+}), which causes tubular damage. The distribution of Cd in the body is MT-dependent, thus, liver and kidney show a high Cd level (Liu *et al.,* 2001; Klaassen *et al.,* 2009). For this reason, most of researches on kidney toxicity by Cd focused on proximal tubular cells. However, kidney damage involves both tubular and glomerular dysfunction. Especially, chronic exposure with low level of Cd is related with chronic kidney disease (CKD), resulting in proteinuria and reduced glomerular filtration rate, as well as is associated with developing hypertention (Oliver-Williams *et al.,* 2018; Grau-Perez *et al.,* 2017; Ferraro *et al.,* 2010; Satarug et al., 2018).

Cd-induced kidney toxicity has been extensively studied at the molecular level. Several studies have now demonstrated that Cd exposure is a main cause of renal toxicity , and one of the main mechanisms implicated in renal toxicity is apoptotic cell death, including ER stress-mediated apoptosis, caspase-dependent and -independent apoptosis, and p53-dependent apoptosis (Gobe and Crane, 2010). In porcine renal proximal tubular epithelial cells (LLC-PK1), Cd exposure induced ER stress-mediated apoptosis by inducing ER stress response proteins such as PERK, ATF6, IRE1, and CHOP (Yokouchi *et al.,* 2008). In mouse mesangial cells, Cd exposure triggered mitochondria- and ER stress-mediated apoptosis by inducing caspase-9, caspase-3, and CHOP activation (Liu and Templeton, 2008). Furthermore, Cd induced caspase-dependent and -independent apoptosis in an immortalized kidney proximal tubule cell line (WKPT-0293 Cl.2) by releasing cytochrome *c,* an apoptosis-inducing factor (Lee *et al.,* 2008). In our previous studies, we found that exposure of NRK52E cells to Cd resulted in phospho-p53 accumulation due to DNA damage. Consistently, Cd exposure

also resulted in the accumulation of phospho- γ-H2AX, a DNA damage marker, and activation of caspase-7 and -3, and PARP-1 cleavage.

The tumor suppressor p53 is responsible for the activation of numerous cellular processes in response to a variety of cellular stresses, and leads to cell cycle arrest, apoptosis, and senescence (Levine, 1997). Under unstressed normal condition, cellular p53 protein maintains at very low level. However, p53 protein stabilized by various stresses, including DNA damage. Depending on the extend of the stress, cells lead to different biological responses involved in cell cycle arrest, genome stability, DNA repair, and apoptosis. Therefore, p53 protein level is a critical factor for its function. p53 is stabilized by many post-transloational modification, including ubiquitylation, acetylation, metylation, and sumolyation. The best well-known negative mechanism for p53 protein is MDM2-mediated ubiquitin degradation pathway, wherein MDM2, an E3 ligase, interacts with p53 and promotes ubiquitin-dependent degradation by nuclear and cytoplasmic proteasome (Momand et al., 1992; Haupt et al., 1997; Marki et al., 1996). Therefore, p53 stabilization can be occurred through blocking MDM2 interaction with p53 (Eischen and Lozano, 2014). Additionally, autophagy also involves in p53 degradation, which contributed in promoting cancer (Guo *et al.*, 2013; White 2015; White, 2016). Despite the known roles of autophagy in p53 degradation, its role of p53-autophagy signaling in Cd-induced tocixity remains unclear.

Autophagy helps maintain cellular health by inducing lysosomal degradation of proteins and damaged organelles. Autophagy is induced in response to various stresses, such as starvation (Kroemer, 2010), and plays an essential role in cell survival against metabolic stresses and immunological responses (Kimura, 2017; Liang, 2010). In contrast, hyper-active autophagy signaling can result in severe pathogenic conditions

caused by excessive cell death (Luo, 2016). Many studies have shown that autophagy is associated with Cd-induced kidney damage; however, this is still controversial. Cd-induced autophagy is associated with kidney damage in mouse and mesangial cells (Wang, 2009). In contrast, autophagy is protective against Cd-induced apoptotic and necrotic cell death in rat kidney mesangial cells and proximal tubular cells (Fujishiro *et al.,* 2018). Previously, we found that autophagy is protective against Cd-induced apoptosis in NRK52E rat kidney epithelial cells (So *et al.,* 2016) suggesting that Cd-induced autophagy may be species-specific. Since autophagy can be involved in both cell death and cell survival depending on stress conditions, further studies are needed to clarify functions of autophagy under pathophysiological conditions.

In this study, we aimed to elucidate the role of autophagy-mediated p53 in Cd-induced kidney damage and the underlying molecular mechanisms related to autophagy. We found that Cd-induced apoptosis was regulated by autophagy-mediated nuclear p53 aggregation. Therefore, deficient of p62/sequestosome-1 (SQSTM1) (referred to as p62) and autophagy-related gene ATG5 inhibitted p53 degradation and then apoptosis. Collectively, our results suggested that aggregated p53 protein may be a biomarker for Cd-induced kidney toxicity, and that polyubiquitinated (polyUb)-p62/p53 signaling may be a potential therapeutic target in Cd-induced kidney damage.

2. MATERIALS AND METHODS

2.1. Reagents and antibodies

Cadmium acetate (289159), bafilomycin A1 (B1793), MG132 (M8699), Hoechst 33342 (B2261), and anti-β-actin antibody (ab-8226) were obtained from Sigma-Aldrich. Ubiquitin (sc-8017), p21 (sc-6246), FITC-conjugated goat anti-mouse (sc-2010), and rhodamine-conjugated goat anti-rabbit (sc-2091) antibodies were purchased from Santa Cruz Biotechnology. LC3B (2775, 3868), Sirt6 (12486), PARP-1 (9532), procaspase-3 (9662), cleaved caspase-3 (9661), caspase-8 (4790), cleaved caspase-8 (9749), phospho-p53 (9284), CHOP (2895), and phospho-γ- H2AX (S139, 2577) antibodies were purchased from Cell Signaling Technology. SQSTM1/p62 (H00008878-M01) and APG3L (AP1807a) antibodies were obtained from Abnova and Abgent, respectively. HDAC1 (GTX-100513) antibody was purchased from GenTex.

2.2. Cell culture

Mouse messangial cells (MES13E) were maintained in DMEM (Gibco BRL, Grand Island, NY) supplemented with heat-inactivated 10% fetal bovine serum, 50 μg / ml penicillin, and 50 μg / ml streptomycin at 37°C in a 5% CO_2–95% air-humidified incubator. Bafilomycin A1, chloroquine, proteinase inhibitor cocktail, MG132, and MTT [3-(4,5-dimetylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma-Aldrich (Munich, Germany). Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), were purchased from Calbiochem (Darmstadt, Germany). Hoechst33342 was purchased from Molecular Probes (Invitrogen, Carlsbad,

CA, USA). The other chemicals used were of the purest grade available from Sigma (St. Louis, MO).

2.3. MTT assay

MES13E cells were suspended in complete media, at a concentration of 1 x 10^5 cells / ml, and samples (200 μl) of the cell suspensions were seeded onto 48-well plates and cultured overnight. Cells were then exposed to chemicals for 24 h. After 4 h of incubation with MTT (0.5 mg / ml), and the formazan crystals were dissolved with DMSO. Absorbances were measured at 540 nm using an ELISA microplate reader (Perkin-Elmer).

2.4. Western blot analysis

Proteins were extracted from the collected pellet using lysis buffer (1 % Triton X-100, 150 nM NaCl, 5 mM EDTA and protease inhibitors), quantified using BCA Protein Assay Kit (USA) and the concentration was measured at 595 nm using a Microplate reader. 20-50 μg of protein was electrophoresed using 10-12 % SDS-PAGE gel and transferred to PVDF membrane. In order to inhibit the binding of nonspecific proteins, membranes of 5 % skim milk made in TBS (20 mmol / L Tris, 137 mmol / L NaCl, pH7.6). Primary antibodies were reacted overnight at 4℃. The membrane was washed three times with TBST solution containing 0.05 %. The secondary antibody was reacted at room temperature for 2 h. Finally, the membrane was identified as a Super HR-A (Fuji film, Japan) component of the Fuji Medical X-RAY membrane using a Millipore immobilon Western chemiluminescent HRP substrate.

2.5. Transfection of siRNAs

MES13E cells were washed with OPTI-MEM medium (Gibco BRL, Grand Island, NY), and transfected with control siRNA and *Atg5* and *p62* siRNAs by using lipofectamine^{TM} RNAiMAX (Invitrogen, Carlsbad, CA) in 6-well plates according to the manufacturer's protocol. After 4 h of incubation, the medium was exchanged to a complete medium containing 10 % serum and antibiotics. Cells were incubated for an additional 24 h and treated as indicated in the figure legends.

2.6. Immunoprecipitation (IP)

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.25% Triton X-100, 10% glycerol) with protease inhibitors. Proteins (600 μg) were precleared with 50% protein G Plus-agarose beads (Santa Cruz Biotechnology, sc-2002) for 20 min and centrifuged at 12,000 × *g* at 4℃ for 10 min. The supernatants were incubated with the indicated primary antibodies or mouse IgG (Sigma-Aldrich, 12-371) or rabbit IgG (Sigma-Aldrich, 12-370) overnight at 4℃ on a rocker. The antibody complexes were then captured on protein G Plus-agarose beads and analyzed using SDS-PAGE. Semi-quantification of protein levels was performed using Image J software (National Institutes of Health, Bethesda, USA). The relative amount of each protein was normalized as the ratio between the quantified levels of protein, and the quantified levels of the respective loading control.

2.7. Immunofluorescence (IF)

Cells were cultured on a cover slip (Marienfeld, D111580) and then fixed in 10% neutral-buffered formalin (Sigma-Aldrich, HT501128) for 10 min on ice, and then washed with PBS. Cells were then permeabilized with 0.05% Triton X-100 (Sigma-Aldrich, T8787) for 10 min, washed with PBS, and blocked with 2% bovine serum albumin (Bioshop, ALB001.100). The cells were incubated with primary antibodies and fluorescent conjugated secondary antibodies. Nuclei were stained with Hoechst 33342 (1 μg/ml) and imaged using a fluorescence microscope (Nikon Eclipse TE300).

2.8. Statistical analysis

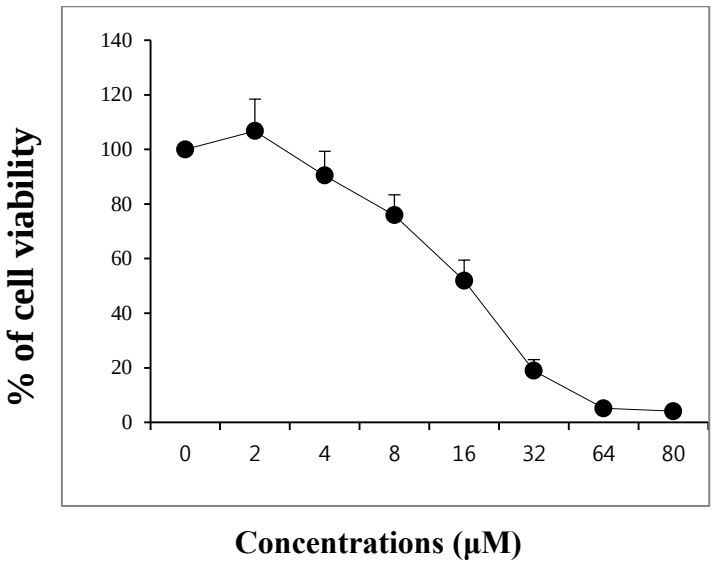
All experiments were repeated at least three times, and values are expressed as means ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test, or one-way ANOVA.). A value of $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Cd induces apoptosis in MES13 cells

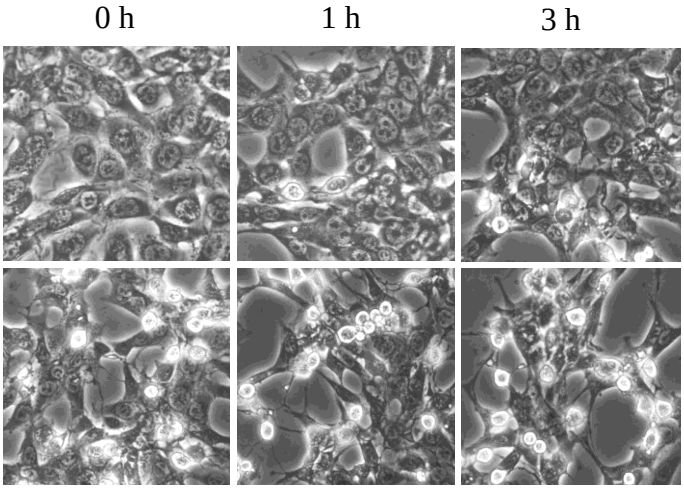
To examine the sensitivity of mouse kidney epithelial cells (SV40 MES13 cells) to Cd, cells were exposed to increasing concentrations of Cd for 24 h and then subjected to MTT assays (Fig. 1A). The half-maximal inhibitory concentration (IC_{50}) of Cd was approximately 24 μM. Cd exposure caused morphological changes, such as rounding up and floating in the medium (Fig. 1B). To determine whether Cd sensitivity was associated with apoptosis in MES13E cells, we first investigated the expression levels of proteins related to apoptosis. The cleavage of caspase-8, caspase-3, and PARP-1 occurred in a concentration-dependent manner. Consistent with these findings, Cd exposure induced phosphorylated (phospho)-γH_2AX (a marker for double-strand DNA breaks), phospho-p53, and p21 (Fig. 1C, D), implying that Cd caused DNA damage-induced apoptosis. These data indicated that Cd-induced cytotoxicity in MES13 cells was caused by apoptosis through the caspase -dependent signaling pathways.

Figure 1A



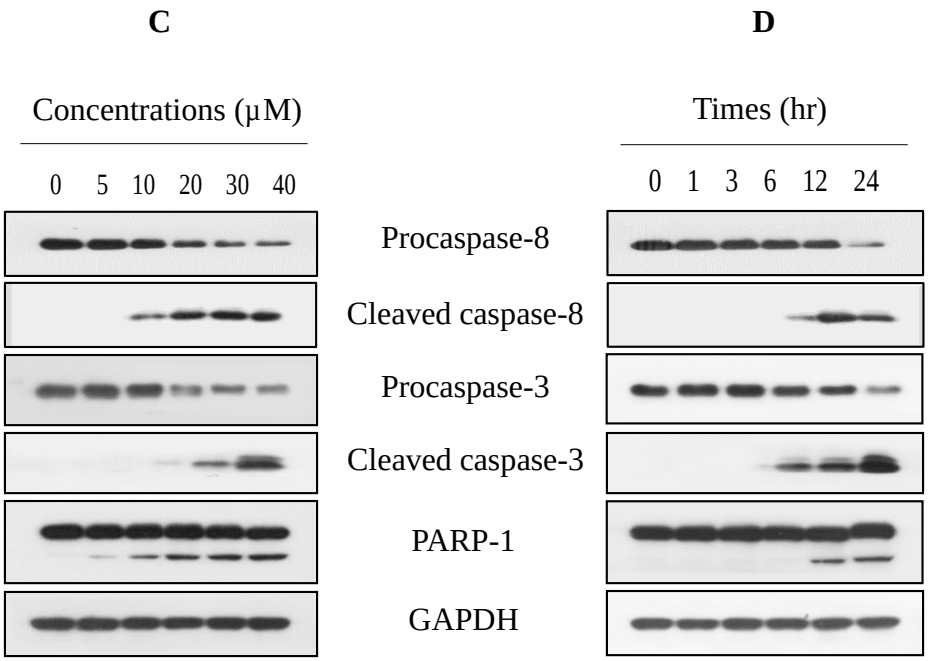
Effects of Cd on the viability in MES13E cells. Cells were seeded onto 48-well plate 1 X 10^6 and treated with the indicated concentrations of Cd for 24 h. Cell viability was determined by an MTT assay. Data were expressed as the mean ± SD of fold-increase compared to the untreated control from three independent experiments performed in triplicate.

Figure 1B



Effects of Cd on cell death in MES13E cells. Cells were exposed to Cd (24 μM), and .captured images at different times.

Figure 1C-D

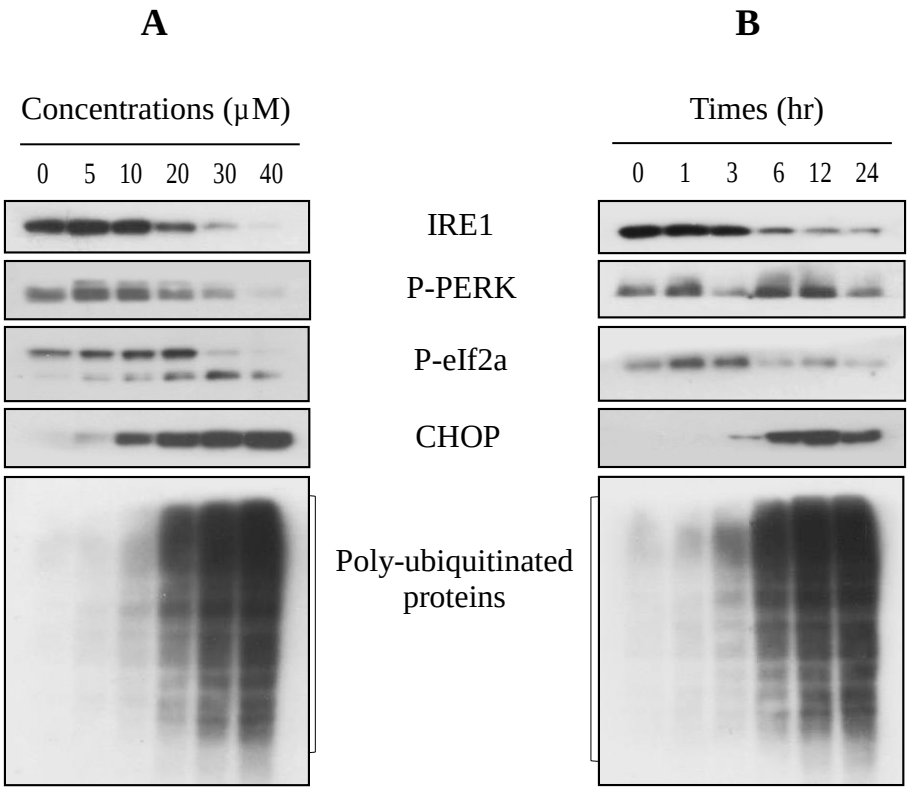


Cd induces apoptosis via caspase activation and PARP-1 cleavage in MES13E cells. Cells were treated with an increasing concentrations of Cd for 18h, or with IC50 concentrations (24 μM) for up to 24h, harvested, lysed, and expressions of indicated proteins were assessed by Western blot analysis. GAPDH was used as the loading control. Data shown are representative of at least three independent experiments.

3.2. Cd induced endoplasmic reticulum (ER) stress-mediated apoptosis in Cd-exposed MES13 cells

Cd toxicity is related to ER stress (10, 19, 20) thus, we examined whether ER stress is involved in the Cd-exposed MES13 cells. In Cd-exposed MES13 cells, ER stress sensor proteins, including IRE1, p-PERK, and p-eIF2a, were decreased in a concentration- and time-dependent manner. By contrast, one of the components of the ER stress-mediated apoptosis pathway is C/EBP homologous protein (CHOP) induced in a concentration- and time-dependent manner. Furthermore, polyUb-proteins levels increased in a concentration- and time-dependent manner, indicating that ER-associated degradation (ERAD) pathway induced in a coordinated manner with the ubiquitin–proteasome system (UPS) (Fig. 2). These results suggest that the unfolded protein response (UPR) and the sustained stress caused apoptotic cell death through CHOP induction.

Figure 2A and B



Cd induces ER stress in MES13E cells. Cells were treated with an increasing concentrations of Cd for 18h (A), or with IC50 concentrations (24 μM) for up to 24h (B), harvested, lysed, and expressions of indicated proteins were assessed by Western blot analysis. GAPDH was used as the loading control. Data shown are representative of at least three independent experiments.

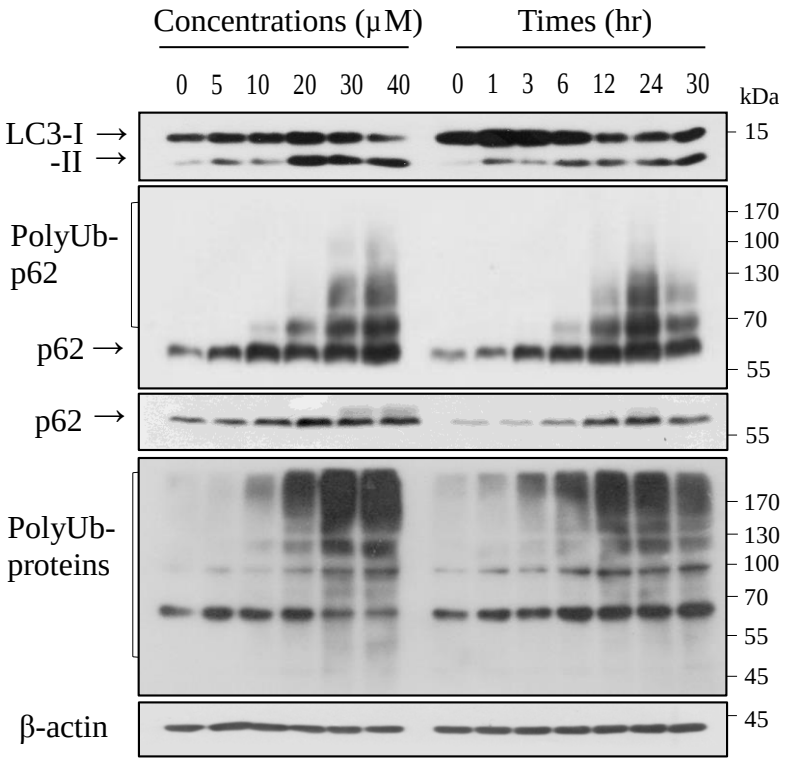
3.3. Cd induced p62-independent autophagy in MES13 cells

Previous studies have shown that autophagy has critical roles in the kidney (Wang *et al.,* 2009; Fujishiro *et al.,* 2018; Luo *et al.,* 2017; Chargui *et al.,* 2017). We observed autophagy induction via LC3B conversion using western blotting in Cd-exposed MES13 cells. LC3-II, an autophagy marker, was induced even in the presence of low Cd concentrations but did not cause morphological changes related to apoptosis. Despite increased LC3-II expression, the autophagy substrate p62 was detected as monomeric and high-molecular-weight multibands that accumulated in a concentration-dependent manner. In a time-course experiment using Cd at the IC_{50}, LC3-II was induced as early as 1 h after treatment. Monomeric and high-molecular-weight forms of p62 accumulated and peaked at 24 h (Fig. 3A). However, long exposure of films showed a few multibands below 55 kDa and a marked single band smaller than 25 kDa (Supplementary Fig. S1), These results indicated that Cd could cause impairment of p62-mediated autophagic degradation or low levels of autophagic flux.

Because LC3-II accumulation can be caused by blockage of autophagic flux or autophagic activation, we examined autophagic flux using bafilomycin A1 (BaF1), an inhibitor of fusion between autophagosomes and lysosomes. Pretreatment with BaF1 before Cd exposure for 12 h upregulated LC3-II compared with that in Cd-treated cells without BaF1 pretreatment. Moreover, BaF1 pretreatment slightly increased, but not significant, monomeric p62 and high-molecular-weight p62 expression, indicating that Cd resulted in impairment of p62-mediated selective autophagic degradation (Fig. 3B, C). These results were further confirmed by gene silencing of *atg5*. Cells were transiently transfected with *atg5* small-interfering RNA (siRNA) and nonspecific

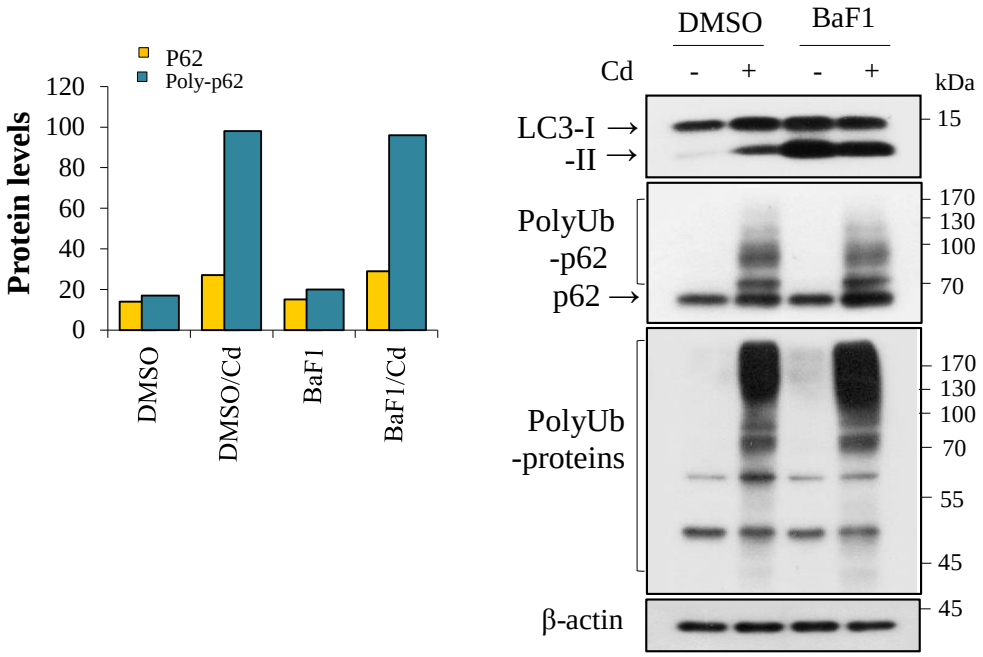
control (NC) siRNA, and transfection efficiency was analyzed by immunoblotting for Atg5 protein levels at 24 h after transfection (Fig. 3D). Knockdown (KD) of *atg5* inhibited autophagy, as demonstrated by decreased LC3-II levels. These modifications resulted in decreased levels of monomeric p62, high-molecular-weight p62, and PolyUb-proteins, indicating that autophagy may be associated with PolyUb-p62 accumulation (Fig. 3E, F). Collectively, these findings demonstrated that Cd exposure in MES13 cells could impair p62-mediated selective autophagy, at least, transiently.

Figure 3A



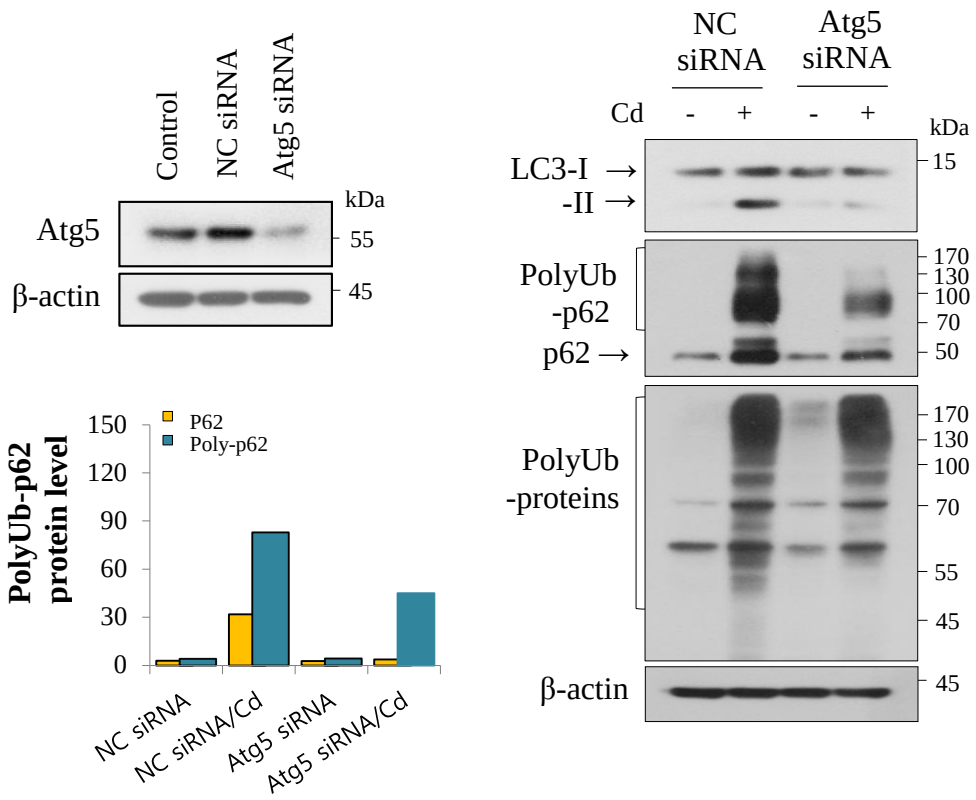
Cd exposure accumulated p62 and polyUb-p62. Cells were treated with an increasing concentrations of Cd for 18h, or with IC50 concentrations (24 μM) for up to 24h, harvested, lysed, and expressions of indicated proteins were assessed by Western blot analysis. GAPDH was used a0s the loading control. Data shown are representative of at least three independent experiments.

Figure 3B and C



The effects of autophagy inhibitor on Cd-induced polyUb-p62 in MES13E cells. Cells were pretreated with BaF1 (20 nM) for 2h, and then continuously exposed with Cd (24 μM). After 12h, cells were harvested, lysed, and expressions of indicated proteins were assessed by western blot analysis. GAPDH was used as the loading control. Data shown are representative of at least three independent experiments.

Figure 3D-F

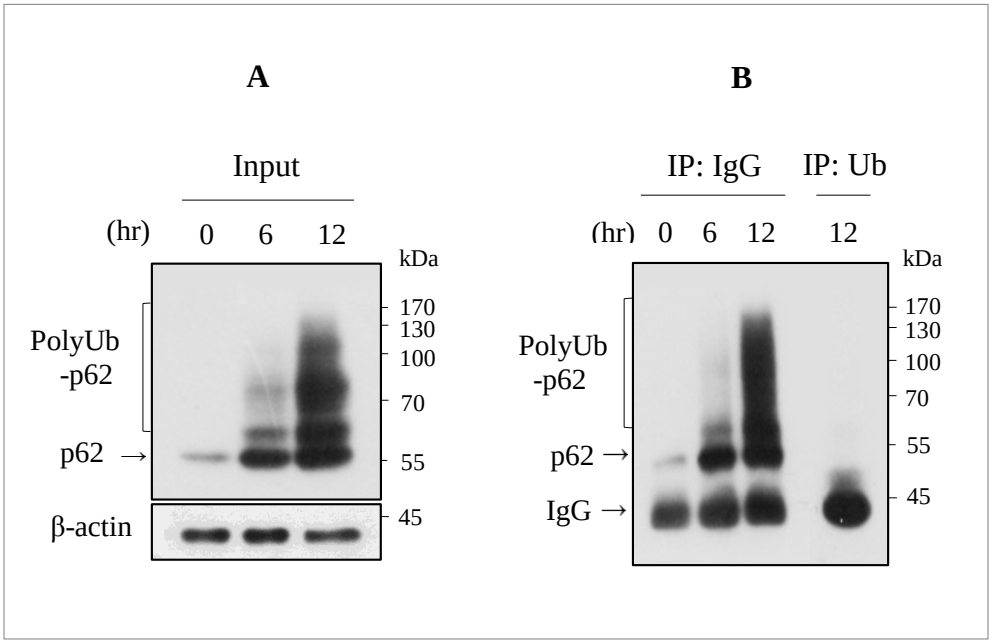


The effect of autophagy inhibition on Cd-induced polyUb-p62 in MES13E cells. (D) After 24 h, knockdown (KD) efficiency using *Atg5*-specific siRNA was evaluated by immunoblotting for Atg5. NC, negative control. (E) Cells transfected with NC and ATG5 siRNA were exposed to 24 μM Cd for 12 h. Cells were harvested, lysed, and expression of indicated proteins was assessed by Western blot analysis, GAPDH was used as the loading control. (F) p62 levels determined by immunoblotting were normalized to β-actin.

3.4. P62 interacts with ubiquitin (Ub) in Cd-exposed MES13 cells

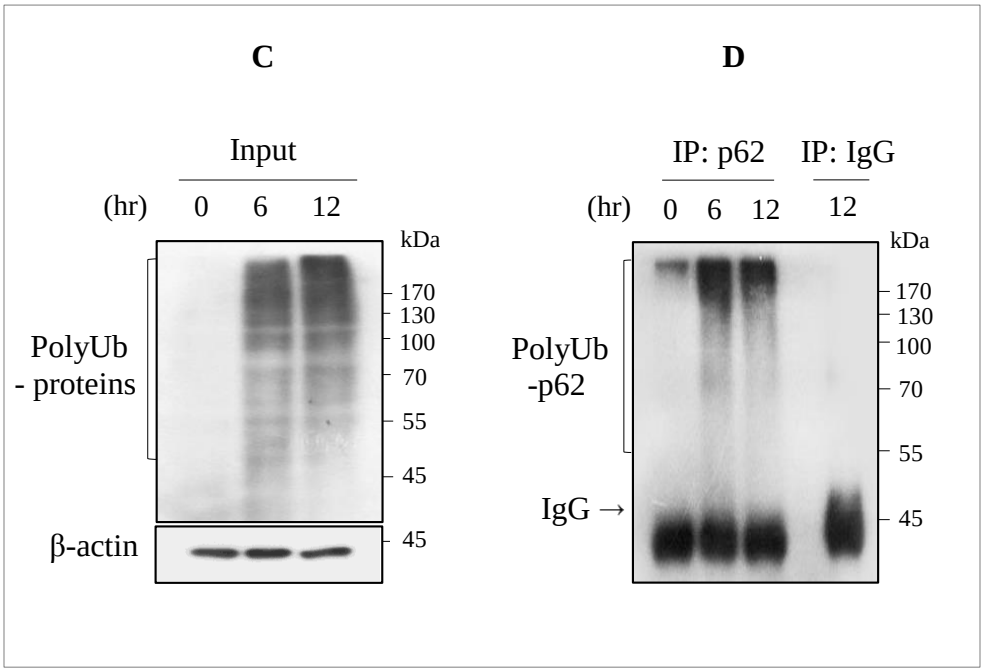
To examine whether high-molecular-weight multibands detected by anti-p62 antibody were PolyUb-p62, immunoprecipitation (IP) for ubiquitin (Ub) and mouse IgG was performed in cells treated with Cd for 6 or 12 h, followed by immunoblotting with anti-p62 antibody (Fig. 4A, B). Inversely, IP analysis for p62 was performed using the same protein lysates, followed by immunoblotting with anti-Ub antibody (Fig. 4C, D). Both results revealed that p62 interacted with Ub. These results indicated that the high-molecular-weight multibands were PolyUb-p62.

Figure 4A-B



p62 interacted with Ub. (A, B) Cells were treated with 24 μM Cd for 6 h or 12 h. Immunoblotting was performed for p62 (input), where immunoprecipitation (IP) was performed with 600 μg of the remaining protein with Ub antibody, followed by immunoblotting for p62.

Figure 4C-D



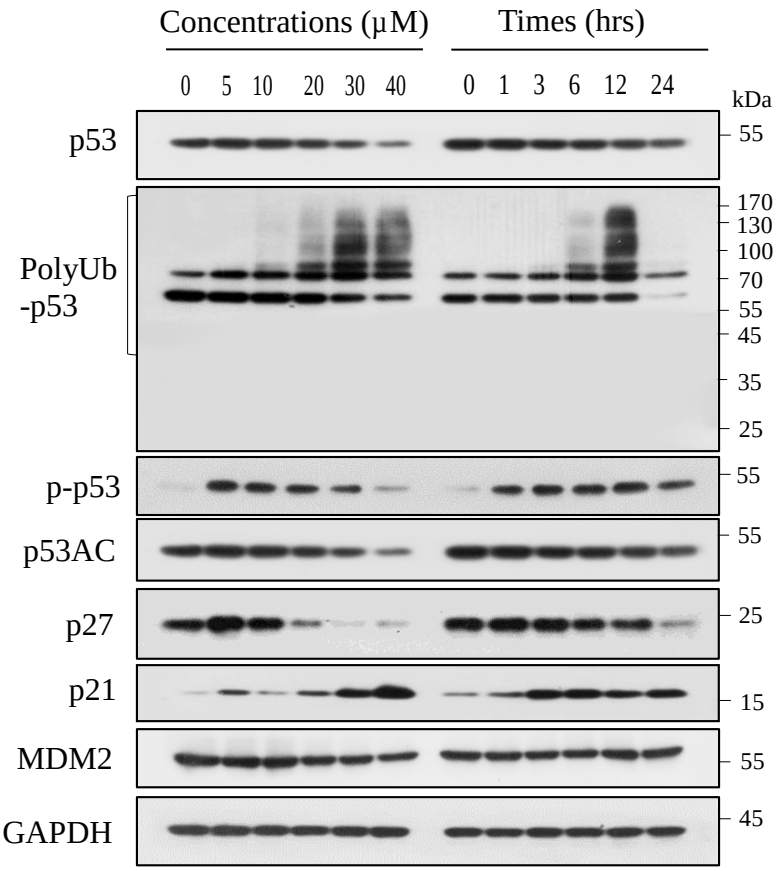
P62 interacted with Ub. (C, D) Cells were treated with 24 μM Cd for 6 h or 12 h. Immunoblotting was performed for Ub (input), where IP was performed with 600 μg of the remaining protein with p62 antibody, followed by immunoblotting for Ub.

3.5. Response of p53 in Cd-exposed Mes13 cells

Previously we found that Cd-exposed MES13 cells induced apoptosis via caspase-dependent PARP-1 cleavage. To determine the response of p53 during Cd-induced apoptosis, MES13 cells were exposed to increasing concentrations of Cd for 18 h and also exposed with half-maximal inhibitory concentration (IC_{50}) of Cd (approximately 24 μM) for different times. To determine whether Cd sensitivity was associated with p53 protein level, we first investigated p53 expression level by immunoblotting. Interestingly, p53 was detected as monomeric and high-molecular-weight forms. Monomeric p53 protein level decreased in a concentration- and exposure time-dependent manner. By contrast, high-molecular-weight forms increased with Cd concentrations- and exposure times. Because p53 protein level is regulated by post-transcriptional modifications, acetylated (Lys379)-p53 (p53Ac) and phosphorylated (Ser15)-p-p53 were evaluated by immunoblotting by using specific antibodies. The p53Ac protein level decreased in parelle with p53 protein level, but p-p53 increased, indicating that Cd caused DNA damage. DNA damage results in cell cycle arrest or apoptotic cell death, we thus examined the expression level of proteins associated with cell cycle arrest and apoptosis. We first examined the cell cycle-regulatory proteins p21 and p27. p21 protein increased in dose- and time-dependent manner. In contrast, p27 expression reduced in line with p53 expression, indicating that Cd-exposed MES13 cells induced G1 cell cycle arrest via p21 expression resulting from DNA damage. Next, we examined the expression of MDM2, a target of p53, and it did not show any changes under Cd-exposed conditions (Fig. 5A). Next, we examined the p53 subcellular localization by immunofluorescence staining. In unstimulated control cells, p53 stainined with rodamin showed diffuse staining pattern

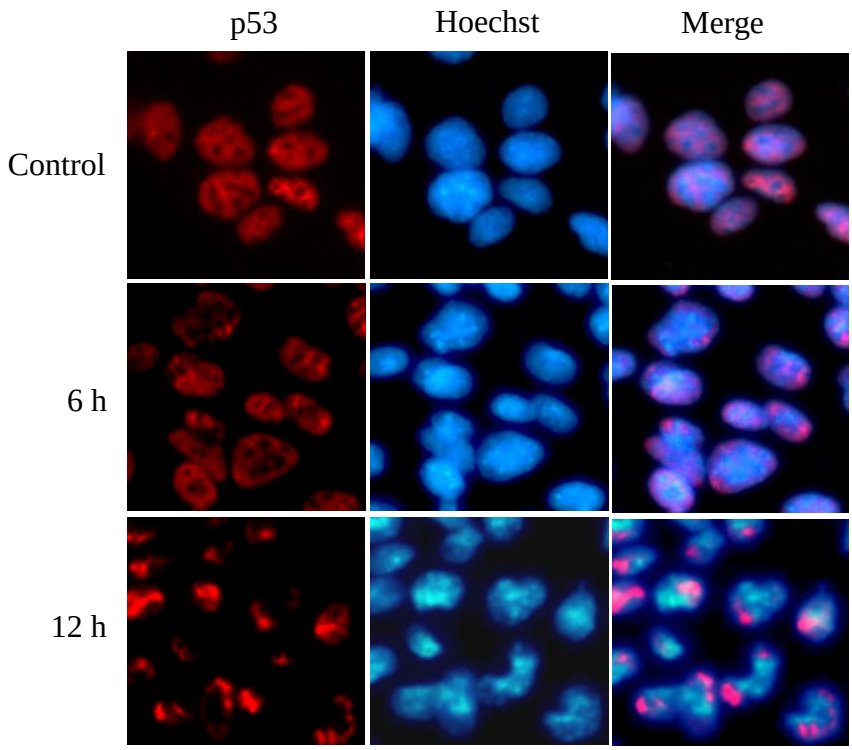
in the nucleus. However, Cd exposure caused p53 aggregation in the nucleus. At 6 hours of Cd exposure, p53 began to aggregate at pheripery of the nuclear membrane (arrows), which was further enhanced at 12 hr, accompanied by nuclear condensation (arrow heads) (Fig.5B). Collectively, p53 activation may be related with high molecular weight forms of p53.

Figure 5A



Effects of Cd on p53 and cell cycle-related proteins in MES13 cells. Cells were treated with increasing Cd concentrations for 18 h or with 24 μM for up to 24 h. Lysates were analyzed by immunoblotting for the indicated proteins.

Figure 5B

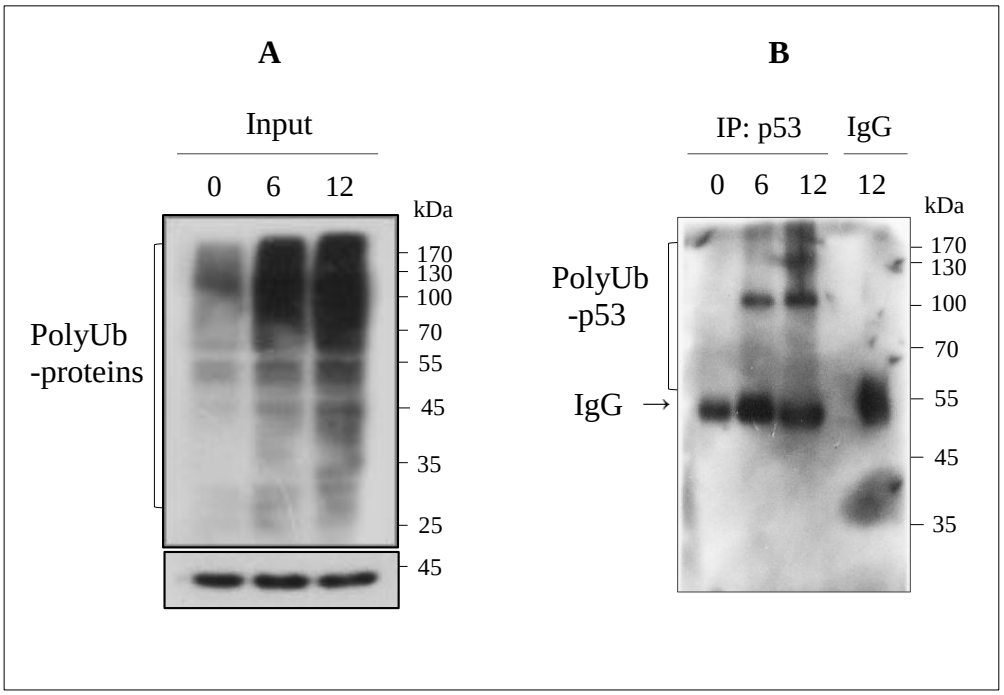


The effects of Cd on subcellular localization of p53. Cells cultured on coverslips were treated with 24 μM Cd, fixed, and then incubated with p53 antibody, and subsequently with rhodamine-conjugated secondary antibodies. Nuclei were counterstained with Hoechst 33342 (blue) and images were acquired with a fluorescence microscope. Scale bar, 25 μm.

3.6. P53 interacts with ubiquitin in Cd-exposed MES13 cells

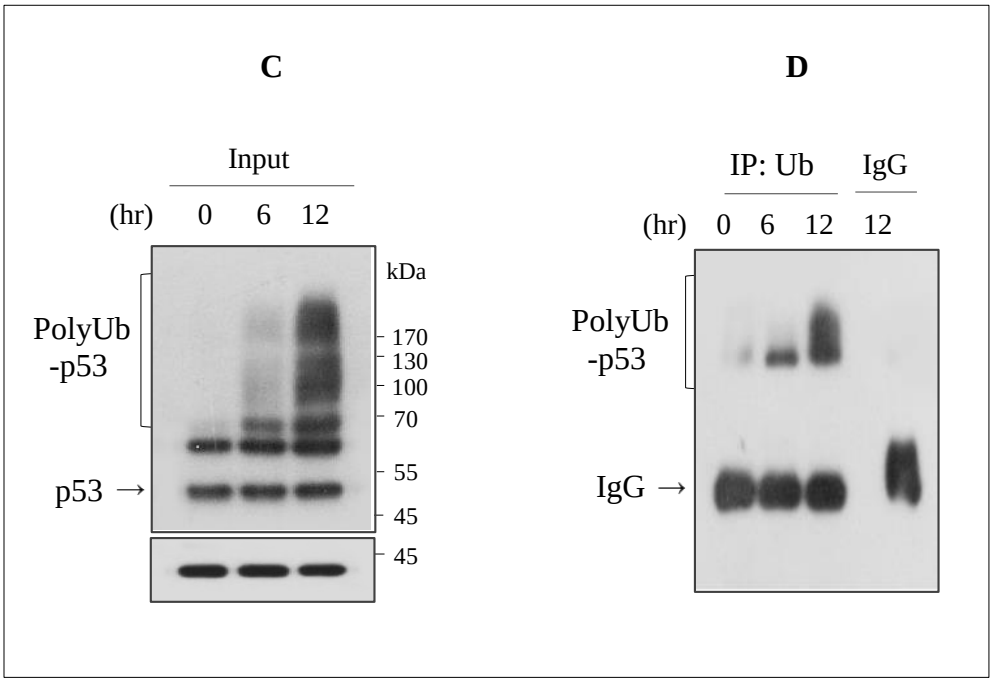
To examine whether high-molecular-weight bands detected by anti-p53 antibody were related with poly-ubiquitination (PolyUb), immunoprecipitation (IP) for p53 and mouse IgG was performed in cells treated with Cd for 6 h or 12 h, followed by immunoblotting with anti-Ub antibody (Fig. 6A, B). IP analysis was also performed for Ub using the same protein lysates, followed by immunoblotting with anti-p53 antibody (Fig. 6C, D). Both results revealed that p53 interacted with Ub These results suggested that p53 protein level may be dependent on ubiquitin-proteasome degradation pathway. To elucidate this, cells were exposed to different concentrations of MG132 with or without Cd. MG132 treatment accumulated Cd-induced polyUb-p62 in Cd-exposed cells and was more effective by lower concentration of MG132. p53 protein accumulated accumulated in MG132 only cells and did not in Cd-exposed cells (Fig. 6F), indicating that p53 protein level was independent on ubiquitin-proteasome pathway. In fact, MDM2 protein level did not show any change by Cd exposure. However, proteasome blocking enhanced Cd-induced apoptosis through caspase-3 and PARP-1 cleavage.

Figure 6A - B



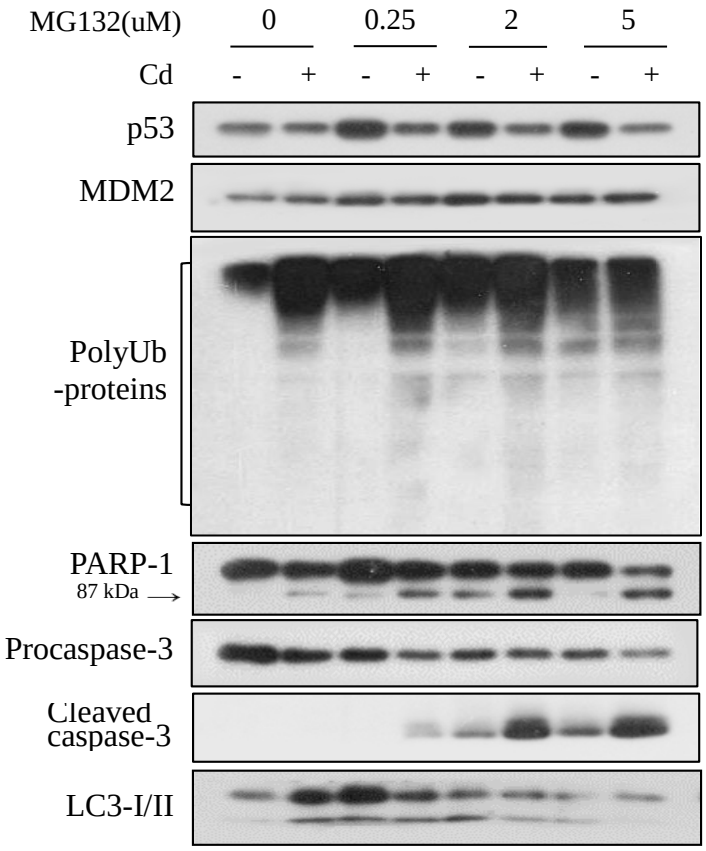
p53 interacted with Ub. p53 interacted with Ub. (A, B) Cells were treated with 24 μM Cd for 6 h or 12 h. Immunoblotting was performed for Ub (input), where IP was performed with 600 μg of the remaining protein with p53 antibody, followed by immunoblotting for Ub.

Figure 6C-D



P53 interacted with Ub. (C, D) Cells were treated with 24 μM Cd for 6 h or 12 h. Immunoblotting was performed for p53 (input), where IP was performed with 600 μg of the remaining protein with Ub antibody, followed by immunoblotting for p53.

Figure 6F

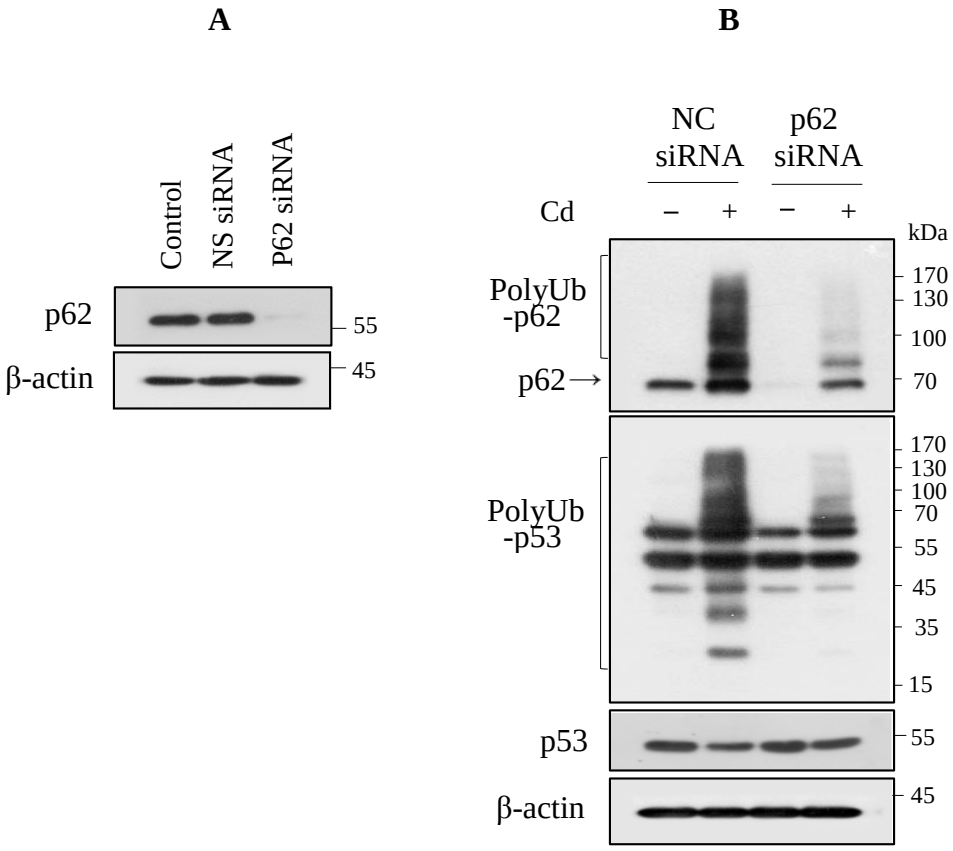


Cd-induced p53 accumulation was independent on proteasome. MES13 cells were pretreated with increasing concentrations of MG132 (0.25~5 μM) for 2 h and followed by Cd (24 μM) treatment for 12 h, harvested, lyzed, and immunoblotted for indicated proteins. GAPDH was used as the loading control.

3.7. Regulation of p53 protein level is related with autophagy in Cd-exposed cells

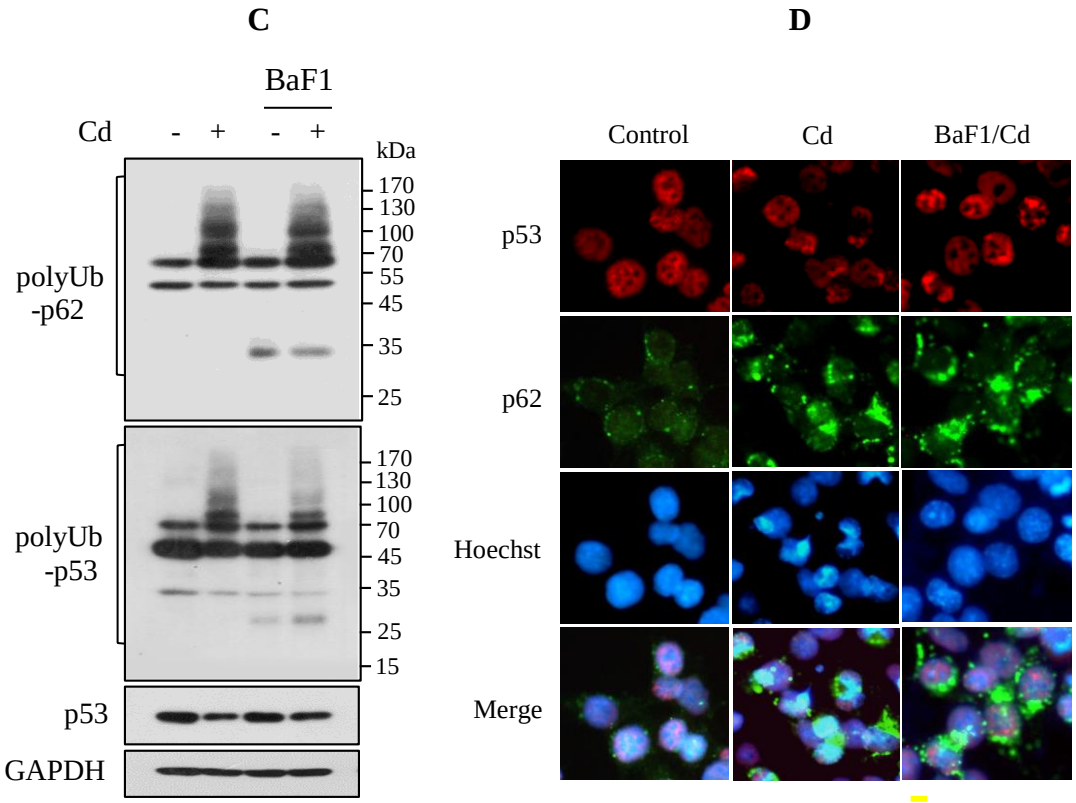
Both p53 function and stability are tightly controlled by Mdm2, which binds to the p53 N-terminus and targets p53 to proteasome for proteolysis (Kubbutat *et al.*, 1997; Haupt *et al.*, 1997). We found downregulation of p53 protein without changing in MDM2 protein level in Cd exposed cells (Fig. 6A). Cd exposure to MES13 cells induced autophagy with increasing monomeric- and polyUb-conjugated p62 forms (Fig. 3A). In this context, we examined the involvement of autophagy in p53 protein regulation in Cd-exposed MES13 cells. Autophagy degrades autophagosome contents via lysosomal-dependent pathway, we thus examined whether p62 targets p53 into autophagosome. p62 knockdown markedly decreased Cd-induced p62 monomer and polyUb p62 level, and p53 level was recovered (Fig. 7A). Next, to elucidate whether p62-targetted p53 recruited into the autophagosome, autophagic flux inhibited by using bafilomycin A1 (BaF1), an inhibitor of autophagosomal and lysosomal fusion. Pretreatment with BaF1 before Cd exposure for 18 h upregulated polyUb-p62, polyUb-p53 and monomeric p53 (Fig.7B). By contrast, inhibition of autophagosome formation by knockdown of *atg5* gene downregulated p62 monomer and polyUb-p62, upregulating p53. These data indicated that p53 protein was regulated by p62-mediated autophagy in Cd-exposed MES13 cells.

Figure 7A - B



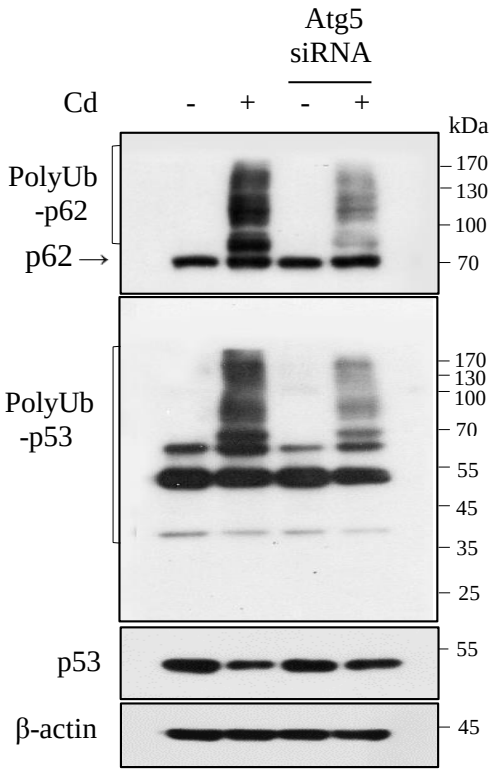
The effects of p62 knockdown on Cd-induced p53 protein. (A) After 24 h, knockdown (KD) efficiency using *p62* specific siRNA was evaluated by immunoblotting for p62. NC, negative control. (B) Cells transfected with NC and p62 siRNA were exposed to 24 μM Cd for 12 h. Cells were harvested, lysed, and expression of indicated proteins.

Figure 7C- D



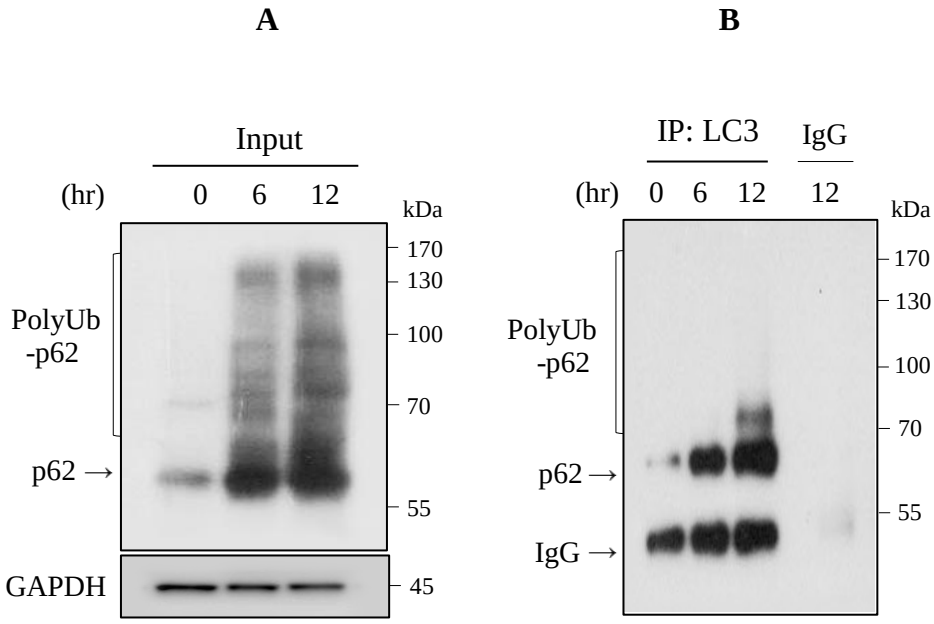
(C) Cells were exposed to 24 μM Cd for 12 h with or without pretreatment with 20 nM BaF1 for 2 h. p62 and p53 levels determined by immunoblotting. GAPDH was used as the loading control. (D) Cells cultured on coverslips were treated with 24 μM Cd, fixed, and then incubated with p53 and p62 antibodies, and subsequently with rhodamine- and FITC-conjugated secondary antibodies. Nuclei were counterstained with Hoechst 33342 (blue) and images were acquired with a fluorescence microscope.

Figure 7E



The effects of autophagy on the Cd-induced p62 and p53 expression. Cells transfected with NC and ATG5 siRNA were exposed to 24 μM Cd for 12 h. p53 and p62 levels determined by immunoblotting. GAPDH was used as the loading control.

Figure 8A-B



LC3 interacted with polyUb-p62 and polyUb-p53. Immunoprecipitation was performed with 600 μg of remaining protein with LC3B antibody or rabbit IgG, and then immunoblotting was performed with p62 or p53 antibodies, respectively. GAPDH was used as the loading control.

DISCUSSION

About 50% Cd in the body accumulates in the kidney (Thevenod, 2003; Nordberg *et al.*, 1975). Thus, kidney is a primary main target of Cd toxicity (Cherian, 1983). Renal accumulation of Cd leads to reduced GFR, polyuria, and tubular dysfunction (Nordberg, 1992; Nordberg, 1975; Felley-Bosco and Diezi, 1987). Cd reabsorbed into proximal tubular cells (Johri *et al.*, 2010) and accumulated, therefore, most of studies for kidney toxicity by Cd focused on proximal tubular cells. However, glomerular damage may lead to reducing tubular reabsorbtion, molecular mechanisms for Cd –induced glomerular damage remains unclear.

Several studies have now demonstrated that Cd exposure is a main cause of renal toxicity , and one of the main mechanisms implicated in renal toxicity is apoptotic cell death, including ER stress-mediated apoptosis, caspase-dependent and -independent apoptosis, and p53-dependent apoptosis (Gobe and Crane, 2010; Shu *et al.*, 201). In porcine renal proximal tubular epithelial cells (LLC-PK1), Cd exposure induced ER stress-mediated apoptosis by inducing ER stress response proteins such as PERK, ATF6, IRE1, and CHOP (Yokouchi *et al.*, 2008). In mouse mesangial cells, Cd exposure triggered mitochondria- and ER stress-mediated apoptosis by inducing caspase-9, caspase-3, and CHOP activation (Liu and Templeton, 2008). Furthermore, Cd induced caspase-dependent and -independent apoptosis in an immortalized kidney proximal tubule cell line (WKPT-0293 Cl.2) by releasing cytochrome *c*, an apoptosis-inducing factor (Lee *et al.*, 2008). In the present study, we found that exposure of MES13 cells to Cd resulted in phospho-p53 accumulation due to DNA damage, and activation of caspase-7 and -3, as shown by western blot using antibodies for their cleaved forms. Caspase activation also led to apoptotic PARP-1 cleavage. CHOP, a

key protein in the induction of ER stress-mediated apoptosis, is involved in cell death through the promotion of protein synthesis and enhancement of oxidative stress in the ER (Marciniak *et al.,* 2004). Furthermore, eIF2α can act as a regulator of either cell death or survival by activating Akt during oxidative stress (Rajesh *et al.,* 2015). In the present study, we found inactivation of eIF2α. IRE1, and p-PERK, which could result in oxidative stress through increased protein synthesis, and ultimately to apoptosis through the induction of CHOP and PARP-1 cleavage. Thus, serious ER stress after Cd exposure promoted apoptosis through inactivation of ER stress sensor with CHOP induction in MES13 cells.

Previous studies have been reported that Cd toxicity is associated with p53-mediated apoptosis in diverse tissues and cells (Fernandez *et al.,* 2003; Aimola *et al*., 2012; Luevano & Damodaran 2014; Tokumoto *et al.,* 2011; Lee *et al.,* 2016). (Tokumoto *et al.,* 2011) reported that p53-mediated apoptosis by Cd in mouse kidney and rat NRK52E cells was associated with down-regulation of ubiquitin-conjugating enzyme Ube2d family, indicating that Cd interrupts Ub-proteasome pathway. However, Cd concentration used might resultin different results in the status of p53 protein; low concentrations of Cd that did not affect on cell viability impaired p53 activity by inhibiting its DNA binding. Therefore, exposure time and p53 concentration used can play a critical role in p53 activity in Cd-exposed cells. In the present study, p53 protein detected as monomeric – and polyUb-conjugated form in Cd-exposed MES13 cells, which was dependent on Cd concentrations and exposure times. Decreasing of monomeric p53 was corresponded by polyUb-p53, indicating that decreasing monomeric p53 may due to poly-ubiquitination of p53. IF staining for p53 revealed that p53 aggregated in the nucleus and cytosol, indicating that nuclear aggregated p53

translocate into the cytosol for degradation. There are both kinds of protein quality control systems in the cells, Ub-proteasome and autophagy. To address the fate of cytoplasmic p53, when we used MG132, proteasome blocker, Cd-induced p53 protein level was not changed, indicating that Cd-induced p53 protein status was not regulated by proteasome activity. In fact, MG132 treatment without Cd upregulated p53 and p27, but did not affect on the Cd-induced cells. In contrast, MG132 upregulated p21 with and without Cd conditions. These results suggested that MG132 blocked proteasome, and p53 and p27 was not under proteasome control.

p62 is a multifunctional protein that contains various protein–protein interaction domains, including N-terminal Phox/Bem1p (PB1), a nuclear localization signal (NLS), a nuclear export signal (NES), an LC3-interacting region (LIR), and a C-terminal Ub-associated (UBA) domain. The most well-characterized function of p62 is a key role in intracellular quality control via selective autophagy, wherein p62 interacts with ubiquitinated cargo through the UBA domain and recruits them into the autophagosome through its interaction with the LIR motif. p62 also facilitates shuttling of substrates to the proteasome. Furthermore, p62 is involved in nuclear quality control by shuttling between the nucleus and cytoplasm via the NLS and NES (Moscat and Diaz-Meco, 2009; Liu et al., 2016). In fact, autophagy is involved in kidney toxicity (Wang et al., 2017; Fujishiro et al., 2018; Luo et al., 2018; Chargui *et al.*, 2011). In our study, despite induction of LC3-II after Cd exposure, p62 levels increased and was polyubiquitinated. In this study, we did not elucidate how p62-mediated autophagic degradation was selectively and transiently impaired, and the underlying molecular mechanisms remain to be determined. Notably, p62 plays critical roles in the formation of ubiquitinated protein aggregates, which act as a

signaling hub for cell death and survival (Moscat and Diaz-Meco, 2009). We hypothesized that polyUb-p62 accumulation after Cd exposure may be associated with Cd-induced cytotoxicity in MES13 cells. We confirmed that p62 KD suppressed Cd-induced apoptosis.

In the present study, polyUb-p53 expression was similar pattern with polyUb-p62 expression. To address whether Cd-induced p53 protein level is regulated by p62, when p62 was knockdowned by using p62 siRNA, polyUb-p53 decreased and upregulated monomeric p53 level, indicating that p53 protein level was regulated by p62. And we found that p53 interacted with p62 via IP analysis. Furthermore, IF staining. revealed that cytosolic p53 colocalized with p62. These results suggested a possibility that p62 might targets p53. Because p62 targets polyUb-proteins to autophagosomes, we examined whether p53 was recruited into the autophagosome by autophagy inhibition.

In this study, Cd exposure induced p53 translocation with p62 into the cytosol, resulting in transient aggregation. Therefore, we investigated how the p53/p62 complex transiently avoided autophagic degradation. Since p62 is an autophagy adaptor protein, p53 can be recruited to the autophagosome by p62. We observed that LC3B interacted with p53, and that autophagy impairment via *Atg5* KD inhibited cytosolic translocation of p53, thereby promoting nuclear accumulation. Furthermore, treatment with BaF1, a late-stage autophagy inhibitor, enhanced Cd-induced p53 and polyUb-p62 aggregation in the cytosol. Therefore, transient accumulation of p53 after Cd exposure could be caused by impaired autophagic degradation. To date, most *in vivo* and *in vitro* studies have evaluated cellular disease mechanisms involved with Cd toxicity. These studies have focused on Cd-induced apoptotic cell death, which is

caused by oxidative stress (Fujiwara *et al.,* 2012; Ravindran *et al.,* 2016). Therefore, we tested whether polyubiquitination of p62 was associated with Cd-dependent reactive oxygen species (ROS) production. We found that treatment with *N*-acetyl cysteine completely suppressed Cd-induced polyubiquitination of p62 and upregulated monomeric p53 expression. Thus, Cd-induced oxidative stress may be critical in regulating p62 polyubiquitination and subsequent p53 translocation. However, further studies are required to confirm these results.

In conclusion, we demonstrated that p62-mediated p53 accumulation and translocation was critical in Cd toxicity in mouse kidneys, where nuclear p53 was targeted by polyUb-p62 to the autophagosome. Therefore, p53-p62 signaling pathway may serve as a therapeutic target for treating kidney diseases.

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감사의 말씀

긴장되고 설레는 마음으로 시작했던 대학원 생활이 어느덧 2 년이라는 시간이 지나게 되었습니다.

물론 어렵고 힘든 시간도 있었지만 하나씩 배워갈 때의 기쁨이 더 컸던 시간들이였던거 같습니다.

처음 대학원에 들어와 제 지도 교수님이신 오선희 교수님을 뵈었을 때를 아직도 잊지 못합니다.

대학교와는 너무도 달랐던 대학원 석사 과정에 힘들어할 때 저를 다잡아 주시고 학교생활뿐만 아니라 여러 사회생활이나 일생에 살아가면서 뼈와 살이 되는 가르침이 있었기에 제가 이렇게 많은 걸 배우며 졸업하게 되었습니다.

그냥 교수님이라는 말보다는 엄마 같은 교수님 이라는 말이 어울리신 존경하는 오선희교수님 너무 진심으로 감사드립니다.

바쁜신데도 불구하고 제 논문을 심사해주신 최철희 교수님 기성환교수님 너무 감사드립니다.

대학원 오기 전 대학교에서 실험실 생활을 많이 가르쳐주시고

지도해 주셨던 최필선 교수님 감사드립니다.

대학원 생활하면서 많은 조언도 해주고 힘들거나 말 못 할 고민 있을 때 높은 선배님이셔서 다가가기 조심스러웠는데 항상 친동생처럼 잘 챙겨주시고 신경 써줬던 수진이언니랑 관환이오빠 감사합니다.

또 많은 시간 동안 옆에서 격려의 말 해주면서 모든 걸 이해해줬던 화연이 가혜 수아 효지 고맙습니다.

마지막으로 평생 네 편이자 늘 잘 되기만을 바라고 옆에서 응원해준 우리 가족들 너무 고맙습니다

비가 오나 눈이 오나 학교 가는 길 편하게 가라고 등하교 시켜주시고 이런저런 일이든 항상 들어주고 같이 고민해줬던 우리 가족 엄마 아빠 그리고 첫째 동생 성진이 우리 막둥이 영일이까지 너무 고맙습니다.

대학원 생활을 하면서 연구를 통한 지식과 더불어 실험실 생활을 통해 한층 더 성장할 수 있었던 거 같습니다.

아직 시작에 불과하지만 앞으로 새 출발이 더 기대되고 가슴 뜁니다.