

Lipocalin 2 acts as a cytoprotective factor against cisplatin toxicity, an *in vitro* study

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ABSTRACT

Background and the purpose of the stud.: Lipocalin 2 (Lcn2, NGAL) is a member of the lipocalin super family which has been known as an early marker for ischemic acute renal injury and cisplatin nephrotoxicity. In this study the ability of Lcn2 to prevent cisplatin-induced toxicity was studied.

Methods: Lcn2 cDNA was isolated from Hep G2 cell line and cloned to pcDNA3.1(+) vector. The construct was transfected to CHO cell line. Stable clones were generated and the expression of Lcn2 was determined by RT-PCR and ELISA. Lcn2 gene in A549 cell line was also down-regulated with the siRNA. CHO and A549 cells were exposed to cisplatin and cell proliferation was determined by MTT assay.

Results: Cell proliferation was higher in CHO expressing Lcn2 at doses of 75, 150, and 200 µg/ml of cisplatin after 48 h compared with control. Cisplatin toxicity increased significantly in A549 cells following treatment with Lcn2 siRNA.

Major conclusion: findings of this study revealed that Lcn2 acts as a cytoprotective factor against cisplatin toxicity and suggests that Lcn2 might have the potential application to ameliorate the cisplatin toxicity.

Keywords: Lcn2/ NGAL, Cisplatin, Toxicity, Cytoprotective factor

INTRODUCTION

The lipocalins constitute a broad but evolutionally conserved family of small proteins. Although the primary function of the lipocalins is thought to be transport of small ligands, they have also been implicated in a variety of different functions such as retinol transport, cryptic coloration, olfaction, pheromone transport, prostaglandin synthesis, regulation of the immune response and cell homeostatic (1). Initially 24p3/lcn2 was identified as a super-induced protein secreted by 3T3 cells in response to fibroblast growth factor and was called SIP24 (2-3). Around the same time that 24p3/lcn2 messenger RNA (mRNA) was cloned, the human homologue NGAL was identified as a 25-kilodalton protein associated with gelatinase/matrix metalloproteinase 9 from human neutrophils (4). Neutrophil Gelatinase-Associated Lipocalin (NGAL) and 24p3/lcn2 have a high degree of identity at the complementary DNA (cDNA) level and have 62% identical amino acid homology (5). The pathophysiological functions of

24p3/Lcn2/NGAL are unclear, but it has been suggested that they may act as an immunomodulator by binding to or inactivation of bacterial products, (6), or through direct actions on the inflammatory cells (7). Lcn2 has been known as an early marker for acute ischemic renal injury and cisplatin nephrotoxicity. Cisplatin is an important antineoplastic drug and particularly effective for the treatment of testicular and ovarian cancers (8-9). However, its dose-limiting nephrotoxicity which is characterized by decreased glomerular filtration and tubular injury and is observed in about 20% of patients despite the use of saline hydration and diuretics, is a major clinical problem (10). Consequently, there is much interest in new methods to abrogate renal damages (11). It has been shown that administration of recombinant Lcn2 mice lacking Lcn2 not only did not enhance ischemic acute renal injury (12), but instead ameliorated it (13). Therefore it was postulated that Lcn2 might have cytoprotective effects and in this study its ability

to prevent in vitro cisplatin-induced toxicity was investigated. For this purpose pcDNA 3.1(+) vector was used which provides the opportunity to clone the desired insert in bacteria and expresses in mammalian cell line. In addition it contains neomycin resistance gene that allows selection of stable transfectants in the presence of geneticin in mammalian cells.

MATERIALS AND METHODS

Plasmids and bacterial strains

Plasmid pcDNA3.1(+) (Invitrogen, USA) was used for construction and expression. Bacterial strain was *E. coli DH5α* (Cinagen, Iran).

Cell culture

Human hepatoma (HepG2) and Chinese Hamster Ovary (CHO) were obtained from National Cell Bank (NCBI), of Pasteur Institute of Iran. These cell lines were grown in RPMI-1640 medium (Gibco-BRL, Germany) with 10% fetal bovine serum (Gibco-BRL, Germany).

RNA extraction

Total RNA from cell lines was extracted by Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The quality of RNA was determined by electrophoresis with 2 µg of RNA.

cDNA synthesis

Reverse transcription was performed by SuperScript III reverse transcriptase according to manufacturer's protocols (Invitrogen, USA) with 500 ng of total RNA followed by DNaseI (Invitrogen, USA) treatment and heat inactivation.

Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

RT-PCR was used for isolation of the full length of Lcn2 and screening of CHO cells for expression of Lcn2. PCR was performed using Pfu and Taq DNA polymerase (Cinnagene, Iran) in a GeneAmp PCR system 9600 (PerkinElmer Life And Analytical Sciences, Inc., Wellesley, MA, USA). Primer set for full length human *Lcn2* containing Kozak sequences and *EcoRI* restriction enzyme site was; forward 5'-ACGAATTCACC ATGGTGGCCCTAGGTCTCCTGTGGCTG-3' and reverse containing *NotI* restriction enzyme site 5'-TAGCGGCCGCTCAGCCGTCGATAC ACTGG.

TC-3. Primer set for a 240 bp fragment of *Lcn2* was, forward 5'-TCA CCT CCG TCC TGT TTA GG-3' and reverse 5'-CGA AGT CAG CTC CTT GGT TC-3'. For normalization, expression of β -actin was examined and the primer set was; forward 5'-TTC TAC AAT GAG CTG CGT GTG G -3' and reverse 5'-GTG TTG AAG GTC TCA

AAC ATG AT-3'. The PCR condition was; initial denaturation at 94 °C for 5 min followed by 30 amplification cycles consisting denaturation at 94 °C for 30 sec., annealing at 60 °C for 30 sec. and extension at 72 °C for 30 sec. PCR annealing temperature was 59 °C for *beta-actin*. PCR products were separated in agarose gel.

Real-time PCR

Real-time PCR analysis was performed to detect reduction in *Lcn2* gene expression in siRNA gene silencing experiment by a BIO-RAD icycler iQ, SA-THK Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Amplification was conducted using AB solute syber green ROX mix (ABgene, UK) (14). The PCR condition was; initial denaturation at 94 °C for 15 min. followed by 40 amplification cycles consisting denaturation at 94 °C for 30 sec., annealing at 60 °C for 30 sec. and extension at 72 °C for 30 sec. Threshold cycle values were normalized by *beta-actin* expression

Plasmid construction and generation of stable Lcn2 - expressing cell lines

To construct the *Lcn2* expression plasmid, the full-length human *Lcn2* cDNA was synthesized by RT-PCR using the primer which was designed according to the sequence of the *Lcn2* gene. The amplified *Lcn2* cDNA containing *EcoRI* and *NotI* restriction enzyme sites was cloned into the mammalian expression vector pcDNA3.1 in the sense orientation. The identity and orientation of this construct were confirmed by DNA sequencing. CHO cells were transfected with 1 µg of linearized DNA of pcDNA3.1-Lcn2, cut with *BglII* restriction enzyme, using the fugene 6 (Roche, Germany) according to the manufacturer's protocol. pcDNA3.1 (+) DNA was used as a control. CHO cells stably expressing the pcDNA3.1-Lcn2 construct were selected in a medium containing 600 µg/ml Geneticin (Roche, Germany) for at least 14 days. Several stable clones were generated by dilution of the cells and culture in 96 well culture plates. The expression of *Lcn2* was determined by RT-PCR and ELISA.

ELISA for human Lcn2 Immunoassay

Medium of stable CHO cells and A549 was used for assessment of *Lcn2* protein expression. It was performed with quantikine kit in accordance with manufacturer's protocol (R& D system, USA). All measurements were performed in triplicates.

Small interfering RNA (siRNA) gene silencing

Lcn2 mRNA was down-regulated with Hs_Lcn2_6_HP validated siRNA (Qiagen, Germany). A siRNA targeted to unknown gene

was used as a negative control. The sequences of control siRNA was; sense: UUC UCC GAA CGU GUC ACG U dT dT and antisense: ACG UGA CAC GUU CGG AGA A dT dT. (Qiagen, Germany). Then 5nM of the synthetic double-stranded siRNA oligonucleotides were delivered into A549 cells using different doses of HiPerFect transfection reagent (Qiagen, Germany) according to the manufacturer's recommended protocol. Reduction in Lcn2 gene expression by Lcn2 siRNA was measured by real-time RT-PCR 72 h post-transfection, and also by assessment of the amount of Lcn2 protein secreted into the medium at this time by ELISA.

Cytotoxicity assays

The cytotoxic effects of cisplatin, on CHO and A549 cell lines were determined by trypan blue dye exclusion and MTT assays (14). For MTT assay, 2×10^4 cells were seeded/well, in a 96-well plate. After 24h, cis platinaly at concentrations of, 25- 250 μ g/ml, (Sigma, Germany) was diluted with 200 μ l of medium and added to each well. After 24, 48 and 72 h the cells were incubated with 10 μ l of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Germany) at concentration of 5mg/ml at 37°C in 5% CO₂ atmosphere for 4h to allow MTT to be converted to formazon crystals through the reaction with metabolically active cells. Reaction was stopped by addition of 10% SDS, 0.01M HCl and the cell viability was measured at 570 nm using a plate reader. The numbers of viable cells also were examined by trypan blue exclusion assay.

Statistical analysis

The results are expressed as mean \pm SD of three independent experiments. Differences between groups were compared using ANOVA with Tukey-Kramer Multiple Comparison Test as a post test.

RESULTS

Isolation and Construction of Lcn2 gene

Specific primers were designed to amplify full length of human Lcn2. The expected size of the product of PCR was found as 625 bp. Then the isolated fragment was cloned in pcDNA3.1(+) vector and transformed to *DH5 α* *E.coli*. pcDNA3.1 (+) vector was used. For both cloning and expression, the bacteria were screened for the existence of recombinant vector, pcDNA-Lcn2, by restriction enzyme analysis and PCR (Fig 1) and finally the accuracy of the nucleotide sequence of the gene and its frame in the vector was confirmed by DNA sequencing.

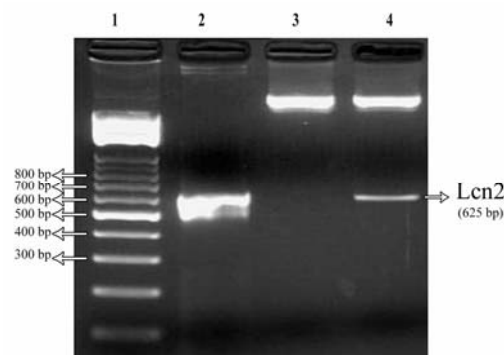


Figure 1. Electrophoresis of PCR and digestion products on 1.5 percent agarose gel. A 625 pb fragment was amplified by specific primers (lane 1). pcDNA3.1 and pcDNA3.1-Lcn2 plasmids, were extracted and cut with *E.corI* and *NotI*. Lane 2, pcDNA3.1. Lane3, pcDNA3.1-Lcn2, In addition to linear plasmid a 625 bp band can be observed indicating the presence of the insert (lane 3).M; 100 bp ladder marker.

Expression of Lcn2 in CHO cell line

To express Lcn2, CHO cell line was trasfected with linearized pcNDA3.1- Lcn2. After culture of the cell in the presence of geneticin for tow weeks, stable cell lines were generated. Then the stable cells were screened for Lcn2 expression. To investigate whether the stable cells express Lcn2, RT-PCR was performed. While stable clones expressed Lcn2 mRNA, there was no expression in the CHO cell transfected with pcDNA3.1 (Data not shown). The cell culture medium of stable cells was used for detection of Lcn2 protein by ELISA. Again the medium of CHO transfected with pcNDA3.1-Lcn2 expressed Lcn2 protein while CHO cell transfected with pcDNA3.1 vector was negative indicating the CHO cells ectopically expressed Lcn2 (Table 1). Several stable clones expressing Lcn2 were established of which clones expressing 0.25 μ g/ml of recombinant Lcn2 was used for cytotoxicity study following treatment with cisplatin.

Table 1. ELISA for human NGAL Immunoassay

Samples	OD 450
Lcn2 ^a , 10 ng/ml	1.752 \pm 0.123
Lcn2 ^a , 5 ng/ml	0.912 \pm 0.165
Lcn2 ^a , 2.5 ng/ml	0.533 \pm 0.116
Lcn2 ^a , 1.25 ng/ml	0.294 \pm 0.094
CHO – pcDNA3.1/Lcn2 ^b	1.85 \pm 0.162
CHO – pcDNA3.1	0.095 \pm 0.0032
A549(control siRNA)	0.482 \pm 0.072
A549(siRNA)	0.182 \pm 0.117

^a, Standard concentrations of human Lcn2 was provided in the ELISA kit.

^b, 25 – fold dilution

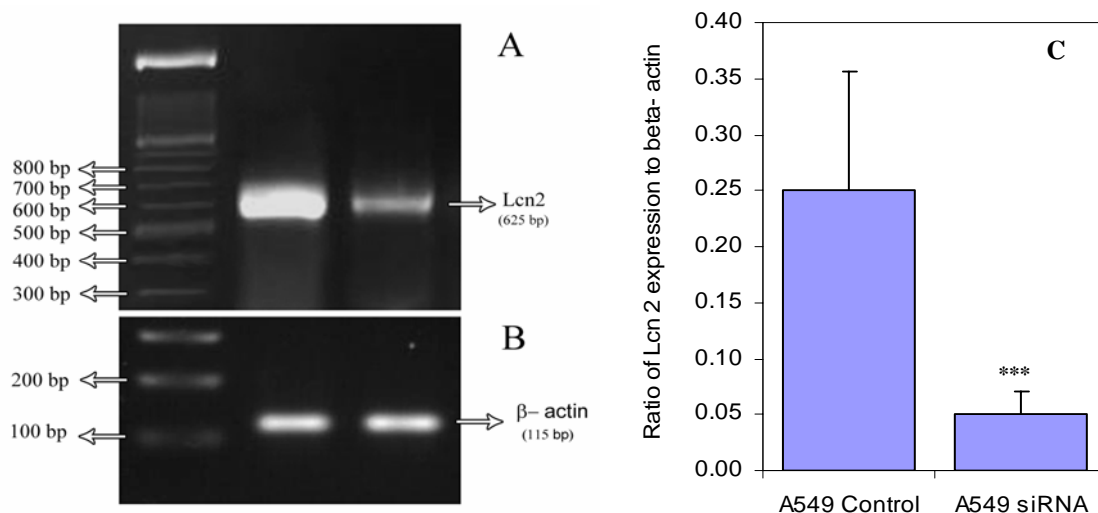


Figure 2. Down-regulation of Lcn2 gene. RNA was extracted from cells transfected with the siRNA and control. cDNA was synthesized and RT-PCR was performed. **A**; PCR products were electrophoresed in 2 percent of agarose gel. Lcn2 in A549 cell transfected with siRNA (lane 2) was suppressed compared with the control (Lane 1). **B**; indicating the expression of beta actin. M; 100 bp ladder marker. **C**; Down-regulation of Lcn2 expression in A549 cells also were measured by real time PCR. (Mean±SD, ***: P<0.001)

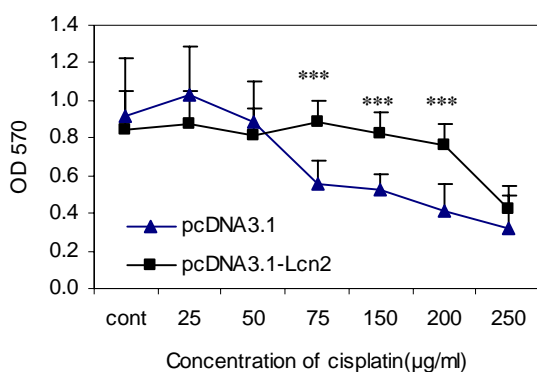


Figure 3. Cytotoxicity of different concentrations of cisplatin on stable CHO cells transfected with pcDNA3.1-Lcn2 or pcDNA3.1 for 48 h in MTT assay. (Mean±SD, ***: P<0.001)

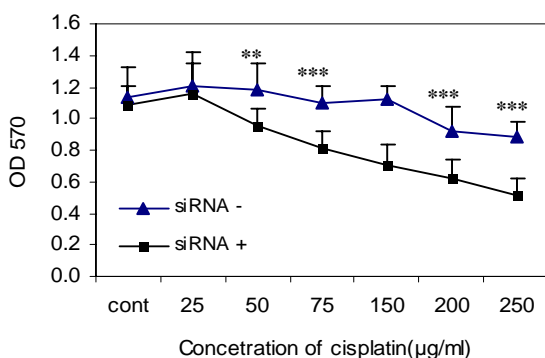


Figure 4. Cytotoxicity different concentrations of cisplatin on A549 cell in MTT assay. A549 cells in which Lcn2 was down-regulated with siRNA and A549 cell transfected with control siRNA were treated with different doses of cisplatin for 48 h followed by cytotoxicity assay. (Mean±SD, **: P<0.001, ***: P<0.001)

Down-regulation of Lcn2 gene

RT-PCR was used to analyze Lcn2 gene silencing. About %80 reduction in Lcn2 expression was detected compared with control siRNA (Fig 2A and 2B). The reduction in Lcn2 expression also was verified by Real time PCR (Fig 2 C) and ELISA (Table 1).

CHO cells expressing Lcn2 decreased toxicity of Cisplatin.

To test the involvement of Lcn2 in cell survival after treatment with cisplatin, the stable CHO cells expressing Lcn2 and a control transfectant clone were treated with different doses of cisplatin and then assayed for cytotoxicity. Cell proliferation was higher in CHO expressing Lcn2 at doses of 75, 150, and 200 µg/ml of cisplatin after 48 h compared to control suggesting NGAL acts as a protective factor against cisplatin (Fig 3).

Down-regulation of Lcn2 increases cell sensitivity to cisplatin toxicity

The hypothesis that Lcn2 exerts protective activities was tested further by down-regulating the expression of this gene with siRNA. A549 cells in which Lcn2 was down-regulated with siRNA and A549 cells transfected with control siRNA were treated with different doses of cisplatin and then cytotoxicity and proliferation were determined. Cisplatin toxicity was significantly increased in A549 cells after treatment with Lcn2 siRNA compared with the control (Fig 4).

DISCUSSION

A fundamental goal of the cancer treatment is to enhance the therapeutic index of chemotherapy in such a way that toxicity to normal cells becomes minimized. A major limiting factor in successful cancer therapy is development of resistance to the drugs used for treatment. A second fundamental problem is the toxic effects of the drugs to the normal tissues. Cisplatin (*cis*-dichlorodiammine-platinum (II)) is one of the major therapeutic compounds in the treatment of gynecological cancers. Its activity is greatly restricted by low bioavailability and high toxicity. Cisplatin damages liver and, especially, renal functions. The current study was designed to determine whether in an in vitro model Lcn2 acts as a cytoprotective factor against cisplatin toxicity. The recombinant pcDNA 3.1 -Lcn2 was constructed and transfected to CHO cell line. This cell line does not express human Lcn2, so after ectopically expression of Lcn2 any change in cell viability after treatment with cisplatin would be due to the recombinant Lcn2. Our hypothesis was further examined by down- regulation of Lcn2 expression in A549 cell line by gene silencing technology. Results revealed that Lcn2 acts as a cytoprotective factor against cisplatin toxicity.

While a number of functions have been postulated for Lcn2, its precise biological roles is not fully understood. For example, elevated Lcn2 expres-

sion in certain human neoplastic diseases (15-17), and its association with MMP-9 (18), suggests its role in tumor progression. Up- regulated Lcn2 expression in the colon epithelium is associated with a variety of inflammatory conditions (7), and its induction by IL-1 β in A549 cells demonstrates Lcn2 involvement in immunomodulation. (19). Lcn2 also represents an early and quantitative urinary biomarker for cisplatin nephrotoxicity and renal ischemia (13). Recently, It has been reported that ROS can induce Lcn2 (20), and one of the reasons for induction of Lcn2 in cisplatin nephrotoxicity could be due to generation of free radicals by cisplatin (21-22) and as a result cytoprotective activity of Lcn2 could be due scavenging of free radical which requires further studies. It has been reported that Lcn2 acts as a surviving factor against xenobiotics, MK886, OSU03012 and OSU03013, (23). More recently, the preventive effect of *Thea sinensis* melanin (TSM) against cisplatin-induced nephrotoxicity in ICR mice has been reported and it has been found that TSM pretreatment significantly decreased the level of Lcn2 expression which had increased to 15 folds (24). Thus induction of Lcn2 in response to cisplatin may be a compensatory response that involves cell defense pathways.

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