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Annexin A5 as a New Potential Biomarker for Cisplatin-Induced Toxicity in Human Kidney Epithelial Cells

Yeo-Jung Kwon[1], Jin-Joo Jung[1], Na-Hee Park[1], Dong-Jin Ye[1], Donghak Kim[2], Aree Moon[3] and Young-Jin Chun[1,*]

[1]College of Pharmacy, Chung-Ang University, Seoul 156-756,
[2]Department of Biological Sciences, Konkuk University, Seoul 143-701,
[3]College of Pharmacy, Duksung Women's University, Seoul 132-714, Republic of Korea

Abstract

Cisplatin is a member of platinum-containing anti-cancer drugs that causes cross-linking of DNA and ultimately cancer cell apoptosis. The therapeutic function of cisplatin on various types of cancers has been widely reported but the side effects have been discovered together and nephrotoxicity has been regarded as major side effect of cisplatin. To select candidates for new sensitive nephrotoxicity biomarker, we performed proteomic analysis using 2-DE/MALDI-TOF-MS followed by cisplatin treatment in human kidney cell line, HK-2 cells, and compared the results to the gene profile from microarray composed of genes changed in expression by cisplatin from formerly reported article. Annexin A5 has been selected to be the most potential candidate and it has been identified using Western blot, RT-PCR and cell viability assay whether annexin A5 is available to be a sensitive nephrotoxic biomarker. Treatment with cisplatin on HK-2 cells caused the increase of annexin A5 expression in protein and mRNA levels. Overexpression of annexin A5 blocked HK-2 cell proliferation, indicating correlation between annexin A5 and renal cell toxicity. Taken together, these results suggest the possibility of annexin A5 as a new biomarker for cisplatin-mediated nephrotoxicity.

Key Words: Cisplatin, Annexin A5, Nephrotoxicity, Biomarker, Proteomic analysis

INTRODUCTION

Cisplatin (*cis*-diammine-dichloro-platinum^{II}) is the first member of metal-containing chemotherapeutic drugs and its first clinical treatment was performed for testicular cancer in 1979 (Prestayko *et al.,* 1979). Since the first clinical trial, cisplatin has been widely used as an intravenously administered anti-cancer agent that is available to be applied to various types of cancers such as carcinomas of testis, ovary, neck and head. This practical anti-cancer drug binds and interacts with DNA to form adducts, mainly causing intrastrand cross-links (Sherman and Lippard, 1987; Siddik, 2003). DNA adducts formed by cisplatin activate several signaling pathways that ultimately trigger apoptosis such as those of ATR, p53 and MAPK (Wang *et al.,* 2000; Damia *et al.,* 2001), which makes cisplatin possible to be used as an anti-cancer drug.

In spite of various abilities of cisplatin as a chemotherapeutic drug, limits in clinical use still exist due to serious side effects such as oto-, myelo-, neuro- and nephrotoxicity (Laurell and Jungnelius, 1990; Harmers *et al.,* 1991; Treskes and van der Vijgh, 1993). Among them, nephrotoxicity is known as representative dose-limiting side effect of cisplatin that induces promotion of apoptosis and necrosis of renal epithelial cells (Hanigan and Devarajan, 2003) and considerable efforts have been contributed to overcome it. To establish effective way that makes reduction of side effects possible, studies to identify the level or mechanism of nephrotoxicity occurred by cisplatin have been widely investigated.

It has been significantly emphasized that the biomarkers sensitive for nephrotoxicity are essential to detect the level of toxicity and reduce it. For this reason, various types of nephrotoxic biomarkers such as NGAL (neutrophil gelatinase-associated lipocalin) and NAG (*N*-acetyl-β-D-glucosaminidase) have been discovered and some of them are in clinical use (Devarajan, 2007; Franke *et al.,* 2010). Despite serious necessity and much effort, widely accepted and sensitive biomarker for nephrotoxicity still has not been discovered yet and it needs to be investigated further.

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***Corresponding Author**
E-mail: yjchun@cau.ac.kr
Tel: +82-2-820-5616, Fax: +82-2-825-5616

Annexin A5 has specific properties that it requires higher calcium concentration than other types of annexin proteins for phospholipid binding and it forms two-dimensional networks on lipid bi-layers through binding with phosphatidylserine to activate its function (Raynal and Pollard, 1994; Reviakine *et al.,* 2000). Recently, it also has been identified that this networks are available to open a new portal for cellular entry, which is able to suggest the possibility for contribution of annexin A5 to apoptosis (Kenis *et al.,* 2004). These properties of annexin A5 make it possible to suggest that annexin A5 may be involved in cisplatin-induced toxicity and could be used as a new biomarker for side effect of cisplatin.

In present study, we performed experiments to find new sensitive biomarker for nephrotoxicity. Screening for selection of candidates, proteomic analysis was performed. Because annexin A5 was selected as one of the most potential candidates, validation of sensitivity of annexin A5 for cisplatin-induced nephrotoxicity has also been implemented.

MATERIALS AND METHODS

Reagents

Cisplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM medium, penicillin or streptomycin was obtained from Welgene (Daegu, Korea). Fetal bovine serum (FBS) or enhanced chemiluminescence (ECL) detecting reagent was purchased from Thermo Fisher Scientific (Rockford, IL, USA). BCA protein assay kit was from Pierce Chemical Co. (Rockford, IL, USA). EZ-CYTOX cell viability kit was purchased from Daeil lab service Co., Ltd (Seoul, Korea). Rabbit polyclonal antibody for annexin A5 was obtained from Sigma-Aldrich (St. Louis, MO, USA). HRP-conjugated goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Santa CRUZ, CA, USA). Maloney murine leukemia virus (M-MLV) reverse transcriptase and RNase inhibitor (RNasin) were obtained from Promega (Madison, WI, USA). *Ex Taq* DNA polymerase was purchased from TaKaRa Bio (Shiga, Japan). Other reagents and chemicals were commercially available of the highest quality.

Cell culture

Immortalized human kidney epithelial cells (HK-2) was obtained from American Type Culture Collection (ATCC, Manassa, VA, USA) and cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated FBS, 100 unit/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Cell treatment and harvest

Cisplatin was dissolved in sterile 0.9% NaCl solution. Cisplatin solutions were used for no longer than 3 days. Cells were treated with cisplatin and incubated at 37°C in a humidified atmosphere of 5% CO_2 for the designated time. After incubation, cells were harvested by scrapping and washed with phosphate-buffered saline (PBS). Cells were centrifuged at 1,000x*g* for 4 min at 4°C and the pellets were stored in -70°C.

2DE/MALDI-TOF-MS analysis

For total cell lysates, cells were solubilized with ice-cold 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1% nonidet P-40, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin (pH 7.4). Extracted proteins (20 μg) were prepared in adequate volumes determined by BCA protein assay reagents and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels. Seperated proteins were dyed using silver staining. For protein identification by peptide mass fingerprinting, protein spots were excised, digested with trypsin (Promega, Madison, WI, USA), mixed with α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA, and subjected to MALDI-TOF analysis (Ettan MALDI-TOF Pro, Amersham Biosciences, Piscataway, NJ, USA) as described (Fernandez *et al.,* 1998). Spectra were collected from 350 shots per spectrum over m/z range 600-3,000 and calibrated by two point internal calibration using trypsin auto-digestion peaks (m/z 842.5099, 2211.1046). Peak list was generated using Ettan MALDI-TOF Pro Evaluation Module (version 2.0.16). Threshold used for peak-picking was as follows: 5,000 for minimum resolution of monoisotopic mass, 2.5 for S/N. The search program MASCOT (http://www.matrixscience.com/) was used for protein identification by peptide mass fingerprinting. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. PMF acceptance criteria is probability scoring.

Western blot

Whole cell lysates were prepared and protein concentration was determined using BCA Protein Assay Reagents (Pierce). Cellular extracts (20 μg) were separated on 10% SDS-PAGE at 100 V and transferred onto 0.45 μm PVDF membrane. Nonspecific binding was blocked with 5% nonfat milk in tris-buffered saline containing 0.1% tween-20 (TBS-T) for 2 h at 4°C. Membranes were then incubated for overnight with primary antibody specific for annexin A5 at a 1:500 dilution in TBS-T. Horseradish peroxidase (HRP)-conjugated secondary antibody was done at 4°C for 2 h. Proteins were visualized by an ECL method (Thermo) and the band intensity was analyzed by ChemiDoc XRS densitometer and quantified by Quantity One software (Bio-Rad).

RT-PCR

Total RNA was extracted using Ribospin™ (GeneALL, Seoul, Korea). Total RNA (500 ng) was transcribed at 37°C for 1 h in a volume of 20 μl containing 5x RT buffer, 10 mM dNTPs, 40 units of RNase inhibitor, 200 units of M-MLV reverse transcriptase, and 100 pmole of oligo-dT primer. Subsequently, 0.8 μl of the reaction mixtures from each sample were amplified with 10 pmole of each oligonucleotide primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 1.25 units of *Ex Taq* DNA polymerase in a final volume of 25 μl. PCR was performed as follows: one cycle of 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 63°C for 15 s, and extension at 72°C for 15 s. The number of cycles for amplification was optimized in preliminary experiments to ensure that the PCR has not reached its plateau. PCR products were applied to a 2% (w/v) agarose gel electrophoresis, and analyzed by ChemiDoc XRS (Bio-Rad, Hercules, CA). Human annexin A5 cDNA was amplified using a sense primer (5'-CAGTCTAGGTGCAGCTGCCG-3') and an antisense primer (5'-GGTGAAGCAGGACCAGACTGT-3'). Human GAPDH was amplified using a sense primer (5'-TGAACGGGAAGCTCACTGG-3') and an antisense primer (5'-TCCACCACCCTGTTGCTGTA-3').

Transient transfection and cell viability assay

HK-2 cells (2×10^4 cells/well) were plated onto well of 96-well plate after transfection with 400 ng of plasmid DNA (pcDNA3.1zeo-annexin A5) containing annexin A5 cDNA using Neon™ transfection system (Invitrogen, Carlsbad, CA, USA). Following microporation, transfected cells were stabilized in antibiotics free DMEM medium contained 10% FBS for designated times at 37°C. After incubation, 10 μl of CCK solution was added and incubated for 2 h at 37°C. The absorbance at 450 nm was measured using a Sunrise™ microplate reader (Tecan, Männedorf, Switzerland). The percentages of surviving cells relative to control in each group were calculated.

Statistical analysis

Using one-way analysis of variance, statistical analysis was performed followed by Dunnett's Multiple Comparison *t*-test using Graph-Pad Prism Software (GraphPad Software Inc., San Diego, CA) when appropriate. The difference was considered statistically significant at $p<0.05$.

RESULTS

Proteomic analysis for cisplatin-induced nephrotoxicity biomarkers

To find valuable biomarker for cisplatin-induced nephrotoxicity, human kidney epithelial cells (HK-2) were treated with 20 μM of cisplatin for 24 h or 48 h. To identify which proteins showed changes in expression, we performed proteomic analysis by two-dimensional gel electrophoresis (2DE)/MALDI-TOF-MS. As shown in Fig. 1A, considerable amounts of spots that reflect the expression of proteins were elevated in density when cells were treated with cisplatin for 24 h or 48 h.

Selection of new biomarker candidate for nephrotoxicity

From the results of proteomic analysis, 160 spots showing significant increase of expression were selected as candidates for nephrotoxic biomarkers. To identify whether the

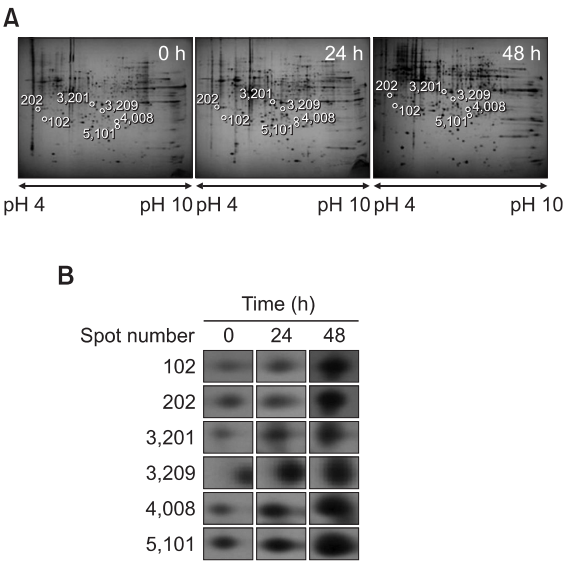

Fig. 1. Changes in expressions of various proteins after cisplatin treatment in HK-2 cells. HK-2 cells were treated with 10 μM of cisplatin for indicated times (0, 24 or 48 h). Using 2-DE, various types of proteins were separated. Six differentially expressed protein spots were selected for further study. (A) 2-DE gel analysis. (B) Differentially expressed proteins by cisplatin.

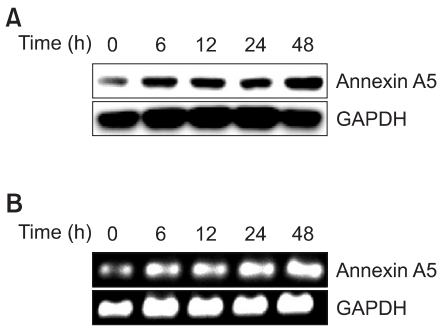

Fig. 2. Cisplatin induces annexin A5 mRNA and protein expression in HK-2 cells in time-dependent manner. (A) Western blot analysis. HK-2 cells were treated with 20 μM cisplatin for the indicated times (0, 6, 12, 24 or 48 h). After harvesting, total cellular lysates were prepared for Western blot analysis using antibody against annexin A5. GAPDH was used as a loading control. (B) RT-PCR. HK-2 cells were treated with 20 μM cisplatin for conducted times (0, 6, 12, 24 or 48 h). After harvesting, total RNA was isolated and amplified using specific primers. The PCR products were separated on 2% agarose gel. Expression of GAPDH mRNA was determined as a RNA control.

Table 1. Identification of six differentially expressed proteins by mass analysis

Spot no. (Fig. 1B)*	NCBI gene identifier	Protein name	Molecular mass (kDa)	pI	% Coverage**	Matched Peptide No.†
102	17943181	Micro-calpain form 1	21.398	4.90	43%	10
202	4502107	Annexin A5	35.971	4.94	41%	14
3201	13786847	Lactate dehydrogenase H chain	36.769	5.72	12%	5
3209	30410796	Proteasome activator complex subunit 3	31.038	5.79	33%	7
4008	119569783	Peroxiredoxin 3	11.158	6.06	47%	4
5101	14286220	Human mitochondrial enoyl CoA hydratase	31.835	8.34	47%	11

Spots of differentially expressed proteins were isolated and proteomic analysis was performed to identify proteins. *Spot numbers refer to those in Fig. 1B. **Amino acid sequence coverage for the identified protein. †Number of peptide masses matching the top hit from MASCOT peptide mass.

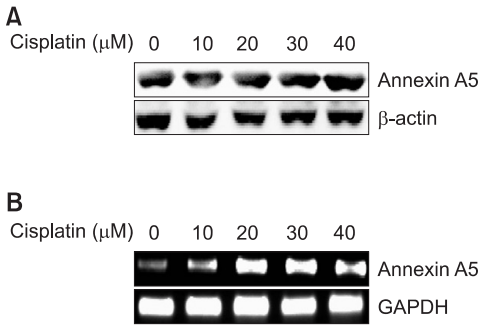

Fig. 3. Cisplatin promotes annexin A5 mRNA and protein expression in HK-2 cells in concentration-dependent manner. (A) Western blot analysis. HK-2 cells were treated with designated concentrations of cisplatin for 24 h. After harvesting, total cellular lysates were prepared for Western blot analysis using antibody against annexin A5. GAPDH or β-actin was used as a loading control. (B) RT-PCR. HK-2 cells were treated with conducted concentrations of cisplatin for 24 h. After harvesting, total RNA was isolated and amplified using specific primers. The PCR products were separated on 2% agarose gel. Expression of GAPDH mRNA was determined as a RNA control.

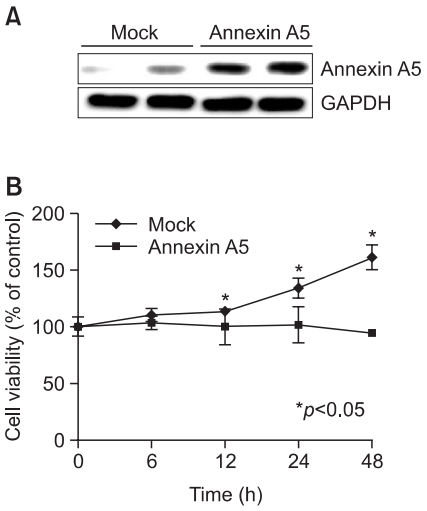

Fig. 4. Over-expression of annexin A5 prevents cellular proliferation in HK-2 cells. (A) Western blot analysis. HK-2 cells were transfected with pcDNA3.1zeo-annexin A5 and stabilized on 100 mm-dish for 24 h at 37°C. After harvesting, total cellular lysates were prepared for Western blot analysis using antibody against annexin A5. GAPDH was used as a loading control. (B) Cell viability assay. HK-2 cells were transfected with pcDNA3.1zeo-annexin A5 or pcDNA3. 1zeo (mock) and plated onto 96-well plate. Cell viability was determined after incubating for 0, 6, 12, 24 or 48 h. The absorbance at 450 nm was measured and percentage of cells surviving each group relative to the untransfected controls was expressed as the mean ± S.D. of three separate experiments. *p<0.05 compared with untransfected control cells.

corresponding results were observed *in vivo*, we compared proteins each spots represented to the previously reported DNA microarray results (Wang *et al.,* 2008). In this paper, male rats were intravenously treated with cisplatin for 1, 3 and 5 days. Various types of genes reported to be associated with cisplatin-induced nephrotoxicity were examined and their level of change in expression were profiled. As a result of comparison, several proteins were matched and narrowed for 5 candidates in Table 1. Annexin A5, one of matched proteins, was selected as the most potential new nephrotoxic biomarker candidate due to significant changes in both studies and its property to accumulate in kidney. Furthermore, it has been recently reported that annexin A5 mediates internalization on cell surface and this phenomenon is activated early after initiation of apoptosis (van den Eijnde *et al.,* 1997; Kenis *et al.,* 2004). This potential ability of annexin A5 was also considered as proper reason for selection.

Effect of cisplatin on annexin A5 expression in HK-2 cells

To identify whether annexin A5 is increased by cisplatin, HK-2 cells were treated with 10, 20, 30 or 40 μM of cisplatin and expression of annexin A5 was investigated using Western blot and RT-PCR. As shown in Fig. 2-3, expression of annexin A5 was significantly promoted due to cisplatin treatment in time- and concentration-dependent manners. Moreover, to explore the effect of annexin A5 on kidney cells, viability of HK-2 cells was detected after annexin A5 over-expression. Protein level of annexin A5 in HK-2 cells was measured after transfection with pcDNA3. 1zeo-annexin A5 for 24 h. As shown in Fig. 4A, we found that annexin A5 was significantly increased by transfection. Over-expression of annexin A5 inhibited cell proliferation in Fig. 4B. At 48 h, mock-transfected cells showed about 160% of viability but 94% of viability was shown in annexin A5-transfected cells. These results elucidate that annexin A5 may play an important role in cisplatin-induced toxicity in HK-2 cells.

DISCUSSION

Platinum-based anti-neoplastic agents have been widely used as anti-cancer drugs. Among them, cisplatin, a member of first generation of this group, is applied to various types of tumors including ovarian, lung, head and neck, bladder and less severe breast and gastric cancer (Lokich and Anderson, 1998). Despite usefulness of cisplatin, the treatment of this agent is limited due to serious side effects including nephrotoxicity (Donzelli *et al.,* 2004). The most significant mechanism underlying cisplatin-induced nephrotoxicity is reported to be apoptosis (Hanigan and Devarajan, 2003). Considerable efforts have been made for decades to eliminate the toxicity of cisplatin and several ways were identified that are available to reduce cisplatin-induced nephrotoxicity such as pre-hydration of patients and dual use of diuretics (Quasthoff and Hartung, 2002). However, nephrotoxicity still remains as the major dose-limiting side effect of cisplatin and the mechanism or solution for cisplatin-induced nephrotoxicity has not been yet clarified. For these reasons, the investigations to develop the ways for reduction of nephrotoxicity have to be more implemented.

Annexins are proteins that bind to cellular membranes containing phospholipids with negative charge in Ca^{2+}-dependent manner (Markoff and Gerke, 2005). These proteins are expressed in various types of animals and plants and classified into 5 groups, A, B, C, D, and E (Moss and Morgan, 2004). Twelve types of annexins have been identified in human with tissue- and cell type-specific expressions (Markoff and Gerke, 2005). Among them, annexin A1, A2, A3 and A5 are expressed

in digestive or ductal organs, especially in kidney (Pepinsky *et al.,* 1988; Dreier *et al.,* 1998).

Annexin A5, a member of annexin protein family, is basically known as Ca^{2+} and negatively charged phospholipid binding protein and widely used as a detector for apoptosis due to ability of binding to phosphatidylserine exposed to outside of cells during apoptosis (Vermes *et al.,* 1995). Recently, it has been reported that annexin A5 forms two-dimensional network covering phosphatidylserine-expressed cell surface through trimerization using intramolecular salt bridges (Kenis *et al.,* 2004; Ungethum *et al.,* 2011). The network formed by annexin A5 induces bending of cell membrane and subsequent formation of endocytic vesicle, which makes a new portal for cell entry open (Kenis *et al.,* 2004; Kenis *et al.,* 2007). This endocytic pathway induced by annexin A5 was observed to be activated early after initiation of apoptosis along with appearance of phosphatidylserine molecules on cell surface and reported not to operate commonly in healthy tissues (van den Eijnde *et al.,* 1997; Kenis *et al.,* 2004). Although the contribution of annexin A5 on apoptosis is unclear, these properties of annexin A5 make it possible that annexin A5 has potential to participate in apoptosis and this potential ability of annexin A5 may play an important role in kidney malfunction due to accumulation of annexin A5 in kidney.

In this study, we explored a new biomarker for cisplatin-induced nephrotoxicity. To select proteins that show changes in expression followed by cisplatin treatment, proteomic analysis using 2DE/MALDI-TOF-MS was applied. The proteins altered in expression level were compared to the profile of genes that changed in expression due to cisplatin treatment *in vivo* model (Wang *et al.,* 2008). As outcome of comparison between proteomic results and genomic DNA microarray results, we found that annexin A5 was increased in both studies. Due to one of the properties of annexin A5 that it is accumulated in kidney (Markoff and Gerke, 2005), we selected annexin A5 as one of the potential candidates in proteins obtained from the results. To evaluate whether annexin A5 is able to reflect nephrotoxicity sensitively, we performed Western blot and RT-PCR on expression of annexin A5 in HK-2 cells and it has been resulted in increase of annexin A5 expression followed by cisplatin treatment in both protein and mRNA levels. Direct evidence for correlation between annexin A5 expression and cytotoxicity was obtained by measuring cell viability. According to our results, over-expression of annexin A5 significantly inhibited proliferation of HK-2 cells. Recently, it has been reported that over-expression of annexin A5 increases apoptosis elicited by basic calcium phosphate crystals in articular chondrocyte (Ea *et al.,* 2008). Although the mechanism has not been yet identified, we suggest that annexin A5 may play an important role in apoptosis.

In summary, our study suggests annexin A5 as a new sensitive biomarker for cisplatin-induced nephrotoxicity. Based on the report that cisplatin-induced nephrotoxicity is elicited by various pathways (Yao *et al.,* 2007), annexin A5 has possibility to be a general biomarker for nephrotoxicity obtained from various types of kidney malfunction. Formerly, it has been reported that increase of excreted annexin A5 in urine of patients with acute renal impairments is available to detect using ELISA (Matsuda *et al.,* 2000). This report and our study could suggest that annexin A5 may be useful as clinical biomarker of cisplatin-induced nephrotoxicity. Although *in vivo* biomarkers are more useful in clinical aspects, finding *in vitro* biomarkers is also important to explore basic molecular mechanism of nephrotoxicity and predict more precisely and rapidly *in vivo* toxicity. Based on the ability of annexin A5 inducing endocytic pathway, we suggest that the potential role of annexin A5 in apoptosis may be related to pro-apoptotic pathways with pore formation. Recently, it has been reported that annexin A4 is up-regulated by cisplatin and over-expression of annexin A4 decreases cell viability (Yamashita *et al.,* 2012). Due to the common property of annexin A4 and A5 that both annexins are more closely related to ion channel regulation than other annexins, the effects of cisplatin-induced annexin A4 and A5 on cell viability may be linked with their ability as regulator of ion channels. Further studies need to be conducted to identify the exact mechanism exploring the contribution of annexin A5 to renal cell apoptosis and whether annexin A5 could be used as general biomarker for wide range of renal failure.

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