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석사학위논문

Studies on the enhanced cytotoxic effect of cisplatin in epidermal growth factor-primed kidney epithelial cells

조선대학교 대학원

약 학 과

남 현 정

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상피세포 성장인자 노출 신장상피세포 에서 Cisplatin 독성 증폭에 관한 연구

2012년 2월 24일

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Figure 3. Up-regulation of Pin1 expression by EGF in NRK-52E cells. After serum starved for 18h NRK-52E cells were incubated with growth factors, EGF, IGF-1, PDGF for 24h and Pin1 expression was detected by Western blot analysis as described under Material and methods. Equal loading amounts of proteins were verified by actin immunoblot.

Figure 4. Significant role of Pin1 on cell viability of cisplatin-exposed NRK-52E cells. (A) Reduction of cell death by Pin1 inhibition. NRK-52E cells were incubated with cisplatin for 24h in existence or absence of juglone(10uM). (B) Decrease of cell survival in condition of Pin1 induction. We established Pin1-overexpressing NRK-52E cells by multiple infections (15

국문 초록

상피세포 성장인자 노출 신장상피세포 에서 Cisplatin 독성 증폭에 관한 연구

남현정

지도교수: 유진욱

조선대학교 대학원 약학과

Cisplatin (CIS – Diamminedichloroplatinum (II))은 화학 요법에 사용되는 매우 효과적인 항암제이다. 특히, cisplatin과 doxorubicin을 병용시 고환, 난소, 방광, 및두경부종양에 효과적이다. Cisplatin은 주로 세포의 DNA와의 반응에서 부가물을 형성하면서 DNA를 손상시키는 항암제로 간주된다. 그러나 치료에 있어서 이 약물의 사용에 몇 가지 제한이 있다. 여기에는 특히, 유소아환자에서 정상적인 세포의 DNA에 부작용을 일으키는 것, 암 세포 내부에 cisplatin 축적의 감소 등이 포함되며, 가장 심각한 부작용은 신독성으로 알려져 있다. 지금까지 이러한 부작용 및 세포독성 현상에 대한 연구가 많이 진행됐지만 아직 기전에 대해 명확하게 밝혀지지 않았다. Cisplatin의 여러 부작용들 가운데, 본인은 연령과 관련된 신독성에 중점을 두었다. 본인은 미성숙및 성숙

흰쥐에 Cisplatin을 처치한 후 신장조직표본으로부터 Cisplatin 독성이 미성숙개체에서 증가함을 밝혔다. 그 기전을 규명하기 위해서 각종 세포성장인자 (Epidermal growth factor, Platelet-derived growth factor, Insulin-like growth factor1) 처치후 Cisplatin 세포독성을 NRK-52E, Rat 신장상피세포에서 평가하였다. 그 결과, EGF 처치시 Cisplatin의 세포독성이 유의성 있게 증가함을 규명하였다. 이는 미성숙 흰쥐에서 다량 분비되는 EGF가 Cisplatin의 증가된 독성과 관련이 있음을 시사한다.

Pin-1은 세포증식과 생존, 면역체계를 조절하는 데 관여하는 물질로서 주로 암세포에서 과다 발현된다. 흥미롭게도, 신장상피세포에서 EGF처치는 Pin-1의 발현증가를 일으켰으며, Pin-1 억제시 Cisplatin의 세포독성이 유의성 있게 감소되었다. 반대로 Pin-1 과발현 NRK-52E 세포에서는 GFP 과발현 세포에 비해서 세포독성이 유의성 있게 증가하였다. 이 결과는 EGF에 의한 Pin-1 발현증가가 Cisplatin 독성과 밀접한 관련이 있음을 제시한다. Cisplatin에 대한 NRK-52E 세포 독성은 apoptosis(Bax, PARP, Cleaved caspase-3)및 autophagy(LC-3B, Beclin-1, ATG7) marker 단백질의 발현변화를 일으켰으며, Pin-1은 이들 단백질들의 발현에 영향을 미치고 있었다. 이상의 결과는 EGF 노출시 신장 상피세포의 Cisplatin 독성에 대한 민감성이 증가하며, 이는 Pin-1 발현에 의한 apoptosis 및 autophagy 신호 교란과 관련이 있음을 시사한다.

ABSTRACT

Studies on the enhanced cytotoxic effect of cisplatin in epidermal growth factor-primed kidney epithelial cells

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Cisplatin is a very effective antitumor agent used in chemotherapy. Particularly, it is effective to treat tumors in testis, ovary, bladder, and head and neck when used it in conjunction with doxorubicin. Cisplatin is considered a DNA-damaging anticancer drug, mainly forming adducts in its reaction with cellular DNA. However, there are several limitation in the use of the agent in clinics including an adverse effect on normal cellular DNA, especially in young patients, and a reduction in cisplatin accumulation inside cancer cells, so on. It has been well known that the most serious side effect is nephrotoxicity. Until now, a lot of researches on these side-effects of cisplatin have progressed, but the exact molecular mechanism is still not clearly unveiled. Among the several side-effects of cisplatin, I focused on the age-related nephrotoxicity. After injection of cisplatin to immature and mature-rats, I found out

that nephrotoxicity of cisplatin increased in immature object. After incubation with various cell growth factors (Epidermal growth factor (EGF), Platelet-derived growth factor (PDGF), Insulin-like growth factor-1 (IGF-1)), Cisplatin cytotoxicity was evaluated in NRK-52E, a rat kidney epithelial cells. As a result, I identified that the cytotoxicity of cisplatin significantly potentiated when it was cotreated with EGF. This suggests that the EGF secreted in a large amounts in immature rat is associated with increased cisplstin cytotoxicity. As a substance which is involved in control of cell proliferation, survival and immune system, Pin1 is often overexpressed in cancer cells. Interestingly, EGF treatment in kidney epithelial cells caused increased expression of Pin1 and cytotoxicity of cisplatin was significantly reduced by Pin1 inhibition. On the contrary, cisplatin cytotoxicity was significantly enhanced in Pin1 overexpressing NRK-52E cells compared to GFP overexpressing cells.The data suggests that Pin1 expression increase by EGF is closely related with the increased cisplatin toxicity. Cisplatin treatment in NRK-52E cells induced changes in expression of apoptosis (Bax, PARP, Cleaved caspase-3) and autophagy (LC-3B, Beclin-1, ATG7) marker proteins and the Pin1 altered the expression of these proteins. These results imply a susceptibility of kidney epithelial cells to cisplatin toxicity may be dependent on exposure of cells to EGF.

Keywords : Cisplatin, Pin1, Epidermal growth factor, Kidney, Apoptosis, Autophagy.

1. Introduction

Cisplatin (cis-Diamminedichloroplatinum(II)) is one of the widely used chemotherapeutic agent. Cisplatin has a structure that the platinum atom is coordinated by two chlorines and ammonias ([Pt (NH3)2Cl2]) (1). Cisplatin stably binds to guanine in DNA and RNA, and causes unwinding and shortening of DNA helix (2). As a combined treatment agent with vinblastin, this is particularly effective in testicular tumors. When used in conjunction with doxorubicin, it is also effective in ovarian, bladder, breast tumors (3, 4). In many cancers, the effectiveness of cisplatin-chemotherapy has been proved, but unfortunately the agent has several kinds of side-effects. They include nephrotoxicity, nausea and vomiting, ototoxicity, and electrolyte disturbance. Among them, the dose-limiting side effect is damage to kidney (5, 6).

Clinical sudies have shown that cisplatin at doses in the range 30-100 mg/㎡ is tolerable in young patient. However, the dose should be reduced in case of kidney impairment (7, 8, 9). Recent clinical records show that severe nephrotoxicities were found in pediatric patients rather than old patients (10). So, there seem to be specific causality between age and nephrotoxicity. But, so far, there are no clearly identified mechanisms for the reason why this events happen. In the present study, we tried to investigate what mechanism is involved in age-dependent cisplatin-induced nephrotoxicity. First, we assessed histopathological condition in cisplatin-treated kidney tissues from 3 weeks and 20 weeks old rats.

Considering high levels of growth factors are secreted in young-aged group, we hypothesized that growth factors might affect the kidney toxicity of cisplatin (12, 13). Using diverse growth factors-treated NRK-52E cells (a rat kidney epithelial cell line), we found that EGF (Epidermal Growth Factor)is closely related to the cisplatin-induced toxicity**.** EGF plays an important role in the regulation of cell differentiation, cell proliferation, cell survival and cell growth by binding to EGFR (EGF receptor) (12, 13).

Pin 1, peptidyl-prolyl cis/trans isomerase, functions as a post phosphorylation controller of regulating protein function, binding to a subset of proteins (14, 15, 16). Many Studies showed

that up or down-regulation of Pin1 may play an important role in various diseases like cancer, Alzheimer's disease (17, 18). Alterations in the level of Pin1 would affect the state of activation of cells death-related proteins and signaling pathways (15, 17). Interestingly, we found that Pin1 expression was selectively up-regulated by EGF treatment in NRK-52E cells.

We then tried to identify which cell death pathways are responsible for cisplatin-induced nephrotoxicity in associated with enhanced Pin1. There are several studies showing apoptosis and autophagy pathways are involved in cisplatin-mediated cell death (19, 20). As a form of programmed cell death, apoptosis is organizational control and the normal process that takes place in the cells (19). Autophagy plays a role as a protective substance mediating recycling of intracellular components and becoming an alternative source of energy when nutrients are scarce (19). In this study, cisplatin resulted in cell death via either apoptosis (Bax, PARP, Caspase-3 related) or autophagy(LC-3B, ATG-7, Beclin-1 related) in NRK-52E cells.

We further found that Pin1 inhibition reduced cisplatin-induced cell death by blocking apoptosis or autophage pathways. Vise versa, Pin1 overexpression increased cisplatin-induced cell death via apoptosis pathway. Overall, our results indicated that increase in EGF level arouses susceptibility of kidney epithelial cells to cisplatin-induced cytotoxicity via Pin1 pathway. Clinicaly abundance of EGF in children can cause a increase in Pin1 level, which would affect the susceptibility of cells to cisplatin.

2. Materials and Methods

2-1. Materials

The Pin1, Bax antibody were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Bcl2, LC-3B, Beclin-1, Cyclin D1, ATG12, ATG7, PARP, Cleaved Caspase-3 and horseradish peroxidase-conjugated anti-rabbit, anti-mouse IgGs were purchased from Cell Signaling Technology (Beverly, MA). Alkaline phosphatase-conjugated donkey anti-mouse IgG was provided by Jackson Immunoresearch Laboratories (West Grove, PA). PDGF, EGF were purchased from PeproTech (Rocky hill, NJ). Anti-actin antibody, IGF-1, Juglone, most of the reagents used for molecular studies were obtained from Sigma (St. Louis, MO). Cisplatin was supplied by Korea United Pharm.INC.

2-2. Cell culture and Pin1 retroviral infection

NRk-52E cells were plated in a culture dish, and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 g/ml streptomycin. Pin1 was stably expressed in NRK-52E using an MSCV-GFP retrovirus system. Pin1 cDNA was subcloned into MSCV-GFP retroviral vector and phoenix cells (a packaging cell line) were transfected with MSCV-GFP (control) or MSCV-Pin1-GFP (Pin1 overexpressed) plasmid. Supernatants containing ecotrophic replication-incompetent retroviruses were collected and then stored at -80℃ until required. 20% confluent NRK-52E were multiply infected (15 times) with retrovirus particles. Intensities of infection were monitored by Western blot analysis using a specific antibody.

2-3. MTT assay for cell viability

Viable adherent cells were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (2 mg/ml) for 4 h. Media were then removed and the formazan crystal-stained cells were dissolved in 200 □l dimethylsulfoxide. Absorbance was assayed at 540 nm using a microtiter plate reader (Berthold Tech., Bad Wildbad, Germany).

2-4. Protein extract and immunoblot analysis

After washing sterile PBS, the NRK-52E cells were lysed in EBC lysis buffer containing 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β- glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 □g/ml leupeptin. Cisplatin injected rat kidneys were grinded into small piece of tissue and they were lysed in EBC lysis buffer having the same composition. The cell lysates were centrifuge at 10,000*g* for 10 min to remove the debris, and the proteins were then fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper, and the proteins were immunoblotted with the specific antibodies. Horseradish peroxidise- or alkaline phosphatase-conjugated anti-IgG antibodies were used as the secondary antibodies. The nitrocellulose papers were developed using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/4-nitroblue tetrazolium (NBT) or an ECL chemiluminescence system. For chemiluminescence detection, the LAS3000-mini system (Fujifilm, Tokyo, Japan) was used.

2-5. Cisplatin treatment & histopathology

Cisplatin (10 mg/kg) was intraperitoneally injected to 3 weeks-(n=5) and 20 weeks-old (n=5) male Sprague Dawley (SD) rats and 3 days after kidneys were dissected, fixed immediately for 12 h in 10% neutralizing formaldehyde and embedded in paraffin. Tissue sections (4 μm) were mounted on common slides for staining with hematoxylin and eosin. A certified pathologist scored samples in a blinded fashion. In order to biochemically assess kidney toxicity, the levels of total protein, glucose and lactate dehydrogenase (LDH) in urine were determined.

2-6. Data analysis

One way analysis of variance (ANOVA) procedures were used to assess significant differences between treatment groups. When treatment was found to have a significant effect, the Newman-Keuls test was used to compare multiple group means. Statistical significance was accepted at either $p<0.05$ or $p<0.01$.

3. Results

3-1. Cisplatin induced more severe toxicity in young rather than in old rat kidney.

Histological examination showed that single injection of 10 mg/kg cisplatin increased necrotic cell death more in kidneys from 3 wks old rats than those from 20 wks old rats (Fig. 1A). In comparison to 20 wks old rat samples, levels of total protein, glucose and LDH in urine were also more enhanced in 3 wks old rats injected with cisplatin (Fig. 1B).

3-2. Cisplatin-induced cytotoxicity in EGF treated NRK-52E Cells.

We assessed whether cisplatin cytotoxicity in kidney epithelial cells is affected by exposure of growth factors. EGF, PDGF and IGF-1 were used as representative growth factors. NRK-52E cells were serum-starved for 18 h, incubated in 5% CO2, 37℃ condition, and pretreated with 0.3-30 EGF for 24 h, and then treated with various concentrations of cisplatin for an additional 24 h. Cisplatin-induced cytotoxicity was assessed by MTT assay. 1-10 μM cisplatin treatment without growth factor exposure caused no significant change in the cell viability of NRK-52E cells. In contrast, EGF and cisplatin co-treated group showed significant decrease in the cell viability at the concentrations of 1-10 μM cisplatin. Cell viability was decreased in a concentration-dependent manner after treatment with 1-300 μM cisplatin (Fig. 2A). MTT assays were also performed to determine the effect of IGF-1 and PDGF pretreatments on cisplatin-induced cytotoxicity in NRK-52E cells in the same way. Cell viability was decreased in a concentration-dependent manner after treatment with 1-300 μM cisplatin. But, there was no significant difference between single cisplatin treated group and IGF-1/cisplatin and PDGF/cisplatin co-treated groups (Fig. 2B and C).

3-3. Effects of EGF on Pin1 expression.

A previous study has shown that Pin1 plays an important role in the development of cancer and Alzheimer's disease (17). Pin1 regulates several type of signalling pathways involved in the regulation of cell survival (14). Conceived this point, we investigated whether EGF-mediated increase in susceptibility of cisplatin is related with Pin1 expression. When we

monitored the level of Pin1 expression, the protein levels were increased in a concentration-dependent manner by 24 h incubation of 0.3-30ng/ml EGF (Fig. 3).

3-4. Juglone inhibits cisplatin-induced cell death of NRK-52E cells.

We then hypothesized that the enhanced Pin1 is associated with cisplatin cytotoxicity. Juglone selectively inhibits Pin1 activity by inactivating cysteine residue in active site of Pin1 (22). We tested Pin1 inhibition effect using Juglone in NRK-52 cells to identifiy whether Pin1 is involved in cisplatin toxicity. 10 μM juglone treatment significantly reduced cell death in response to cisplatin (Fig. 4A). To confirm this result, we successfully established GFP-overexpressing- and Pin1-overexpressing-NRK-52E cells. Cisplatin-induced cell death was more severe in Pin1-NRK-52E cells than GFP-NRK-52E cells (Fig. 4B). These data demonstrate that higher Pin1 expression is closely related with cisplatin sensitivity in kidney epithelial cells.

3-5. Increase of cell death markers in NRK-52E cells.

Autophagy appears as a programmed mechanism to control cell survival and death. A previous study has shown that autophagy occurs ahead of apoptosis and apoptosis is not expressed until 12 h after cisplatin exposure (19).

We then confirmed the expression tendency of marker proteins related with apoptosis and autophagy in NRK-52E cells treated with cisplatin. As autophagy markers, LC-3B, beclin-1, ATG-7 expression increased by 10 h incubation of cisplatin in a concentration-dependent manner (Fig. 5A). Whereas, the cells were exposed to cisplatin for 48 h, the apoptosis markers; Bax, PARP, cleaved caspase-3 expression were all enhanced in the same manner. μThese results imply that early event caused by cisplatin is autophagy and then the apoptosis system would be activated as exposure time is prolonged (19) (Fig. 5B).

3-6. Pin1 enhanced cisplatin-induced apoptosis.

Because both autophagy and apoptosis are activated by cisplatin treatment, we then wondered which cell death pathways are more significantly related with the function of Pin1. Cisplatin was treated in Pin1-NRK-52E cells for 48 h and we assessed expression levels of apoptosis markers, Bax, PARP and cleaved caspase-3 by Western blot. They are all increased in cisplatin-treated Pin1-NRK-52E cells compared to GFP-NRK-52E cells. Under the expectation

that Pin1 inhibition may attenuate apoptosis, we determined the effect of juglone (10μM) on the Bax expression. Result showed that Bax expression was decreased by juglone (Fig. 6A).

We then compared expression level of autophagy markers, LC-3B, ATG-7, beclin-1 between GFP-NRK-52E and Pin1-NRK-52E cells. The protein expression levels of LC-3B, ATG-7, beclin-1 in Pin1-NRK-52E cells are lower than those in GFP-NRK-52E cells. We also assessed the LC-3B expression levels in untreated and juglone pretreated NRK-52E cells. LC-3B is more increased in juglone-pretreated cells than control cells (Fig. 6B).

3-7. Pin1 expression is elevated in kidney of cisplatin-injected 3 wks old rats.

Based on previous results, we assessed the level of Pin1 expression in kidney from 3 wks old rats and 20 wks old rats by Western blot analyses. We found a significant difference between control groups. The Pin1 expression in 3 wks old rats control group is a higher extent than those in 20 wks old rats control group (Fig. 7).

4. Discussion

Cisplatin has anti cancer effect on tumors in ovary, bladder, testis, breast (3, 4). However, there are several weakness in clinical use. Especially, nephrotoxicity is one of the severe side-effects induced by cisplatin. According to a previous study, its hydrolysis process is essential to react with proteins, DNA and RNA (1). However it would not happen frequently in blood because of the fact that chloride ion concentration is a quite high and it disturbs that cisplatin binds to proteins. In contrary to this, after diffusing through the cell walls hydrolysis process would occur easily due to the lower chloride ion concentration inside the walls.

Water is involved in hydrolysis reaction equilibria of cisplatin (1). Cisplatin may be changed into an active and dominant form under the water abundant condition and it can cause the cytotoxicity in kidney cells. One of the recent studies presents that cisplatin induces nephrotoxicity more commonly in pediatric patients than in adult patients (10, 11). However, the exact mechanism for cisplatin-induced cytotoxicity in the kidnev of younger patients has not been cleared. In this study, we focused on the reason and mechanism of cisplatin-induced nephrotoxicity related to age.

Biochemical markers and pathological data showed that necrotic cell death was more severe in kidneys from cisplatin-injected 3 wks old rats than those from 20 wks old rats. In this point, we hypothesized that some factors related to young-age would effect seriously on increase of cisplatin-induced nephrotoxicity.

To identify which factor would be involved in toxicity event of cisplatin in NRK-52E cells, we tested the effect of EGF, IGF-1 under the hypothesis that growth factors are secreted much more in young than old person (12, 13).

The results showed that cell viability was sharply decreased in EGF and cisplatin cotreated group and we assumed that EGF played a critical role in cisplatin-induced cytotoxicity. However IGF-1 or PDGF treatement had no effect on cytotoxicity of cisplatin in the renal epithelial cells.

Next, we tried to identify that there a potential signalling molecule stimulated by EGF. After several attempts to search it, we found that EGF treatment increased the level of Pin1 expression in NRK-52E cells. As a post phosphorylation controler of regulating protein

function (14, 15, 16), Pin1 may play an important role in cancer and Alzheimer's disease (17). Many studies showed that Pin1 overexpression is closely related with angiogenesis required for cancer growth and is critical to protection from anticancer agents in cancer cells (23, 24).

From these previous studies, we tested whether Pin1 expression is associated with cell death induced by cisplatin. We found that cisplatin-mediated cell viability change was dependent on the Pin1 expression levels. When Pin1 expression was up-regulated cisplatin-cytotoxicity was potentiated. In contrast, cisplatin sensitivity in NRK-52E cells was diminished in Pin1 inhibitor-treated condition.

We also determined the level of Pin1 expression in kidney from 3 wks old and 20 wks old rats by Western blot analyses. We confirmed Pin1 was highly expressed in kidney samples from 3 wks old rat than 20 wks old rat samples. Unlike many studies on cancer cellls, our results showed that Pin1 is a key factor for cytotoxicity increase of cisplatin in rat kidney epithelial cells. It could be possible that EGF-stimulated Pin1 expression would stimulate cell death pathway in kidney epithelial cells. We then wondered that how Pin1 could connect next step to cell death.

Previous studies have reported that cisplatin-mediated cell death is closely related to autophagy or apoptosis pathway (19, 20, 21). We confirmed whether cisplatin induced cell death via apoptosis or autophagy pathway and how it changed that the tendency of expression of apoptosis marker proteins(Bax, PARP, Cleaved caspase-3) and autophagy marker proteins(LC-3B, Beclin-1, ATG-7) by Western blot assays.

The results revealed that autophagy marker proteins are increased in a concentration-dependent manner by 10h incubation of cisplatin. And there was no change in the expression of apoptosis marker proteins at that time point (data not shown). Vise versa, the apoptosis marker proteins exhibited increasing tendency in expression 48h after cisplatin treatment and autophagy marker proteins showed no change in their expression at that time point (data not shown).

It would be needed to check both the additional apoptosis markers such as M30, BV2, P41, P53, survivin and autophagy markers such as PTEN, TSC1/TSC2, ATG5, ATG12, ATG16 for the future study on cell death pathways related to cisplatin-induced cytotoxicity.

We also initially guessed that cisplatin-incuced cytotoxicity event might happen through 5' adenosine monophosphate-activated protein kinase (AMPK) pathway. We investigated the effect of cisplatin treatment on the expression of phosphorylated acetyl CoA carboxylase (P-

ACC) and phosphorylated AMP-activated protein kinase(P-AMPK). However, we found no significant changes in the expression of them compared to that of apoptosis and autophagy marker proteins (data not shown). Hence, cisplatin induced cell ceath in kidney epithelial cells seems not to appear via AMPK pathway.

We then hypothesized that Pin1-overexpression affect the expression of those markers. In comparison to GFP-NRK-52E cells, apoptosis marker, Bax was remarkably increased and representative autophagy marker, LC-3B was a little increased when the Pin1 overexpressing NRK-52E cells were exposed to cisplatin. On the contrary, Bax expression was decreased or not increased and LC-3B was increased compared to control when Pin1 was inhibited by juglone.

Considering the previous studies showing the role of Pin1 in autopahgy and apoptosis (24, 25, 26), Pin1 may be involved in both pathways, stimulated by cisplatin. We thought there would be a need to study searching for that. In addition, we would need to perform further detailed studies about the proteins and pathways such as MAPKinase or PI3Kinase pathway associated with the relation between Pin1 and autophagy or apoptosis (27, 28, 29, 30).

In summary, increased Pin1 by EGF potentiated the cytotoxicity of cisplatin to a significant extent in kidney epithelial cells and inhibition of Pin1 may be the significant point in the reduction of cisplatin-induced cytotoxicity (31, 32, 33).

5. References

1. Reedijk J, Lohman PHM. Cisplatin: synthesis, antitumour activity and mechanism of action. Pharm Weekbl [Sci]. 1985;7:173-80.

2. Sedletska Y, Giraud-Panis MJ, Malinge JM. Cisplatin is a DNA-damaging antitumour compound triggering multifactorial biochemical responses in cancer cells: importance of apoptotic pathways. Curr Med Chem Anticancer Agents. 2005;5:251-65

3. Ikeda M, Matsumoto K, Niibe Y, Satoh T, Fujita T, Iwamura M, Ishiyama H, Kotani S, Hayakawa K, Baba S. The radiotherapy with methotrexate, vinblastine, doxorubicin, and cisplatin treatment is an effective therapeutic option in patients with advanced or metastatic bladder cancer. J Radiat Res. 2011;52:674-9.

4. Davidson D, Grenier J, Martinez-Marignac V, Amrein L, Shawi M, Tokars M, Aloyz R, Panasci L. Effects of the novel DNA dependent protein kinase inhibitor, IC486241, on the DNA damage response to doxorubicin and cisplatin in breast cancer cells. Invest New Drugs. 2011: Epub.

5. Miller RP, Tadagavadi RK, Ramesh G, Reeves WB. Mechanisms of Cisplatin nephrotoxicity. Toxins (Basel). 2010;2:2490-518.

6. Hanigan MH, Townsend DM, Cooper AJ. Metabolism of cisplatin to a nephrotoxin. Toxicology. 2009;257:174-5.

7. Kovac V, Zwitter M, Rajer M, Marin A, Debeljak A, Smrdel U, Vrankar M. A phase II trial of low-dose gemcitabine in a prolonged infusion and cisplatin for malignant pleural mesothelioma. Anticancer Drugs. 2011: Epub.

8. Cartei G, Contessi E, Comelli M, Cattaruzzi E, Galletti D, Bramezza M. Early impairment of kidney function after cisplatin administration. J Chemother. 1989;1:1285-6.

9. Moon HH, Seo KW, Yoon KY, Shin YM, Choi KH, Lee SH. Prediction of nephrotoxicity induced by cisplatin combination chemotherapy in gastric cancer patients. World J Gastroenterol. 2011;17:3510-7.

10. Fujieda M, Matsunaga A, Hayashi A, Tauchi H, Chayama K, Sekine T. Children's toxicology from bench to bed--Drug-induced renal injury (2): Nephrotoxicity induced by cisplatin and ifosfamide in children. J Toxicol Sci. 2009;34:251-7.

11. Pietilä S, Ala-Houhala M, Lenko HL, Harmoinen AP, Turjanmaa V, Mäkipernaa A. Renal impairment and hypertension in brain tumor patients treated in childhood are mainly associated with cisplatin treatment. Pediatr Blood Cancer. 2005;44:363-9.

12. Carpenter G, Cohen S. Epidermal growth factor. The Journal of Biological Chemistry. 1990;265:7709–12.

13. Herbst RS. Review of epidermal growth factor receptor biology. International Journal of Radiation Oncology, Biology, Physics. 2004;59:21–6.

14. Lu KP, Hanes SD, Hunter T. A human peptidyl-prolyl isomerase essential for regulation of mitosis. Nature. 1996;380:544-7.

15. Lu KP, Finn G, Lee TH, Nicholson LK. Prolyl cis-trans isomerization as a molecular timer. Nat Chem Biol. 2007;3:619-29.

16. Lu KP, Liou YC, Zhou XZ. Pinning down proline-directed phosphorylation signaling. Trends Cell Biol. 2002;12:164-72.

17. Lee TH, Pastorino L, Lu KP. Peptidyl-prolyl cis-trans isomerase Pin1 in ageing, cancer and Alzheimer disease. Expert Rev Mol Med. 2011;13:e21.

18. Lu KP, Hanes SD, Hunter T. A human peptidyl-prolyl isomerase essential for regulation of mitosis. Nature. 1996;380:544-7.

19. Kaushal GP, Kaushal V, Herzog C, Yang C. Autophagy delays apoptosis in renal tubular epithelial cells in cisplatin cytotoxicity. Autophagy. 2008;4:710-2.

20. Inoue K, Kuwana H, Shimamura Y, Ogata K, Taniguchi Y, Kagawa T, Horino T, Takao T, Morita T, Sasaki S, Mizushima N, Terada Y. Cisplatin-induced macroautophagy occurs prior to apoptosis in proximal tubules in vivo. Clin Exp Nephrol. 2010;14:112-22.

21. Mukhopadhyay P, Horváth B, Kechrid M, Tanchian G, Rajesh M, Naura AS, Boulares AH, Pacher P. Poly(ADP-ribose) polymerase-1 is a key mediator of cisplatin-induced kidney inflammation and injury. Free Radic Biol Med. 2011;51:1774-88.

22. Chao SH, Greenleaf AL, Price DH. Juglone, an inhibitor of the peptidyl-prolyl isomerase Pin1, also directly blocks transcription. Nucleic Acids Res. 2001;29:767-73.

23. Kim MR, Choi HS, Yang JW, Park BC, Kim JA, Kang KW. Enhancement of vascular endothelial growth factor-mediated angiogenesis in tamoxifen-resistant breast cancer cells: role of Pin1 overexpression. Mol Cancer Ther. 2009;8:2163-71.

24. Namgoong GM, Khanal P, Cho HG, Lim SC, Oh YK, Kang BS, Shim JH, Yoo JC, Choi HS. The prolyl isomerase Pin1 induces LC-3 expression and mediates tamoxifen resistance in breast cancer. J Biol Chem. 2010;285:23829-41.

25. Shen ZJ, Esnault S, Schinzel A, Borner C, Malter JS. The peptidyl-prolyl isomerase Pin1 facilitates cytokine-induced survival of eosinophils by suppressing Bax activation. Nat Immunol. 2009;10:257-65.

26. Lv L, Zhou Z, Huang X, Zhao Y, Zhang L, Shi Y, Sun M, Zhang J. Inhibition of peptidyl-prolyl cis/trans isomerase Pin1 induces cell cycle arrest and apoptosis in vascular smooth muscle cells. Apoptosis. 2010;15:41-54.

27. Lim JH, Liu Y, Reineke E, Kao HY. The MAPK ERK2 phosphorylates and promotes Pin1-dependent promyelocytic leukemia protein (PML) turnover. J Biol Chem. 2011: Epub.

28. Ryo A, Hirai A, Nishi M, Liou YC, Perrem K, Lin SC, Hirano H, Lee SW, Aoki I. A suppressive role of the prolyl isomerase Pin1 in cellular apoptosis mediated by the death-associated protein Daxx. J Biol Chem. 2007;282:36671-81.

29. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev. 2001;81:807-69.

30. Rashed LA, Hashem RM, Soliman HM. Oxytocin inhibits NADPH oxidase and P38

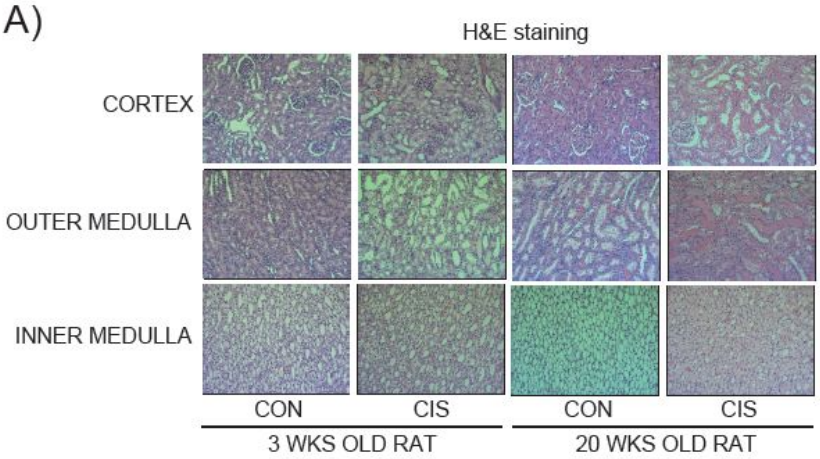
MAPK in cisplatin-induced nephrotoxicity. Biomed Pharmacother. 2011;65:474-80.

31. Melenhorst WB, Mulder GM, Xi Q, Hoenderop JG, Kimura K, Eguchi S, van Goor H. Epidermal growth factor receptor signaling in the kidney: key roles in physiology and disease. Hypertension. 2008;52:987-93.

32. Zeng F, Singh AB, Harris RC. The role of the EGF family of ligands and receptors in renal development, physiology and pathophysiology. Exp Cell Res. 2009;315:602-10.

33. Arany I. Dual role of the activated epidermal growth factor receptor in renal tubular cells during stress. Kidney Int. 2008;73:5-7.

6. Figure Legends



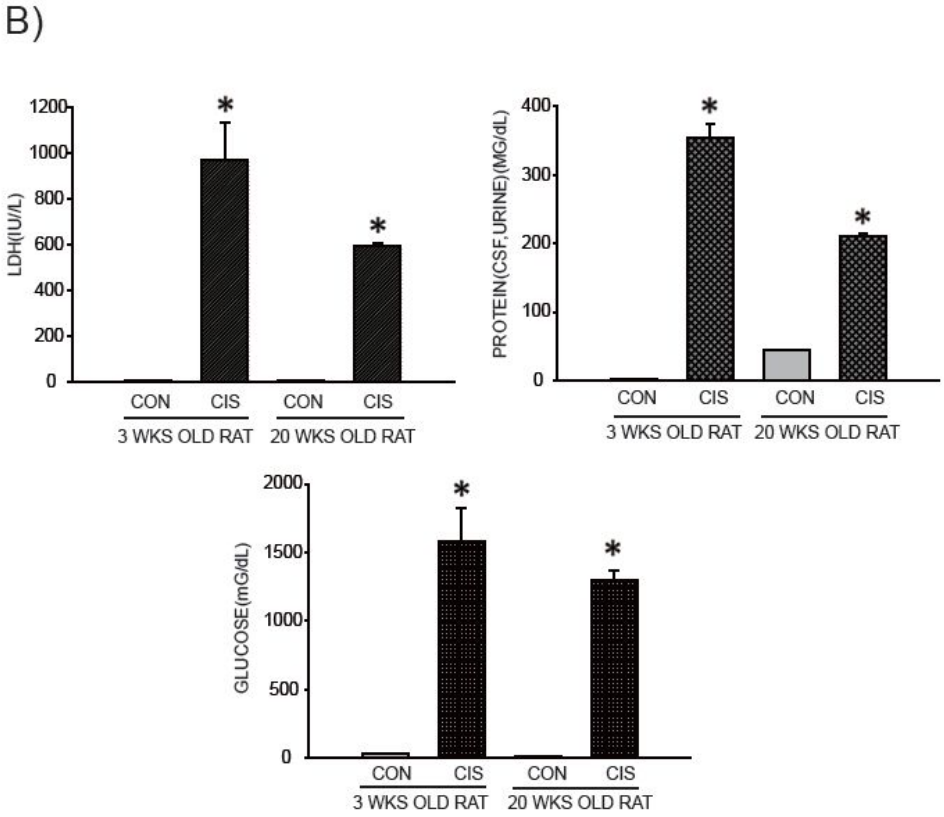


Figure 1. Cisplatin-induced cytotoxicity in kidney epithelial cells. We identified how cisplatin would cause cytotoxicity effect on kidney tissue. (A) Histopathological picture of kidney tissue samples. we injected cisplatin to 3 wks and 20 wks old male Sprague Dawley (SD) rats and 3 days after we dissected the kidney and then fixed it in 10% formaldehyde and embedded in paraffin. We checked the condition of tissues by hematoxylin and eosin staining assay as described under Materials and Methods. (B) Biochemical factor assess related with cisplatin-induced nephrotoxicity. We determined urine levels of total protein, glucose and lactate dehydrogenase (LDH). Data represented the mean± SD with 4 different samples. (significant as compared to control groups,*$p < 0.05$)

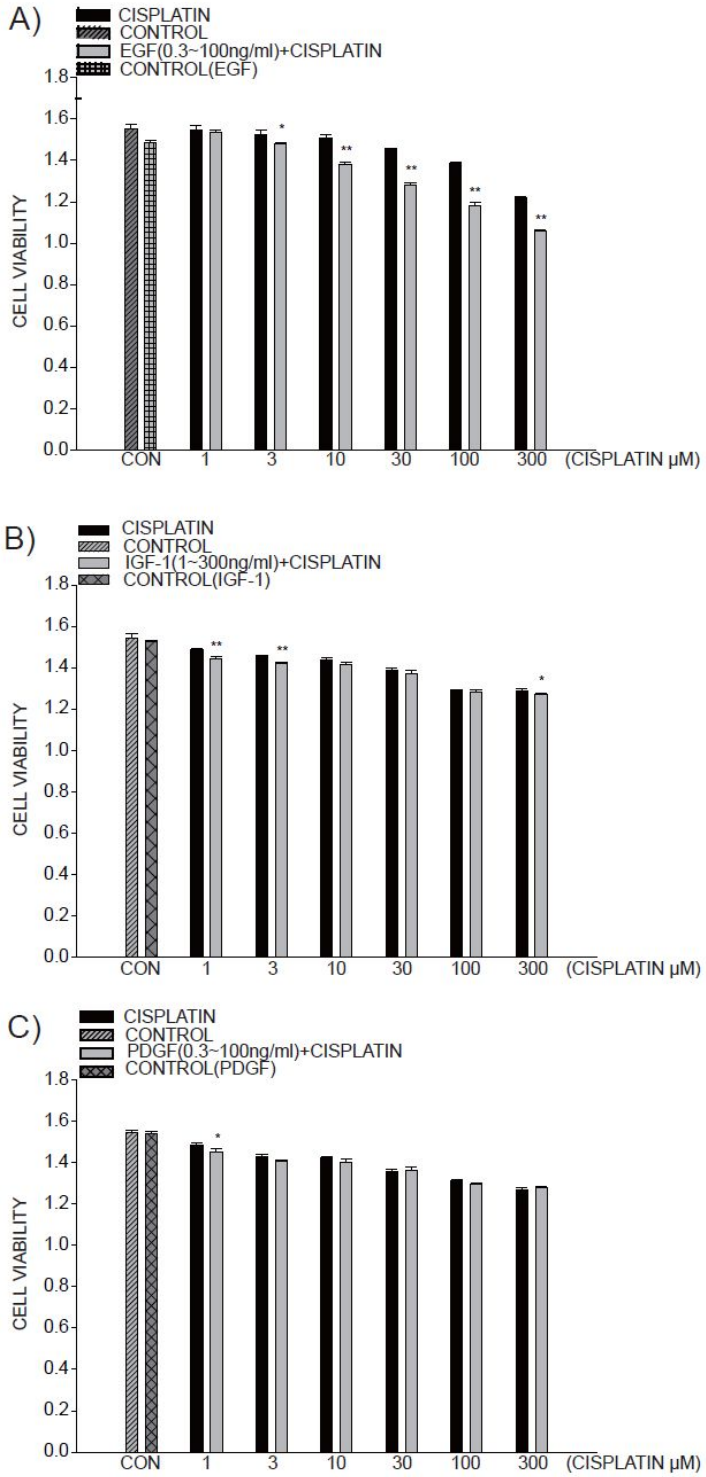

A)
CISPLATIN
CONTROL
EGF(0.3~100ng/ml)+CISPLATIN
CONTROL(EGF)
CELL VIABILITY
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CON
1
3
10
30
100
300
(CISPLATIN μM)
B)
CISPLATIN
CONTROL
IGF-1(1~300ng/ml)+CISPLATIN
CONTROL(IGF-1)
CELL VIABILITY
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CON
1
3
10
30
100
300
(CISPLATIN μM)
C)
CISPLATIN
CONTROL
PDGF(0.3~100ng/ml)+CISPLATIN
CONTROL(PDGF)
CELL VIABILITY
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CON
1
3
10
30
100
300
(CISPLATIN μM)

Figure 2. The significant growth factor related with cytotoxicity of cisplatin especially in young patient. (A) Induction effect of EGF on cell viability. We divided the cells into two groups. One was pretreated with EGF(0.3-100ng/ml) for 24h and incubated with cisplatin(1~300uM) for additional 24h. The other one was not pretreated and incubated with cisplatin for 24h. (B) Comparison of cell viability between IGF-1(1~300ng/ml) and cisplatin co-treated group and single cisplatin treated group. (C) Comparison of cell viability between PDGF(0.3~100ng/ml) and cisplatin co-treated group and single cisplatin treated group. Data represented the mean± SD with 4 different samples. (significant as compared to control groups,*$p < 0.05$, **$p < 0.01$)

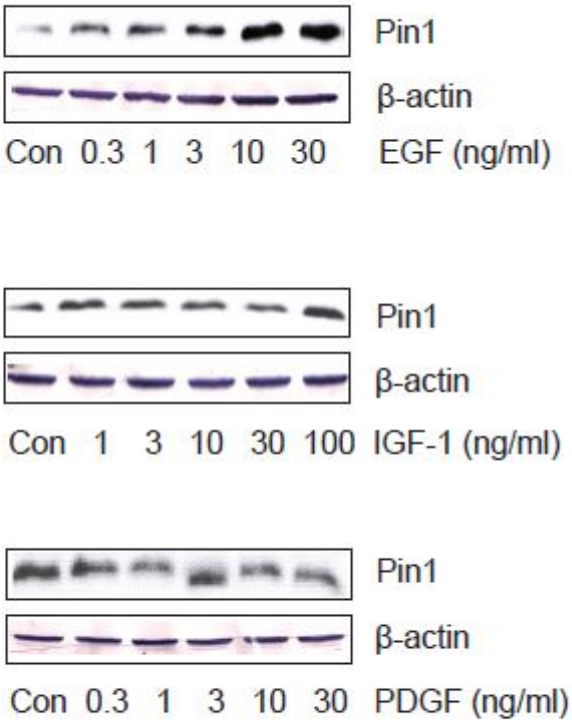
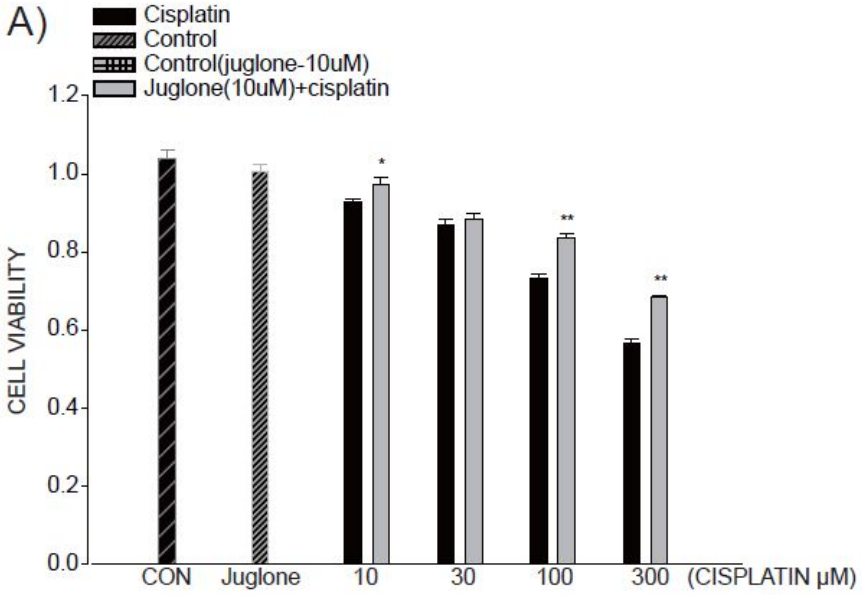

Pin1
β-actin
Con 0.3 1 3 10 30 EGF (ng/ml)
Pin1
β-actin
Con 1 3 10 30 100 IGF-1 (ng/ml)
Pin1
β-actin
Con 0.3 1 3 10 30 PDGF (ng/ml)

Figure 3. Up-regulation of Pin1 expression by EGF in NRK-52E cells. After serum starved for 18h NRK-52E cells were incubated with growth factors, EGF, IGF-1, PDGF for 24h and Pin1 expression was detected by Western blot analysis as described under Material and methods. Equal loading amounts of proteins were verified by actin immunoblot.


A)
Cisplatin
Control
Control(juglone-10uM)
Juglone(10uM)+cisplatin
CELL VIABILITY
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
**
CON
Juglone
10
30
100
300
(CISPLATIN μM)

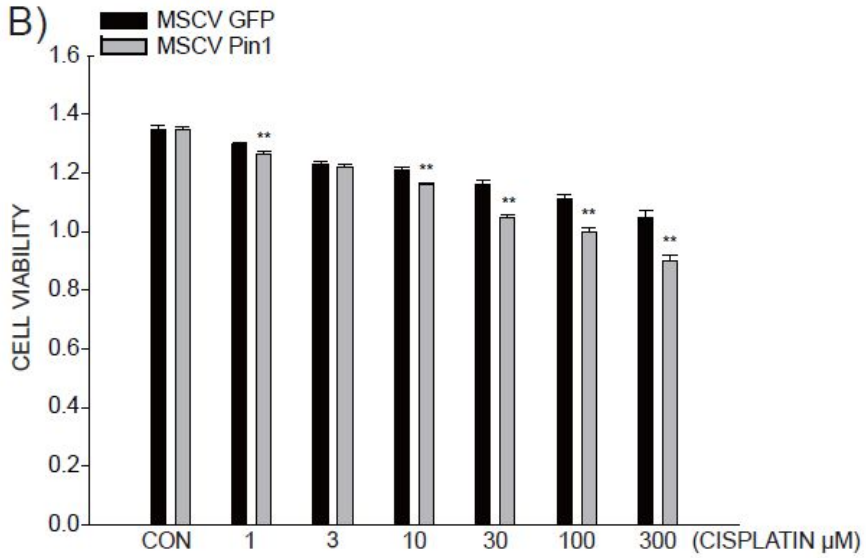
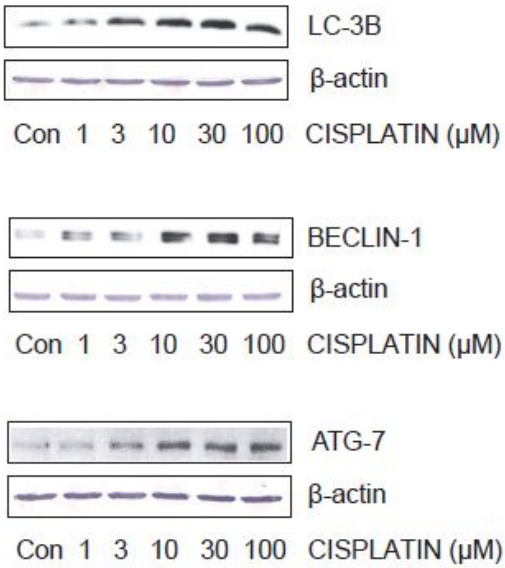

B)
MSCV GFP
MSCV Pin1
CELL VIABILITY
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
**
**
CON
1
3
10
30
100
300
(CISPLATIN μM)

Figure 4. Significant role of Pin1 on cell viability of cisplatin-exposed NRK-52E cells. (A) Reduction of cell death by Pin1 inhibition. NRK-52E cells were incubated with cisplatin for 24h in existence or absence of juglone(10uM). (B) Decrease of cell survival in condition of Pin1 induction. We established Pin1-overexpressing NRK-52E cells by multiple infections (15 times) of MSCV-GFP and MSCV-Pin1 retrovirus. Data represented the mean± SD with 4 different samples. (significant as compared to control groups,*$p < 0.05$, **$p < 0.01$)

A)


LC-3B
β-actin
Con 1 3 10 30 100 CISPLATIN (μM)
BECLIN-1
β-actin
Con 1 3 10 30 100 CISPLATIN (μM)
ATG-7
β-actin
Con 1 3 10 30 100 CISPLATIN (μM)

B)

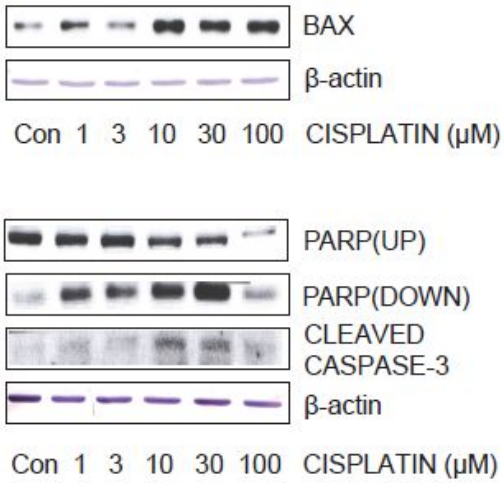
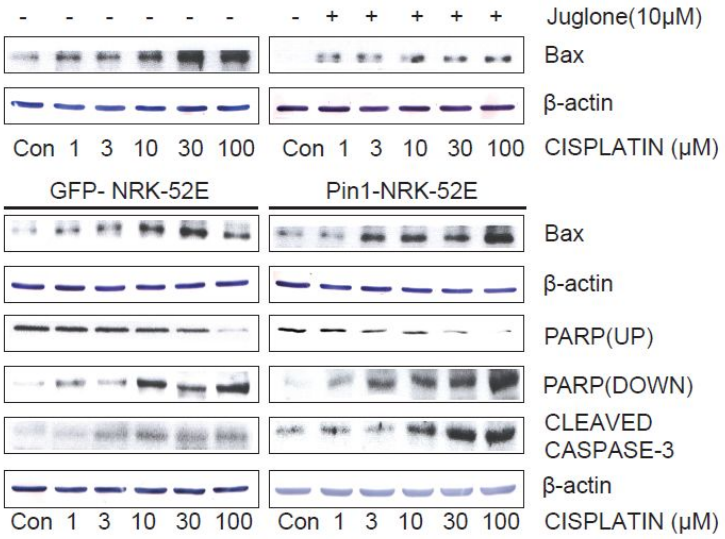
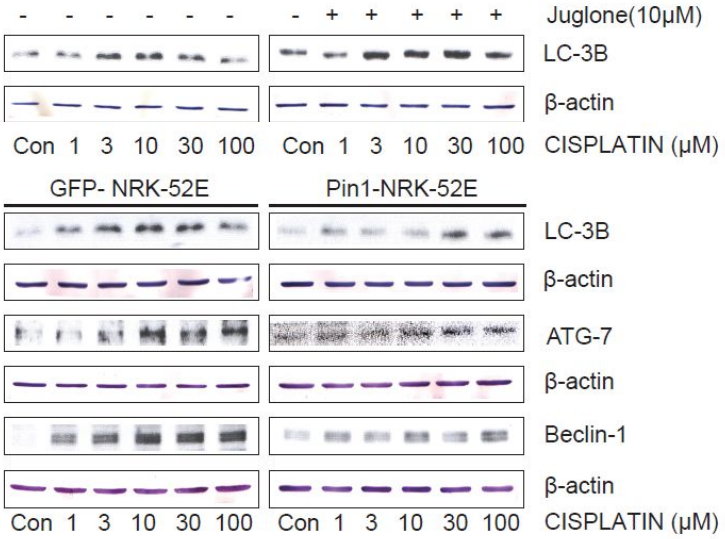

BAX
β-actin
Con 1 3 10 30 100 CISPLATIN (μM)
PARP(UP)
PARP(DOWN)
CLEAVED
CASPASE-3
β-actin
Con 1 3 10 30 100 CISPLATIN (μM)

Figure 5. Induction of autophagy and apoptosis marker expression by cisplatin. (A) Increase of LC-3B, Beclin-1, ATG-7 expression. After serum starved for 18h NRK-52E cells were treated with cisplatin(1 to 100uM) and incubated for 10h in the absence of serum. Representative immunoblots show LC-3B, Beclin-1, ATG-7 protein expression levels. Equal loading of proteins were verified by actin immunoblot. (B) Increase of Bax, PARP, cleaved caspase-3 expression. After serum starved for 18h NRK-52E cells were treated with cisplatin(1 to 100uM) and incubated for 48h in the absence of serum. Representative immunoblots show Bax, PARP, cleaved caspase-3 protein expression levels. Equal loading of proteins were verified by actin immunoblot.

A)


- - - - - - - + + + + +
Juglone(10μM)
Bax
β-actin
Con 1 3 10 30 100 Con 1 3 10 30 100
CISPLATIN (μM)
GFP- NRK-52E
Pin1-NRK-52E
Bax
β-actin
PARP(UP)
PARP(DOWN)
CLEAVED CASPASE-3
β-actin
Con 1 3 10 30 100 Con 1 3 10 30 100
CISPLATIN (μM)

B)


- - - - - - - + + + + +
Juglone(10μM)
LC-3B
β-actin
Con 1 3 10 30 100 Con 1 3 10 30 100
CISPLATIN (μM)
GFP- NRK-52E
Pin1-NRK-52E
LC-3B
β-actin
ATG-7
β-actin
Beclin-1
β-actin
Con 1 3 10 30 100 Con 1 3 10 30 100
CISPLATIN (μM)

C)

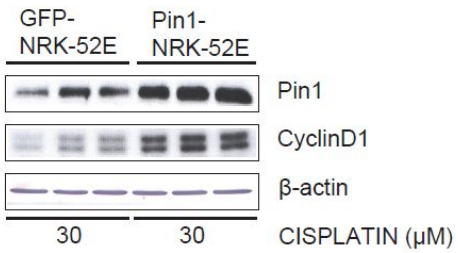

GFP-NRK-52E
Pin1-NRK-52E
Pin1
CyclinD1
β-actin
30 30
CISPLATIN (μM)

Figure 6. Identification of autophagy and apoptosis marker expression in cisplatin-treated NRK-52E cells. (A) Comparison of apoptosis marker protein expression between GFP- and Pin1-NRK-52E cells and. NRK-52E cells were pretreated with or without juglone for 30mim and incubated with cisplatin for additional 48h. We checked Bax, PARP, cleaved caspase-3 protein expression using the established GFP- and Pin1-NRK-52E cells. Equal loading of proteins were verified by actin immunoblot. (B) Comparison of autophagy marker protein expression between GFP- and Pin1-NRK-52E cells. NRK-52E cells were pretreated with or without juglone for 30mim and incubated with cisplatin for additional 10h. We checked LC-3B, ATG-7, Beclin-1 protein expression using the established GFP- and Pin1-NRK-52E cells. Equal loading of proteins were verified by actin immunoblot. (C) Confirmation of establishing GFP- and Pin1-NRK-52E cells. Representative immunoblots show Pin1, CyclinD1 protein expression levels. Equal loading of proteins were verified by actin immunoblot.

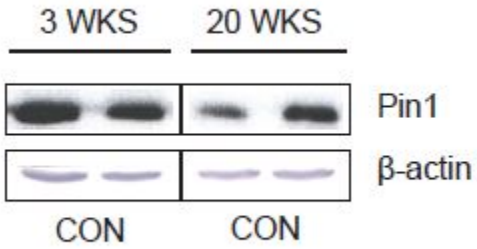

3 WKS
20 WKS
Pin1
β-actin
CON
CON

Figure 7. Comparison of Pin1 expression between kidney tissues from 3weeks old rat and those from 20weeks old rat. Kidneys were grinded into small pieces of tissue and lysed in EBC lysis buffer having the composition as described under Material and methods. Pin1 expression was detected by using Western blot analysis. Equal loading of proteins were verified by actin immunoblot.

저작물 이용 허락서

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논문제목	한글 : 상피세포 성장인자 노출 신장상피세포에서 Cisplatin 독성 증폭에 관한 연구 영어 : studies on the enhanced cytotoxic effect of cisplatin in epidermal growth factor-primed kidney epithelial cells				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

2012 년 2 월 25 일

저작자: 남 현 정 (서명 또는 인)

조선대학교 총장 귀하

감사의 글

2년 전 새하얀 종이마냥 아무것도 그려지지 않았었던 저 스스로가 떠오릅니다. 그때는 모든 것이 새롭고 낯설고 걱정스럽기만 했는데 시간이 이렇게 흐른 지금은 여러 면에서 아쉬움이 크게 남습니다. 지금도 많이 부족하지만, 지금까지 저를 이렇게 이끌어주시고 보듬어 주신 강건욱 교수님이 계셨기에 오늘의 제가 있는 듯하고 그 고마움은 달리 표현할 길이 없는 것 같습니다. 교수님께서 저에게 보여주셨던, 훌륭한 과학자 이전에 인간적인 모습들을 저는 앞으로 잊지 못할 것이고 또 제가 살아가는데 있어서 큰 가르침이 될 것입니다. 앞으로도 서울대학교를 빛낼 훌륭하고 멋진 교수님이 되시길 진심으로 바라며 연구생활에 있어서도 언제나 훌륭한 성과가 있길 바랍니다. 또한 몇 번 찾아 뵙지 못하고 제자로서의 도리를 다하지 못해 죄송스러운 유진욱교수님, 기성환교수님, 최홍석교수님, 최후균교수님 께도 감사의 말씀을 드리고 싶습니다.

올 한해 혼자 지내다 보니, 작년 한해 즐거운 분위기에서 실험과 연구를 할 수 있게 도와준 원영이, 정운이 그리고 미라누나! 선배들이 정말 생각이 많이 났고 그때 당시 힘이 많이 되었고 감사하다는 말을 전하고 싶습니다. 그리고 제가 대학원 총무 일을 하면서 여러모로 도움을 줬던 광모, 해국이, 힘든 회장 일을 도맡아 해오며 논문작업에 큰 도움을 준 준형이, 실험실의 실세이자 정신적 지주, 엄마와 같았던 옥이누나에게 감사의 말씀을 드리고 싶고, 또 그 누구보다도 올해 초 동기와 후배가 없어 걱정만 가득했던 저에게 웃음을 가득 안겨주셨던 멋지고 예쁜 상희누나!

누나가 없었으면 저는 정말 힘들었을 겁니다. 앞으로도 약국 크게 번창하시어 훌륭한 약사님이 되시길 바랄게요. 그리고 생화학실의 안방마님 효정누나, 요새 한창 상승세를 타고 있는 가람이, 그리고 이제 새로운 첫 발을 디딘 물약실의 은영이와 정필이, 그리고 저의 행정적인 부분을 많이 도와주신 수지씨에게도 감사하다는 말을 전하고 싶습니다 그리고 하늘 같은 선배이지만, 편하고 인간적으로 절 대해주셨던 승식이형! 학문과 진로에 있어서 많은 도움 주신 것 정말 평생 잊지 못할 것 같고 진심으로 감사하다는 말씀 드리고 싶습니다.

그리고 항상 그 이름만 떠올려도 감사와 죄송함의 눈물이 흐를 것 같은, 사랑하는 부모님이야 말로 이 한 권의 석사 눈문을 끝마칠 수 있게 해준 세상에서 하나밖에 없는 가장 소중하고 빛나는 보물입니다.