Identification of Noninvasive Biomarkers for Nephrotoxicity Using HK-2 Human Kidney Epithelial Cells

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The kidney is an important site of xenobiotic-induced toxicity. Because the traditional markers of renal injury indicate only severe renal damage, new biomarkers are needed for a more sensitive and reliable evaluation of renal toxicity. This study was designed to identify in vitro noninvasive biomarkers for efficient assessment of nephrotoxicity by using cisplatin as a model of nephrotoxic compounds. To this end, a comparative proteomic analysis of conditioned media from HK-2 human kidney epithelial cells treated with cisplatin was performed. Here, we identified pyruvate kinase M1/M2 isoform M2 (PKM2) and eukaryotic translation elongation factor 1 gamma (EF-1γ) as potential biomarker candidates for evaluation of nephrotoxicity. PKM2 and EF-1γ were increased by cisplatin in a kidney cell-specific manner, most likely due to cisplatin-induced apoptosis. The increase of PKM2 and EF-1γ levels in conditioned media was also observed in the presence of other nephrotoxic agents with different cytotoxic mechanisms such as CdCl₂, HgCl₂, and cyclosporine A. Rats treated with cisplatin, CdCl₂, or HgCl₂ presented increased levels of PKM2 and EF-1γ in the urine and kidney tissue. Taken together, this study identified two noninvasive biomarker candidates, PKM2 and EF-1γ, by comparative proteomic analysis. These new biomarkers may offer an alternative to traditional renal markers for efficient evaluation of nephrotoxicity.

Key words: nephrotoxicity; biomarkers; PKM2; EF-1γ; HK-2; conditioned media.

The kidney, one of the most important organs where exogenous toxic substances and their metabolites are transformed to nonpoisonous compounds (Pfaller and Gstraunthaler, 1998), is an important site of xenobiotic-induced toxicity. Acute renal damage and chronic kidney diseases are closely related to drug-induced nephrotoxicity (Rached et al., 2008). However, minor damage of renal function is hardly detected because of the kidney functional reserve (Rached et al., 2008). The levels of blood urea nitrogen (BUN) and serum creatinine, the most commonly used traditional markers of renal injury, only indicate severe renal damage. Thus, renal toxicity assessment requires more sensitive and reliable biomarkers.

Potential biomarkers with sensitivity, specificity and safety are very useful in the progression of drug development from preclinical into human studies (Marrer and Dieterle, 2010). Proteomic analysis has facilitated biomarker discovery and improved detection of toxicity (Collins et al., 2007). Proteomic analysis using media or biofluids such as plasma and serum is considered particularly useful because it allows the identification of noninvasive biomarkers (Veenstra et al., 2005). Protein identification in cell media or animal urine after exposure to cytotoxic agents is a promising approach for toxicity assessment (Shao et al., 2011). Several urinary biomarkers, including kidney injury molecule (KIM)-1, tissue inhibitory metalloproteinase (TIMP)-1, neutrophil gelatinase-associated lipocalin (NGAL), N-acetyl-β-D-glucosaminidase (NAG), β2-microglobulin (β2-M), urinary clusterin, and cystatin C have been developed and approved as noninvasive biomarkers for in vivo assessment of acute rodent renal toxicity (Hoffmann et al., 2010; Kim and Moon, 2012; Marrer and Dieterle, 2010).

Recently, the evaluation of various toxic compounds using in vitro assays or alternative methods to animal testing has drawn a lot of interest (Ranganatha and Kuppast, 2012). The in vitro assays present a valuable approach for the development of biomarkers because they are cost-effective, convenient, eco-friendly, and allow mechanistic studies (Fox et al., 2010).

The NRK-52E cell line, derived from rat proximal tubule epithelium, has been used for in vitro evaluation of nephrotoxicity (Rached et al., 2008). However, these cells present limitations for human toxicity assessment such as interspecies differences in drug metabolism and mechanism of action (Gildea et al., 2010; Gunness et al., 2010). Thus, the HK-2 cells derived from human kidney proximal tubule epithelial cells were established and used to investigate the drug-induced nephrotoxicity as the more acceptable in vitro model (Guinness et al., 2010; Ryan et al., 1994). KIM-1, calbindin, and TIMP-1 were previously identified as potential in vitro biomarkers for nephrotoxicity assessment in HK-2 cells (Sohn et al., 2013).

Recently, conditioned media have been analyzed for biomarker discovery under well-established in vitro systems (Dowling and Clynes, 2011). Analysis of the proteins released by cultured cells in the media in vitro has been proven useful for biomarker identification because it presents advantages com-
pared with serum or plasma protein analysis. First of all, ~20 species of proteins in serum accounts for 99% of the total protein mass which can mask a relatively small amount of proteins representative of the cell status and prevent the finding of novel biomarker candidates (Dowling and Clynes, 2011). In addition, the collection process of conditioned media can be fast and low-cost compared with serum or plasma (Pin et al., 2013).

Cisplatin, a platinum-based DNA alkylating agent, is one of the most effective anticancer drugs (Choi and Kim, 2006). Although cisplatin has potential for cancer therapy, its use has been limited due to serious side effects, especially nephrotoxicity (Choi and Kim, 2006; Rodriguez-Garcia et al., 2009). This study was designed to identify sensitive and noninvasive in vitro biomarker candidates for cisplatin-induced nephrotoxicity. To this end, two-dimensional gel electrophoresis (2DE) followed by matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF-MS) analysis of proteins in the conditioned media of HK-2 human kidney epithelial cells treated with cisplatin were performed. Pyruvate kinase isozyme isoform M2 (PKM2) and eukaryotic translation elongation factor 1 gamma (EF-1γ) were identified as noninvasive biomarkers for cisplatin-induced nephrotoxicity. We further showed that other nephrotoxic agents with various cytotoxic mechanisms also caused increased levels of PKM2 and EF-1γ both in vitro and in vivo.

MATERIALS AND METHODS

Cell culture. Human normal kidney epithelial cells, HK-2, and rat normal kidney epithelial cells, NRK-52E, were purchased from ATCC (American Type Culture Collection). HK-2 cells were cultured, as recommended by the supplier, in keratinocyte-serum free medium with 50 μg/ml bovine pituitary extract (BPE) and 5 ng/ml recombinant epithelial growth factor (Invitrogen, Carlsbad, CA). Rat normal kidney epithelial cells, NRK-52E, were cultured in Dulbecco’s Modified Eagle’s Medium with 4mM l-glutamate, 4.5 g/l sodium bicarbonate, and supplemented with 5% bovine calf serum and 100 μg/ml penicillin-streptomycin. MCF10A human breast epithelial cells were established, as previously described (Kim et al., 2000). Primary hepatocytes isolated from male Sprague Dawley (SD) rats, according to the previously published method with slight modifications (Kang et al., 2003), were kindly provided by Dr. Sang Geon Kim (Seoul National University, Seoul, Korea). The cells were maintained in humidified atmosphere with 95% air and 5% CO₂ at 37°C.

Reagents. Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]fluoromethylketone (Z-VAD-FMK) was purchased from R&D Systems (Minneapolis, MN). Caspase 1 (ICE) inhibitor I (YVAD-CHO) was purchased from AnaSpec (Fremont, CA), and Brefeldin A isolated from Eupenicillium brefeldianum (brefeldin) was purchased from Calbiochem (EMD Chemicals, San Diego, CA). Cisplatin, cadmium chloride (CdCl₂), mercuric chloride (HgCl₂), and cyclosporine A were purchased from Sigma-Aldrich Co. (St Louis, MO).

Sample preparation. For proteomic analysis, HK-2 cells were seeded and grown up to 70% confluency, and they were incubated in serum-free condition for 24 h in the presence of increasing concentrations of cisplatin. Conditioned media were harvested and centrifuged to remove cells debris and then concentrated by centrifugation using Amicon Ultra Centrifugal Filters-3K (Millipore, Bedford, MA). For proteomic analysis, samples were prepared using the 2-D Clean-Up Kit (Amerham Biosciences, Uppsala, Sweden), according to the manufacturer’s recommendations.

Proteomic analysis. Proteomic analysis, such as 2DE, MALDI-TOF-MS analysis, and Mascot database search, was performed as previously described (Jeon et al., 2013).

Immunoblot analysis. Immunoblot analysis was performed as previously described (Jeon et al., 2013) using antihuman PKM2, anti-EF-1γ, antitumor rejection antigen 1 (GRP 94), anti-YWHAZ protein, antitransferrin, antialkaline phosphatase (ALP) antibodies (Santa Cruz Biotechnology, Dallas, TX); antirat PKM2 and anti-PARP antibodies (Cell Signaling Technology, Beverly, MA) and anti-β-actin antibody (Sigma-Aldrich, St Louis, MO). Protein expression was detected using enhanced chemiluminescence (ECL, Amersham-Pharmacia, Buckinghamshire, UK). Relative band intensities were determined by quantification of each band with Image Lab Software (Bio-Rad Laboratories, Inc., Hercules, CA).

ELISA. In order to quantitatively detect and compare the amount of PKM2 in different conditions, ELISAs using ELISA kit for human PKM2 in the cell supernatant (MyBioSource, Inc., San Diego, CA) were performed according to the manufacturer’s instructions. Samples for ELISA were harvested after cell debris removal and prepared to contain the same amount of protein using the bicinchoninic acid (BCA) protein assay without concentration.

Reverse transcription (RT)-PCR. RNA extraction from cisplatin-treated HK-2 cells and RT-PCR were performed as previously described (Cha et al., 2012). Primers for PKM2 were 5′-GAGTACCATGCGGAGACCATT-3′ (forward) and 5′-GGTTATCCAGCGTGATTTT-3′ (reverse). Primers for EF-1γ were 5′-TCAGACCTTTCAGTGCTGCA-3′ (forward) and 5′-TACTCTCGAACCAGCGTGTCG-3′ (reverse). Primers for β-actin were 5′-ATCTCCTCTGATCCTGTGTC-3′ (forward) and 5′-ACTCTCCAGCTCCTCTCAC-3′ (reverse). For RT-PCR analysis, the following amplification conditions were applied: 95°C, 5 min for 1 cycle, 95°C for 30 s, 56°C for 30 s, 72°C for 45 s for 25 cycles, with a final step at 72°C for 10 min.
Equal volumes of each PCR product were analyzed by agarose gel electrophoresis, and PKM2 (197 bp), EF-1γ (249 bp), and β-actin (175 bp) bands were detected.

Animal experiments. Male SD rats (5-week-old) were purchased from Charles River Laboratories (Orient, Seoul, Korea). The animals were housed under a controlled temperature (22 ± 2%), relative humidity (50–60%), and lighting (12 h, light-dark cycles) environment. The animals were given food (PMI, Brentwood, MO) and tap water ad libitum. The animals were maintained according to the national guidelines and experimental protocols approved by the institutional animal care committee of Pusan National University (Busan, Korea). After 1 week of the acclimatization period, the rats were randomly divided into three experimental groups for each model, six animals in each group. Group I, control rats administered with 0.9% saline as vehicle; Group II, rats intraperitoneally (ip) injected with cisplatin (20 mg/kg body weight, dissolved in 0.9% saline) and sacrificed after 1 day; Group III, rats injected with the same amount of cisplatin and sacrificed after 3 days.

In order to examine in vivo effects of HgCl2 and CdCl2 on the expression of PKM2 and EF-1γ in rat urine and kidney tissue, rats were acclimated for 1 week before use and were randomly allocated into control and drug treatment groups with five rats per group. The rats were orally administered 1 and 5 mg/kg HgCl2 (dissolved in 0.9% saline) for 2 weeks. The rats were orally administered 5 and 25 mg/kg CdCl2 (dissolved in 0.9% saline) for 8 weeks.

Kidneys (right and left) were excised and weighed. The left kidney was immediately frozen in liquid nitrogen and stored at −80°C. The right kidney was fixed overnight in 10% neutral formalin and dehydrated in 70% ethanol. Urine samples were immediately centrifuged at 3000 × g for 15 min and stored in aliquots at −80°C for subsequent analysis. Throughout the study period, each animal was observed at least once daily for clinical signs of toxicity related to the chemical treatment. On working days, all cages were checked in the morning and afternoon for dead or moribund animals.

Immunohistochemical (IHC) analysis. The left kidney tissue was fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin sections (5 μm) were mounted onto slides and deparaffinized with xylene for 7 min (three times). The slides were rehydrated gradually in a graded series of alcohol and washed with deionized water. They were washed with phosphate-buffered saline (PBS) and then treated with 3% H2O2 in methanol for 20 min at room temperature to quench endogenous peroxide activity. To expose antigens, kidney sections were boiled in a target retrieval solution (0.01M sodium citrate, pH 6.0) for 10 min. The slides were then incubated overnight with the anti-PKM2 antibody (1:100, Cell Signaling) and anti-EF-1γ antibody (1:100, OriGene Technologies, Rockville, MD) at 4°C and then washed with PBS. Protein expression in tissue sections was detected using the Histostain-Plus Kits (Invitrogen), according to the manufacturer’s protocol. The sections were observed under a light microscope.

Statistics and bioinformatics. The results were presented as the mean ± SD of three independent experiments run in triplicates and analyzed by Student’s t-test. *: **: statistically different from control at p < 0.05 and p < 0.01, respectively. The biological functions of these proteins were categorized based on the information obtained from the NCBI (http://www.ncbi.nlm.nih.gov/pubmed/) and the Universal Protein Resource (UniProt) (http://www.uniprot.org/) websites.

RESULTS

Identification of Biomarker Candidates of Cisplatin-induced Nephrotoxicity in the Conditioned Media of HK-2 Cells by 2DE and MALDI-TOF-MS Analysis

In order to identify in vitro biomarkers of cisplatin-induced nephrotoxicity, a 2DE-based comparative proteomic analysis was conducted on the conditioned media of HK-2 cells treated with 0, 10, and 25 μM of cisplatin. Conditioned media rather than growth media was used because, in serum-free condition, these media contain the leaked, secreted, and sloughed proteins from the cells (Dowling and Clynes, 2011; Xue et al., 2008). The concentrations of cisplatin used were determined on the basis of our previous report which demonstrated that the IC50 for cisplatin was 28.6 μM when HK-2 cells were treated for 24 h (Sohn et al., 2013). A complete proteomic profile of the conditioned media was obtained from quantitative image analysis and MALDI-TOF-MS analysis. The number of proteins and their expression levels in conditioned media increased as cisplatin concentrations increased in 2DE gels (Fig. 1A). Twenty protein spots showing increased expression upon cisplatin treatment in a dose-dependent manner were identified and categorized using information from the NCBI (http://www.ncbi.nlm.nih.gov/pubmed/), HUGO Gene Nomenclature Committee (http://www.genenames.org/), and the Universal Protein Resource (UniProt) (http://www.uniprot.org/) websites (Fig. 1B and Table 1).

PKM2 and EF-1γ are Biomarker Candidates for Nephrotoxicity

Among proteins that were increased by cisplatin in conditioned media, the levels of five proteins, GRP 94, transferrin, PKM2, YWHAZ, and EF-1γ, were analyzed. Protein levels of GRP 94, transferrin, and YWHAZ were not altered upon cisplatin treatment (data not shown), whereas PKM2 and EF-1γ levels were increased in conditioned media in a dose-dependent manner by cisplatin treatment (Fig. 2A, left). However, PKM2 and EF-1γ levels in cell lysates were not affected by cisplatin (Fig. 2A, center). PKM2 and EF-1γ mRNA levels were also unchanged by cisplatin treatment (data not shown). Treatment of HK-2 cells with 10 μM cisplatin increased the levels of PKM2
## TABLE 1
Classification of Increased Proteins upon Cisplatin Treatment in Conditioned Media of HK-2 Cells

<table>
<thead>
<tr>
<th>Function/protein namea (detailed function)</th>
<th>Gene symbol</th>
<th>Accession no.</th>
<th>Mr/pI</th>
<th>No. of matched peptides</th>
<th>% Coverage</th>
<th>Scoreb</th>
<th>10/0 ratio</th>
<th>25/10 ratio</th>
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<tr>
<td><strong>Signal transduction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Hypothetical protein (regulation of the ubiquitin conjugation pathway)</td>
<td>COPS4</td>
<td>gi</td>
<td>31873302</td>
<td>47.405/7.57</td>
<td>25/60</td>
<td>57</td>
<td>207</td>
<td>3.4</td>
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<tr>
<td>KIAA0465 protein (regulation of Wnt receptor signaling pathway)</td>
<td>MACF1</td>
<td>gi</td>
<td>34328014</td>
<td>505.33/5.22</td>
<td>39/70</td>
<td>11</td>
<td>76</td>
<td>2.5</td>
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<tr>
<td>YWHAZ protein (antiapoptosis signal transduction)</td>
<td>YWHAZ</td>
<td>gi</td>
<td>49119653</td>
<td>30.1/4.72</td>
<td>21/60</td>
<td>59</td>
<td>153</td>
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<tr>
<td>Ubiquitin carboxyl terminal esterase L1 (regulation of the ubiquitin conjugation pathway)</td>
<td>UCHL1</td>
<td>gi</td>
<td>21361091</td>
<td>25.15/5.33</td>
<td>20/60</td>
<td>77</td>
<td>159</td>
<td>0.0</td>
</tr>
<tr>
<td>Eukaryotic translation elongation factor 1 gamma (translation elongation factor activity)</td>
<td>EEF1G</td>
<td>gi</td>
<td>4503481</td>
<td>50.43/6.25</td>
<td>14/60</td>
<td>35</td>
<td>101</td>
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</tr>
<tr>
<td>COP9 complex subunit 4 (regulation of cullin deneddylation)</td>
<td>COPS4</td>
<td>gi</td>
<td>5410300</td>
<td>46.17/5.57</td>
<td>13/76</td>
<td>44</td>
<td>77</td>
<td>0.0</td>
</tr>
<tr>
<td>78 kDa glucose-regulated protein (regulation of protein folding in endoplasmic reticulum and response of unfolded protein)</td>
<td>HSPA8</td>
<td>gi</td>
<td>16507237</td>
<td>72.40/5.07</td>
<td>13/60</td>
<td>21</td>
<td>70</td>
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<tr>
<td><strong>Cell proliferation/metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Phosphoglycerate kinase 1 (glycolysis, carbohydrate degradation)</td>
<td>PGK1</td>
<td>gi</td>
<td>4505763</td>
<td>44.99/8.30</td>
<td>21/60</td>
<td>62</td>
<td>143</td>
<td>2.7</td>
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<tr>
<td>Pyruvate kinase isozymes M1/M2 isoform M2 (glycolysis, programmed cell death)</td>
<td>PKM2</td>
<td>gi</td>
<td>33286418</td>
<td>58.47/7.96</td>
<td>22/60</td>
<td>54</td>
<td>184</td>
<td>1.9</td>
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<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (glycolysis and programmed cell death)</td>
<td>GAPDH</td>
<td>gi</td>
<td>31645</td>
<td>36.20/8.26</td>
<td>22/60</td>
<td>50</td>
<td>164</td>
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<tr>
<td>Tumor rejection antigen 1 (ER-associated protein catabolic process, antiapoptosis)</td>
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<td>gi</td>
<td>61656607</td>
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<td>17/60</td>
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<td></td>
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<tr>
<td>Keratin 1 (ectoderm development, cell structure)</td>
<td>KRT1</td>
<td>gi</td>
<td>11935049</td>
<td>66.15/8.16</td>
<td>11/60</td>
<td>21</td>
<td>56</td>
<td>3.4</td>
</tr>
<tr>
<td>Tubulin, alpha 1B (microtubule-based movement, cytoskeleton organization)</td>
<td>TUBA1B</td>
<td>gi</td>
<td>34740335</td>
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<td>20/60</td>
<td>50</td>
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<td>Chain A, human platelet profilin (actin polymerization in response to extracellular signals)</td>
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<td>gi</td>
<td>5542165</td>
<td>15.01/8.46</td>
<td>8/60</td>
<td>60</td>
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<tr>
<td>PRO1400 (cellular iron ion homeostasis, transferrin transport)</td>
<td>TF</td>
<td>gi</td>
<td>6650772</td>
<td>65.30/6.95</td>
<td>13/60</td>
<td>32</td>
<td>78</td>
<td>1.9</td>
</tr>
<tr>
<td>Serotransferrin precursor (cellular iron homeostasis, transferrin transport, platelet activation and degradation)</td>
<td>TF</td>
<td>gi</td>
<td>4557871</td>
<td>79.28/6.81</td>
<td>31/60</td>
<td>45</td>
<td>274</td>
<td>1.6</td>
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<tr>
<td>Transferrin (cellular iron ion homeostasis, transferrin transport)</td>
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<td>gi</td>
<td>553788</td>
<td>55.21/6.00</td>
<td>27/60</td>
<td>52</td>
<td>256</td>
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<tr>
<td>Hemopexin (cellular iron ion homeostasis, hemoglobin metabolic process)</td>
<td>HPX</td>
<td>gi</td>
<td>13529281</td>
<td>29.07/6.45</td>
<td>8/60</td>
<td>36</td>
<td>61</td>
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<td>gi</td>
<td>3353098</td>
<td>49.95/6.43</td>
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<td>46</td>
<td>126</td>
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</table>

aGrouping of proteins is according to the biological functions based on the information from the NCBI (http://www.ncbi.nlm.nih.gov/pubmed/), HUGO Gene Nomenclature Committee (http://www.genenames.org/), and the Universal Protein Resource (UniProt) (http://www.uniprot.org/) websites.
bProtein scores are $-10\log(P)$, where $P$ is the probability based MOWSE score that observed match is a random event. Protein scores $>63$ are significant ($p < 0.05$).
PKM2 AND EF-1γ AS IN VITRO BIOMARKERS

and EF-1γ in media in a time-dependent manner, but not in lysates (Fig. 2A, right). These data suggest that cisplatin may increase the secretion or leakage of PKM2 and EF-1γ, but not the expression of these proteins, in HK-2 cells.

Similarly, an increase in PKM2 and EF-1γ levels was observed in NRK-52E rat kidney epithelial cells conditioned media upon cisplatin treatment whereas no change was detected in PKM2 and EF-1γ levels in cell lysates (Fig. 2B). No increase in PKM2 and EF-1γ levels was detected in the conditioned media of the primary hepatocytes isolated from male SD rats or MCF10A human breast epithelial cells after cisplatin treatment, respectively (Fig. 2C and 2D), suggesting that the increase in PKM2 and EF-1γ levels in response to cisplatin is specific to kidney cells. We next compared the sensitivities of PKM2 and EF-1γ with those of previously identified nephrotoxicity biomarkers. As shown in Fig. 2E, PKM2 and EF-1γ were more

FIG. 1. Proteomic analysis of the conditioned media of cisplatin-treated HK-2 cells by 2DE and MALDI-TOF-MS. (A) Conditioned media were separated by 2DE. (B) Functional classification of up-regulated proteins in the conditioned media upon cisplatin treatment.
FIG. 2. Increased levels of PKM2 and EF-1γ in media of cisplatin-treated cells. (A) The protein levels of PKM2 and EF-1γ in media (left), lysates (center) in HK-2 cells treated with cisplatin for 24 h were detected by immunoblot analysis. A kinetic study was performed on cells treated with 10μM cisplatin at various time points (right). (B) PKM2 and EF-1γ in cisplatin-treated NRK-52E cells were detected by immunoblot analysis. The protein levels of PKM2 and EF-1γ were detected in the conditioned media of mouse primary hepatocytes (C) and MCF10A cells (D). (E) Detection of PKM2, EF-1γ, KIM-1, TIMP-1, and ALP in conditioned media of HK-2 cells treated with cisplatin for 24 h by immunoblot analysis. Band intensities were quantitated and plotted (*p < 0.05 and **p < 0.01).
sensitive than KIM-1, TIMP-1, or ALP in HK-2 cells upon cisplatin treatment. Our results indicate that PKM2 and EF-1γ proteins may be used as potential sensitive biomarkers for nephrotoxicity induced by cisplatin.

**Cisplatin-induced Apoptosis may be Responsible for the Increases of PKM2 and EF-1γ Levels in Media**

To investigate whether the cisplatin-induced increases of PKM2 and EF-1γ levels in conditioned media was due to a leakage from apoptotic HK-2 cells, apoptosis was inhibited by Z-VAD-FMK, a cell-permeant pan-caspase inhibitor which blocks the apoptosis (Sun et al., 1999). In presence of Z-VAD-FMK, the increase of PKM2 and EF-1γ levels in the media of HK-2 cells treated with cisplatin was almost completely abolished (Fig. 3A, left). However, PKM2 and EF-1γ protein levels in the cell lysates were not affected when cisplatin-induced apoptosis was blocked (Fig. 3A, right). These data indicate that cisplatin-induced apoptosis of HK-2 cells may be responsible for the increased levels of PKM2 and EF-1γ proteins in conditioned media.

To determine whether the increase of PKM2 and EF-1γ levels in the media was the result of cisplatin-induced secretion of these proteins by HK2 cells, cisplatin-treated HK-2 cells were treated with brefeldin, which inhibits the conventional secretion of proteins through the Golgi pathway (Klausner et al., 1992). Brefeldin treatment did not significantly affect the cisplatin-induced increase in PKM2 and EF-1γ levels in conditioned media (Fig. 3B). Similarly, a caspase-1 inhibitor (YVAD-CHO), an inhibitor of the unconventional protein secretion (Keller et al., 2008), did not alter PKM2 and EF-1γ levels in the media (Fig. 3C). These results demonstrate that the conventional and the nonconventional secretory pathways were not involved in the cisplatin-induced increase in PKM2 and EF-1γ levels, excluding the possibility that cisplatin induced the secretion of PKM2 and EF-1γ.

To test if PKM2 and EF-1γ detected in media are from the disrupted cells which were detached from culture plate after cisplatin application, we detected LDH, an indicator of membrane integrity leaked from dead cells into conditioned media (Lennon et al., 1991). As shown in Figure 3D, LDH level in media was increased by cisplatin. In addition, we counted attached cells on the plates upon cisplatin treatment. As shown in Figure 3E, the number of attached cells was decreased by cisplatin treatment in a dose-dependent manner. Furthermore, the microscopic observation (×100) showed that cells attached on the plates were decreased by cisplatin (data not shown). These data Implicate that after cisplatin treatment, PKM2 and EF-1γ may be leaked from the disrupted cells which were detached from culture plate, and thus detected in conditioned media.

**Other Nephrotoxic Agents Increased PKM2 and EF-1γ Levels in HK-2 Cells Conditioned Media**

We next questioned if PKM2 and EF-1γ can serve as protein biomarkers for nephrotoxicity induced by not only cisplatin but also other nephrotoxic agents. To this end, HK-2 cells were treated with CdCl2, HgCl2, or cyclosporine A which have been shown to exert nephrotoxicity (Jennings et al., 2007; Somji et al., 2004; Zalups, 2000). As shown in Figure 5A, treatment of HK2 cells with CdCl2, HgCl2, or cyclosporine A increased PKM2 and EF-1γ levels in the conditioned media in a dose-dependent manner as evidenced by immunoblot analysis. We further determined the level of PKM2 in the conditioned media of HK-2 cells upon treatment with cisplatin, CdCl2, or cyclosporine A by ELISA. The protein level of PKM2 was dose-dependently increased by cisplatin, CdCl2, or cyclosporine A (Fig. 5B). These data demonstrate that PKM2 and EF-1γ can serve as in vitro biomarker candidates for assessment of renal toxicity induced by nephrotoxic agents with various cytotoxic mechanisms.

**In Vivo Evaluation of the In Vitro Biomarker Candidates Using Rat Urine and Kidney Tissue**

The potential for PKM2 and EF-1γ as biomarkers for nephrotoxicity was next evaluated in vivo. PKM2 and EF-1γ were detected in the urine of rats injected with cisplatin at 1 day and 3 days after treatment (Fig. 5A, left). We next examined the expressions of PKM2 and EF-1γ in rat kidney tissues by IHC analysis. As shown in Fig. 5A right, the IHC analysis indicated prominent increases in the levels of PKM2 and EF-1γ in the kidney medulla and cortex of cisplatin-treated rats compared with control rats. Strong positive staining of PKM2 and EF-1γ was observed in both proximal tubule and medulla epithelial cells after cisplatin treatment. In contrast, these proteins were not detected in the liver tissues of cisplatin-treated rats (data not shown).

We then examined histological changes of kidney tissues after cisplatin treatment. It has been previously shown that renal injury induced by cisplatin is due to acute tubular necrosis and is usually reversible (Brillet et al., 1993). As shown in Supplementary figure 1, cisplatin treatment (20 mg/kg) produced mild morphological changes in kidney after 1 day as evidenced by moderate inflammation, and degeneration in proximal and distal tubules. The severity of cisplatin-induced kidney injury was greater at day 3 and included loss of tubular cells, tubular vacuolization, and shrinking of the cytoplasm in the proximal tubule. Tubular degeneration and severe necrosis occurred in some portions of tubules with the presence of pyknotic and karyorrhectic tubular cells.

We further investigated if PKM2 and EF-1γ are elevated after in vivo exposures to CdCl2 or HgCl2. To this end, rats were treated with CdCl2 or HgCl2 for in vivo detection of PKM2 and EF-1γ. PKM2 and EF-1γ were detected in urine and kidney tissue of rats treated with CdCl2 (Fig. 5B) and HgCl2 (Fig. 5C). These results suggest that PKM2 and EF-1γ can be considered as noninvasive biomarker candidates for nephrotoxic assessment.
FIG. 3. Increased levels of PKM2 and EF-1γ in media may be due to cisplatin-induced apoptosis. HK-2 cells were treated with cisplatin and (A) 25μM Z-VAD-FMK, (B) 100 ng/ml brefeldin, or (C) 10μM YVAD-CHO. (D) The protein level of LDH in HK-2 cell media treated with cisplatin for 24 h was detected by immunoblot analysis. (E) Measurement of cell attachment on the plates after cisplatin treatment. Band intensities were quantitated and plotted (*p < 0.05 and **p < 0.01).
DISCUSSION

Early detection of drug-induced nephrotoxicity using more sensitive and reliable biomarkers is critical for drug development. Proteomic approaches have contributed to biomarker identification and development for toxicity assessment. The present study aimed at identifying in vitro noninvasive biomarkers for more sensitive and efficient evaluation of nephrotoxicity using cisplatin as a model of nephrotoxic compounds. Here, we identified that PKM2 and EF-1γ were identified as potential biomarker candidates for evaluation of nephrotoxicity by comparative proteomic analysis of conditioned media of cisplatin-treated HK-2 human kidney epithelial cells.

Changes of BUN, creatinine clearance, ALP, and KIM-1 in blood and urine have been used for the in vivo assessment of renal injury (Emeigh Hart, 2005; Hoffmann et al., 2010). ALP has been used as a traditional enzyme marker for nephrotoxicity (Emeigh Hart, 2005). TIMP-1 and KIM-1 are located in plasma membrane and secreted or shed into media at renal injury (Emeigh Hart, 2005). BUN and creatinine are not considered as appropriate biomarkers because they are not detected at early stage of kidney damage (Rached et al., 2008). In this study, we showed that cisplatin treatment increased the levels of PKM2 and EF-1γ in media with higher sensitivities compared with known nephrotoxicity biomarkers, ALP, KIM-1, and TIMP-1 (Fig. 2E). Of note, we loaded 30 μg of proteins in each lane for immunoblot analysis (Fig. 2E), much less amount loaded for our previous report which was 100 μg (Fig. 3 in Sohn et al., 2013). The present study provided evidence that PKM2 and EF-1γ can be used as sensitive in vitro potential biomark-
FIG. 5. PKM2 and EF-1γ were increased in urine (left) and kidney tissues (right) of rats treated with nephrotoxicants by immunoblot analysis and IHC analysis, respectively. (A) Urine and kidney tissues were obtained from rats injected with 20 mg/kg cisplatin at 1 day or 3 days. (B) Urine and kidney tissues were obtained from rats orally administered 5 or 25 mg/kg CdCl₂ for 8 weeks. (C) Urine and kidney tissues were obtained from rats orally administered 1 or 5 mg/kg HgCl₂ for 2 weeks. The photomicrographs were taken at ×400 magnification. Arrows indicate representative positive staining.
ens for evaluation of nephrotoxicity by immunoblot analysis and ELISA.

Both PKM2 and EF-1α are cytosolic proteins. A large number of cytosolic proteins can be released in the media by secretion (Lee et al., 2004) or leakage through the damaged cell membrane in cell-death-inducing conditions (Lewis et al., 2010). The levels of PKM2 and EF-1α in HK-2 cells conditioned media were increased by cisplatin in a dose-dependent manner whereas the intracellular levels of PKM2 and EF-1α were not altered (Fig. 2A). And the mRNA levels of these molecules were not affected (data not shown). These results implicate that PKM2 and EF-1α may be secreted or leaked out of the cells into the media. The use of inhibitors of the conventional and unconventional secretory pathways indicated that these two pathways were not involved in the cisplatin-induced increase in PKM2 and EF-1α in conditioned media of HK-2 cells (Figs. 3A–C).

When apoptosis was blocked, the effect of cisplatin on the levels of PKM2 and EF-1α in conditioned media was significantly inhibited. These data suggest that cisplatin-induced apoptosis may be responsible for the increased release of PKM2 and EF-1α in the conditioned media.

Upon cisplatin treatment, the level of LDH increased (Fig. 3D) and cell attachment was decreased (Fig. 3E), substantiating that cisplatin may induce leakage of PKM2 and EF-1α from damaged kidney cells. Consistently, these proteins were increased in urine samples of cisplatin-treated rats (Fig. 5A, left). The increased levels of PKM2 and EF-1α were also observed in kidney tissues (Fig. 5A, right). The apparent discrepancy between the in vitro data using cell lysates (Fig. 2A, center) and the in vivo observations using kidney tissues (Fig. 5A, right) may be explained by the possibility that PKM2 and EF-1α are leaked from the damaged kidney tissues and detected by IHC analysis. Importantly, these disrupted/detached cells would not be included in cell lysates because these were rinsed-out during the preparation of cell lysates. Consequently, the total protein levels (conditioned medium and cell lysates) of PKM2 and EF-1α can be changed by cisplatin treatment although the mRNA levels of these molecules were unchanged. These results suggest that PKM2 and EF-1α in the media are from the disrupted cells which were detached from culture plate.

PKM2 is a glycolytic enzyme involved in the supply of energy for cell growth and proliferation. The increase of PKM2 expression is known to depend on anaerobic metabolism, the “Warburg Effect,” especially in cancer cells (Sun et al., 2011). EF-1α is present in the cytoplasm of eukaryotic cells and is involved in the process of protein biosynthesis (Sanders et al., 1992). Although it is highly expressed in pancreatic cancer tissue, its expression level is relatively low in normal organs such as the kidney (Lew et al., 1992). Cisplatin treatment increased the levels of PKM2 and EF-1α in conditioned media of kidney cells but not in liver cells or breast cells (Fig. 2). In addition, when the primary hepatocytes were treated with a hepatotoxic agent, β-galactosamine (Kepler et al., 1968), neither PKM2 nor EF-1α was increased in the media (data not shown). Notably, PKM2 has also been reported to be overexpressed in lung tissue by cisplatin treatment (Guo et al., 2010). The levels of PKM2 and EF-1α in HK-2 cells conditioned media may be elevated by kidney injury, but not liver injury, supporting the potential for PKM2 and EF-1α to be considered as nephrotoxic biomarkers.

The released enzymes and proteins from damaged renal cells excreted into the urine, such as γ-glutamyl transferase, N-acetyl-β-D-glucosaminidase, albumin, and β2-microglobulin, have been regarded as sensitive in vivo indicators (Hart and Kinter, 2005). However, the detection of these proteins in the urine is not easy because of their instability and variable levels (Rached et al., 2008). In the present study, conditioned media of cultured cells were used because they may represent a more convenient and reliable system for the detection of biomarkers. The elevated levels of PKM2 and EF-1α in conditioned media were induced not only by a DNA alkylating agent cisplatin but also by other nephrotoxic agents such as CdCl2, HgCl2, and cyclosporine A, using different cytotoxic mechanisms (Fig. 4). When kidney proximal tubule cells are exposed to CdCl2 or HgCl2, they induced apoptosis by increasing the generation of intracellular ROS (Somji et al., 2004; Zalups, 2000). Cyclosporine A has been shown to induce cellular senescence in kidney cells (Jennings et al., 2007). Our results suggest that PKM2 and EF-1α in the media can be used as in vitro biomarker candidates for nephrotoxic agents, regardless of their cytotoxic mechanisms. Rat urine was used to assess the levels of PKM2 and EF-1α after nephrotoxicants treatment in vivo. Increased levels of PKM2 and EF-1α were detected in the urine of rats treated with cisplatin, CdCl2, or HgCl2. Because urine analysis can provide clear indications of the kidney condition (Shao et al., 2011), our data suggest that PKM2 and EF-1α are suitable biomarkers to assess nephrotoxicity. Taken together, comparative proteomic analysis enabled the identification of two biomarker candidates, PKM2 and EF-1α, which can be used effectively to rapidly and accurately evaluate drug-induced damage in the kidney.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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