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Neutrophil gelatinase-associated lipocalin production negatively correlates with HK-2 cell impairment: Evaluation of NGAL as a marker of toxicity in HK-2 cells

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ABSTRACT

Neutrophil gelatinase-associated lipocalin is an extracellular protein produced mostly in kidney. Recently, it has become a promising biomarker of renal damage in vivo. On the other hand, the validation of NGAL as a biomarker for nephrotoxicity estimation in vitro has not been characterized in detail yet. Since the HK-2 cells are frequently used human kidney cell line, we aimed to characterize the production of NGAL in these cells and to evaluate NGAL as a possible marker of cell impairment. We used heavy metals (mercury, cadmium), peroxide, drugs (acetaminophen, gentamicin) and cisplatin to mimic nephrotoxicity. HK-2 cells were incubated with selected compounds for 1–24 h and cell viability was measured together with extracellular NGAL production. We proved that HK-2 cells possess a capacity to produce NGAL in amount of 2 pg/ml/h. We found a change in cell viability after 24 h incubation with all tested toxic compounds. The largest decrease of the viability was detected in mercury, acetaminophen, cisplatin and gentamicin. Unexpectedly, we found also a significant decrease in NGAL production in HK-2 cells treated with these toxins for 24 h: to 11 ± 5%, 54 ± 5%, 57 ± 6% and 76 ± 9% respectively, compared with controls (= 100%). Our results were followed with qPCR analysis when we found no significant increase in LCN2 gene expression after 24 h incubation. We conclude that extracellular NGAL production negatively correlates with HK-2 cell impairment.

1. Introduction

Neutrophil gelatinase-associated lipocalin (NGAL; also known as lipocalin-2, siderocalin) belongs to the lipocalin superfamily of soluble proteins. These proteins share a similar structure of 8 β-strands arranged in a complex β-barrel representing their binding site. The main feature of neutrophil gelatinase-associated lipocalin seems to be binding of siderophores (Bolignano et al., 2008; Mishra et al., 2005; Schmidt-Ott et al., 2007)

NGAL is a 25 kDa polypeptide covalently bound to gelatinase (Ramirez-Sandoval et al., 2015). It was originally purified from neutrophils but its production was proven also in other cells (e.g. keratinocytes, kidney cells, adipocytes, hepatocytes) (Chakraborty et al., 2012; Wasung et al., 2015). The function of NGAL does not seem to be linked only with sequestration of siderophores from urine because NGAL was proved to play a role also in kidney development and regulation of pro-/anti-apoptotic processes in damaged cells (Bolignano et al., 2008; Kehrer, 2010).

Recently, NGAL has been found to be a marker usable for monitoring kidney injury. It belongs among modern biomarkers of renal function together with cystatin C, kidney injury molecule 1 (KIM-1) and interleukin-18 (Fiseha, 2015; Wasung et al., 2015). Urine NGAL concentration was found to reflect the severity of acute kidney injury (Ho et al., 2015; Wasung et al., 2015), delayed graft function after kidney transplantation (Cantaluppi et al., 2015; Ramirez-Sandoval et al., 2015), contrast induced nephropathy and chronic kidney diseases (Bolignano et al., 2008). Therefore, it may be a suitable specific marker for most of kidney diseases.

Kidney injury has been estimated in a number of in vitro models. Recently, a human kidney cell line (HK-2) has been used in wide range of experiments on nephrology and nephrotoxicity. This cell line was established in 1994 by Dr. Ryan team (Ryan et al., 1994). It was derived from normal adult human proximal tubular cells that were immortalized by human recombinant papilloma virus. It has been found that these cells retain most of functional characteristics, i.e. membrane transporters, enzymes, cytochromes, consistent with in vivo system and human proximal tubular cells (Arbillaga et al., 2007; Racusen et al., 1997) so that they exhibit normal phenotypic characteristics of proximal tubular tissue of human kidney. Based on these outcomes, HK-2 cells have been used for nephrotoxicity testing in a variety of compounds in vitro, i.e. heavy metals (Bao et al., 2013; Fujiki et al., 2014),

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toxins (Quoilin et al., 2012; Wang et al., 2015) and drugs (Genc et al., 2014; Vizza et al., 2013). Therefore, the HK-2 cell line has been predominantly accepted as a suitable in vitro model for studying nephrotoxicity on human kidney cells.

At present, a variety of methods have been used to evaluate the level of nephrotoxicity in HK-2 cells. The studies have assessed cell damage using XTT, MTT or WST-1 viability tests (Ozcan et al., 2015; Vizza et al., 2013; Wang et al., 2015) and trypan blue exclusion (Fujiki et al., 2014; Quoilin et al., 2012), estimation of apoptosis (Kim et al., 2013; Ozcan et al., 2015; Wang et al., 2015) or oxidation products (Bao et al., 2013). In addition, the aim of another study was to find new biomarkers of HK-2 cell injury (Kim et al., 2014). However, any toxicological study did not estimate the relation of toxin treatment and production of NGAL in HK-2 cells although these kidney cells should potentially reflect the level of damage through changing NGAL production. Therefore, the goal of our study was to characterize the change of NGAL extracellular production related to the extent of toxicity and to evaluate if neutrophil gelatinase-associated lipocalin produced to cell medium can be used as a specific marker of cellular impairment in HK-2 cell in comparison to common viability tests.

2. Methods

2.1. Chemicals

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (with/without phenol red), insulin, transferrin and sodium selenite were purchased from Sigma-Aldrich (USA). Fetal bovine serum, pyruvate, penicillin, streptomycin, epidermal growth factor and all other chemicals, if not otherwise specified, were purchased from Invitrogen-Gibco (USA).

2.2. Cell line

Human kidney (HK-2) cells, a proximal tubular epithelial cell line derived from normal adult human kidney cells immortalized by transduction with human papillomavirus (HPV 16) DNA fragment (Ryan et al., 1994), were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12 = 1:1) supplemented with 5% (v/v) fetal bovine serum, 1 mM pyruvate, 50 μg/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml sodium selenite, and 5 ng/ml epidermal growth factor and maintained at 37 °C in a sterile, humidified atmosphere of 5% CO_2. All the experiments were conducted using the HK-2 cells (14.–18. passages).

2.3. Cell viability tests

HK-2 cells were seeded into 96-well plates at density of 3×10^4 cells/well and exposure medium containing tested compounds ($CdCl_2$, $HgCl_2$, acetaminophen, gentamicin, *tert*-butylhydroperoxide, cisplatin). The cells were then incubated for specific time periods (1; 3; 6; 24 h). Cell viability was evaluated by assay of lactate dehydrogenase activity in culture medium after 24 h of treatment using a commercial kit from Roche (Germany) and also using the WST-1 test (Roche, Germany). The WST-1 test measures the activity of intra- and extramitochondrial dehydrogenases. At the required time, the WST-1 reagent was added to the cultured cells (1:10 final dilution). The cells were incubated in a gassed atmosphere (5% CO_2) for 60 min and the absorbance change (0 − 1 h) was measured spectrophotometrically at wavelength of 440 nm using Tecan Infinite M200 plate reader (Tecan Austria).

2.4. Neutrophil gelatinase-associated lipocalin levels measurement

The concentration of human lipocalin-2 (neutrophil gelatinase-associated lipocalin), was evaluated using a commercial human lipocalin-2 ELISA kit (Sigma-Aldrich, USA). Briefly, after seeding of HK-2 cells into 96-well plates (density of 3×10^4 cells/well), the cells were treated with tested compounds for 1–24 h. Cell culture supernatants were then pipetted into 96-well plates coated with specific antibodies for human lipocalin-2 and the experiment was carried out according to the protocol. The concentration of NGAL in a sample was measured at 450 nm and calculated in relation to the calibration curve.

2.5. Analysis of NGAL expression

The expression of NGAL was analyzed after 24 h of treatment of HK-2 cells with toxic compounds. After treatment, the cells were lysed in lysis reagent and the cell lysates were stored at − 80 °C until analyzed. The RNA was isolated using TriReagent (Sigma-Aldrich, USA), the concentration of RNA was determined from absorbance at 260 nm. Maximum 500 ng of RNA was reverse transcribed to cDNA by Reverse Transcription Kit (GENERI BIOTECH, Czech Republic). The stability of reference genes expression was evaluated by geNorm (Vandesompele et al., 2002). The most stable reference genes B2M and TBP were selected from 10 genes contained in the set of qPCR assays for reference genes (GENERI BIOTECH, Czech Republic). Real-time PCR analysis was performed using gb Elite PCR Master Mix, qPCR assays for LCN2, B2M and TBP genes and linearized plasmid standards corresponding to the assays (GENERI BIOTECH, Czech Republic). The concentration of each sequence was determined by extrapolation of Ct values from calibration line from 10^7 to 10^1 copies/μl of sample. The normalized gene expression ratio was assessed according the formula $\mathrm{Exp} = \frac{c_{LCN2}}{GEOMEAN(c_{B2M}, c_{TBP})}$ where c is the concentration in copies/μl of cDNA. The relative expression was determined as $\log_2$ of ratio between *Exp* values of sample and control.

2.6. Statistics

All experiments were repeated at least three times independently; the analysis of LCN2 expression was repeated two-times. The results are expressed as mean ± SD. Statistical significance was analyzed after normality testing using one-way ANOVA test followed by Bonferroni posttest (OriginPro 9.0.0, USA) to compare results with controls at significance level $p = 0.05$ ($^*p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Estimation of relation between cell viability and NGAL production in HK-2 cells

To assess the toxicity in HK-2 cells, we used a variety of model kidney toxins. We used heavy metals ($CdCl_2$, $HgCl_2$), drugs (acetaminophen, gentamicin), *tert*-butylhydroperoxide (tBHP) to induce oxidative stress generally and cisplatin as a typical nephrotoxin. Each compound was tested at two different concentrations. The concentrations of renal toxins were selected according to the toxicological studies of other authors - cisplatin (Gao et al., 2013; Genc et al., 2014; Huang et al., 2015; Kim et al., 2014), cadmium (Fujiki et al., 2014; Kim et al., 2014), mercury (Kim et al., 2014; Sutton & Tchounwou, 2007), acetaminophen (Vrbova et al., 2016; Wu et al., 2009) and gentamicin (Huang et al., 2015; Lee et al., 2013). After 24 h of incubation, the cell viability (WST-1 and LDH activity) and NGAL production to the medium were measured.

We found that almost all toxins caused a significant decrease in cell viability at higher concentrations of each compound after 24 h (Fig. 1). The signal of intracellular dehydrogenases activity in WST-1 test was largely reduced in 10 mM acetaminophen (61 ± 5%, $p < 0.001$), 50 μM