Midkine secretion protects Hep3B cells from cadmium induced cellular damage

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AIM: To evaluate role of midkine secretion during Cd exposure in the human hepatocyte cell line Hep3B cells.

METHODS: Different dosages of Cd (0.5-1-5-10 μg/mL) were applied to Hep3B cells and their effects to apoptosis, lactate dehydrogenase (LDH) leakage and midkine secretion were evaluated as time dependent manner. Same experiments were repeated with exogenously applied midkine (250-5000 pg/mL) and/or 5 μg/mL Cd.

RESULTS: Cd exposure induced prominent apoptosis and LDH leakage beginning from lower dosages at 48h. Cd induced midkine secretion with higher dosages (P < 0.001), (control, Cd 0.5-1-5-10 μg/mL respectively: 1123 ± 73, 1157 ± 63, 1242 ± 90, 1886 ± 175, 1712 ± 166 pg/mL). Exogenous 500-5000 pg/mL midkine application during 5 μg/mL Cd toxicity prevented caspase-3 activation (control, Cd toxicity, 500, 1000, 2500, 5000 pg/mL midkine+ Cd toxicity, respectively: 374 ± 64, 1786 ± 156, 1545 ± 179, 1203 ± 113, 974 ± 116, 646 ± 56, 556 ± 63 cfu) LDH leakage and cell death in Hep3B cells (P < 0.001).

CONCLUSION: Our results showed that midkine secretion from Hep3B cells during Cd exposure protects liver cells from Cd induced cellular damage. Midkine has anti-apoptotic and cytoprotective role during Cd toxicity. Further studies are needed to explain the mechanism of midkine secretion and cytoprotective role of midkine during Cd exposure. Midkine may be a promising therapeutic agent in different toxic hepatic diseases.

Key words: Cadmium; Midkine; Hepatocyte; Apoptosis; Caspase-3; Lactate dehydrogenase

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INTRODUCTION

Cadmium (Cd) exposure occurs widely in the general population, especially low-level chronic exposure through smoking and dietary sources, but it is known as one of the most toxic environmental and industrial pollutants. Cd accumulates in the body because of slow excretion[1]. Cd causes toxicity in different organs. Acute and chronic Cd exposure mostly results in hepatotoxicity[2]. It seems that the level of damage depends on the dosage and duration of Cd application. Exposure of cells to toxic chemicals is known to up-regulate the expression of a number of stress proteins and results in activation of apoptotic pathways and consequently cellular damage. In vivo and in vitro studies showed that inflammation and oxidative damage are main mechanisms of Cd induced toxicity[3-6]. Cd induces mitochondria-dependent apoptotic pathways where caspase-3 and caspase-9 are activated[7].

Midkine is a heparin binding growth factor. It takes part in cancer and inflammation[8]. Although midkine is a mitogenic factor during carcinogenesis, it plays a critical role in ischemia induced inflammatory damage[9]. It was demonstrated that midkine acts as an antiapoptotic factor in HepG2 cells; furthermore, midkine suppressed the activity of caspase-3, which plays a significant role in the apoptotic pathway. Pretreatment with midkine prevents tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis in the HepG2 cells[10]. TRAIL alone triggered massive apoptosis accompanied by caspase activation in tissue explants from patients with liver steatosis or hepatitis C viral
The presence of apoptosis was measured from supernatants. After supernatants were collected, the cell death was measured at the 24th, 48th, and 72nd hours. For evaluation of the effects of dose and time dependent expression of cadmium and midkine on cellular death or proliferation, the percentage of cell death was measured. Cell death was measured as the ratio of cell number of affected group vs control group × 100 at the determined hour.

Biochemical determination of cell death

Hep3B cells were plated in 96 multiwell cell culture plates as 3 × 10^4 cells/mL. LDH is normally present in the cytosol of hepatocytes. In response to cell damage, LDH is released from the cells. Therefore, to determine cell death, we measured secreted and intracellular LDH levels and calculated % released LDH at the 48th h for each group. To do this, the medium was collected to measure enzyme activities. The adherent cells were lysed. Both medium and cell lysates were used for quantitative determination of LDH activity (IU/L) which was performed with an automatic multianalyzer (Roche) using a kit (Roche). Released enzyme fractions for each sample were calculated as the ratio of enzyme present in the medium vs the sum of the levels of same enzyme in the supernatant and in the cells.

Measurement of apoptosis

Caspase-3 levels: The presence of apoptosis was determined by caspase-3 levels. Equal numbers of cells were used for caspase-3 level measurements. Cells were lysed with assay buffer (50 mmol/L HEPES, pH 7.4, 100 mmol/L NaCl, 0.1% CHAPS, 10 mmol/L DTT, 2 mmol/L EDTA, 2 mmol/L EGTA, Triton X-100, 0.1%). Caspase-3 levels were measured by DEVD-R110 Fluorometric HTS Assay Kit from cell lysates. The fluorogenic substrate (Ac-DEVD)-R110 was used for this assay. It is completely hydrolyzed by the enzyme in two successive steps. Cleavage of the first DEVD peptide results in the monopeptide Ac-DEVD-R110 intermediate, which has absorption and emission wavelengths similar to those of R110 (λ_{abs} = 496 nm, λ_{em} = 520 nm), but has only about 10% the fluorescence of the latter. Hydrolysis of the second DEVD peptide releases the dye R110, leading to a substantial fluorescence increase.

Equal volumes of sample and caspase-3 detection buffer were added to assay plate, and then incubated at 37°C for 1 h in an incubator. Results were read with a fluorometer at 470 nm excitation filter and 520 nm emission filter. R110 was used for generating a standard curve to calculate amount of substrate conversion.

Statistical analysis

Results of the experiments were analyzed by One Way ANOVA, followed by a multiple comparison test using SPSS 10.0. P < 0.05 was accepted as statistically significant. Results were given as mean ± SEM.

RESULTS

Cell proliferation and toxicity

We characterized the concentration-dependent cytotoxic effect of Cd on human hepatocyte cell line as a function of time. Cadmium exposure decreased living cell number as well as associated cytokines, are responsible for clinical expression and tissue damage observed with cadmium-induced hepatotoxicity. It was shown that midkine expression was upregulated in a marine gastropod limpet patella caerulea after they were exposed to sublethal doses of Cd. But, whether midkine takes part in Cd induced mechanisms in human cells is still unknown. In this study we aimed to evaluate effects of Cd induced midkine secretion in the human hepatocyte cell line Hep3B cells, and its effects to cellular proliferation, apoptosis and biochemical parameter of cellular integrity during Cd exposure.
cell exposure to 1 μg/mL Cd for up to 2 h only slightly affected cell viability as revealed by MTT measurements compared to control values estimated in untreated cells, but it becomes apparent at the 24th and 48th h.

Cytotoxicity was more prominent with higher dosages at the 24th and 48th h (P < 0.001, Figure 1). Regarding to these data, 5 μg/mL CdCl₂ concentration which with moderate-high toxic impact was chosen for subsequent experiments with different dosages of midkine.

Midkine treatment caused proliferation of Hep3B cells in a dose and time dependent manner compared to control group. The highest increase in cell number was at the 48th h and 5000 pg/mL midkine concentration (P < 0.001, Figure 2). Midkine treatment during Cd toxicity prevents cell death, even with the lowest dosages (P < 0.001, Figure 2).

**Determination of apoptosis**

Increased apoptosis was seen in Cd treated cells, which was confirmed with increased caspase-3 levels. Lowest dosage of Cd application did not increase caspase-3 levels compared to untreated cells. Activation of caspase-3 started at the 1 μg/mL Cd dosage (P < 0.001, Figure 3).

Midkine treatment decreased caspase-3 levels in the Hep3B cells. It prevents Cd induced apoptosis prominently starting from 500 pg/mL concentration of midkine application (P < 0.001, Figure 4).

**Cytotoxic effects of Cd in the hepatocytes**

Incubation of Hep3B cells with Cd resulted in cytotoxicity as assessed by LDH released into the incubation media. LDH release in the Hep3B cells to media started at the 1 μg/mL Cd dosage (Figure 5).

Midkine treatment at the same time with 5 μg/mL Cd exposure decreased LDH release in the Hep3B cells (Figure 6).

**Midkine secretion**

Measurable basal midkine secretion was found in the Hep3B cells under normal conditions. Cd treatment induced midkine secretion in the Hep3B cells in a dose dependent manner (Figure 7).

**DISCUSSION**

Acute/chronic Cd exposure mostly results in hepatotoxicity, where it is a good model to study toxic substance-induced liver damage. Midkine family has strong anti-apoptotic function so they are obviously considered non specific (i.e., for Cd) mechanisms of defence. Intense midkine expression has also been found in increased various human tumors and level of midkine expression correlates...
studies are needed to evaluate LDH leakage (%) and released in to medium as a defense mechanism. It seems that midkine is produced endogenously in a dose and time dependent manner. It has cytoprotective, decreased apoptosis and increased cellular proliferation in a basal midkine secretion. In our study, midkine treatment still unclear. Midkine is multifunctional heparin-binding growth factor and cytokine and has anti-apoptotic and Cd and/or repair Cd induced lesions. Our studies showed that midkine is beneficial for liver regeneration[29]. ERK, JNK signal pathways disturbed during Cd toxicity are activated by midkine[18,19,22,30]. Beneficial effects of exogenous midkine to minimize Cd induced damage would provide a new perspective for innovation in the treatment of Cd intoxications and in NonAlcoholic Fatty Liver Disease (disease near exclusively characterized by apoptotic process), in Drug Induced Liver injury and in the combined form, illnesses far long more evident than Cd intoxication in the every day practice of gastroenterologists and hospitals. But further acute and chronic *in vitro* and *in vivo* studies are needed to evaluate which intracellular pathway(s) is activated during these processes.

### COMMENTS

**Background**

Acute/chronic Cadmium (Cd) exposure mostly results in hepatotoxicity, where it is a good model to study toxic substance-induced liver damage. Midkine is expressed around the damaged neuronal site after cerebral infarction[17], suggesting a role for midkine in tissue repair. The results of the present study supports the idea that Cd exposure causes cytotoxicity and apoptosis in the Hep3B cells. In both time and dose-response studies, LDH leakage, which is very important parameter to detect hepatocellular integrity, was greater in Cd treated cells. These effects were more prominent at the 48th h. During Cd exposure, activation of caspase-3 was detected in Hep3B cells, suggesting a caspase-dependent pathway is involved in Cd toxicity. Cd can upregulate the expression of a number of genes that produce products that can detoxify Cd and/or repair Cd induced lesions. Our studies showed that midkine is one of them. The induction pathways or receptors of midkine expressed by Cd exposure is still unclear. Midkine is multifunctional heparin-binding growth factor and cytokine and has anti-apoptotic and cell-protecting activities[8]. Untreated Hep3B cells have also a basal midkine secretion. In our study, midkine treatment decreased apoptosis and increased cellular proliferation in a dose and time dependent manner. It has cytoprotective, anti-apoptotic effects against Cd toxicity in Hep3B cells. It seems that midkine is produced endogenously and released in to medium as a defense mechanism of Hep3B cells against Cd toxicity. Among midkine receptors, receptor-type protein tyrosine phosphatase z (PTP z) has been studied extensively. Midkine stimulates phosphorylation of specific members of the JAK/STAT pathway, namely JAK1, JAK2, and STAT1α[18,19]. In addition, low density lipoprotein receptor-related protein (LRP) has also been identified as a receptor[20]. The midkine receptor is considered to be a molecular complex containing these proteins. The downstream signaling systems of these receptors include ERK, which participates in the reduction of necrotic and apoptotic cell death[21]. Internalization of midkine in to cell and nuclear targeting is important for its antiapoptotic function[22]. Activation of these receptors and intracellular pathways might take part in cytoprotective effects of midkine during Cd toxicity. Human and experimental studies have shown that apoptosis plays a role in hepatocyte death in alcoholic liver disease and nonalcoholic steatohepatitis and apoptosis levels correlate with the severity of the liver disease[23-26]. LDL receptor-related protein (LRP) is another midkine receptor. LRP is important for lipid and lipoprotein uptake to cells[27]. Lipid profiles of steatohepatitis patients were found disturbed[28]. It was shown that midkine takes part in the inflammatory and repair processes after partial hepatectomy. They suggested that midkine is beneficial for liver regeneration[29]. It was shown that midkine is one of them. The induction pathways or receptors of midkine expressed by Cd exposure is still unclear. Midkine is multifunctional heparin-binding growth factor and cytokine and has anti-apoptotic and cell-protecting activities[8]. Untreated Hep3B cells have also a basal midkine secretion. In our study, midkine treatment decreased apoptosis and increased cellular proliferation in a dose and time dependent manner. It has cytoprotective, anti-apoptotic effects against Cd toxicity in Hep3B cells. It seems that midkine is produced endogenously and released in to medium as a defense mechanism

\[\text{LDH leakage (%) vs control group.}\]

\[\text{Figure 5} \quad \text{Cd induced cytotoxicity at the 48th h of experiment determined by % LDH released to medium. Starting from the 1 μg/mL dosage Cd treatment caused prominent LDH release from hepatocytes at the end of 48h (P < 0.001). Data are presented as mean ± SEM. } ^b P<0.001 \text{ vs control group.}\]

\[\text{Figure 6} \quad \text{Effects of 48 h midkine (250-5000 pg/mL) and/or 5 μg/mL Cd treatment on the LDH leakage in the Hep3B cells. Data are presented as mean ± SEM. } ^b P<0.001 \text{ vs control group.}\]

\[\text{Figure 7} \quad \text{Effects of 0.5-10 μg/mL Cd treatment on midkine secretion in the Hep3B cells. With 0.5 and 1 μg/mL Cd exposure we obtained similar midkine secretion as untreated cells. Midkine secretion was highest as a response to 5 μg/mL Cd treatment dosage. Data are presented as mean ± SEM. } ^b P<0.001 \text{ vs control group.}\]
of midkine expression correlates negatively with the patients' prognosis. Midkine is cytoprotective and has anti-apoptotic effect.

**Research frontiers**

Beneficial effects of exogenous midkine to minimize Cd induced damage would provide a new perspective for innovation in the treatment of Cd intoxications and other liver diseases.

**Innovations and breakthroughs**

Cd is well known environmental toxic substance which mainly damages liver. In this study we showed that Cd treatment induces midkine secretion from hepatocytes. Midkine might have protective role during Cd toxicity.

**Applications**

These finding may be used in the different liver diseases such as alcoholic, toxic and non-alcoholic liver disease models.

**Peer review**

In this experimental in vitro study, the authors showed that midkine secretion from HepG2 cells during Cd exposure protects liver cells from Cd induced cellular damage. Midkine has anti-apoptotic and cytoprotective role during Cd toxicity. Midkine may be a promising therapeutic agent in different toxic hepatic diseases.

**REFERENCES**

1. Nishijo M, Nakagawa H, Morikawa Y, Tabata M, Senma M, Miura K, Takahara H, Kawanami H, Nishi M, Mizukoshi K. Mortality of inhabitants in an area polluted by cadmium: 15 year follow up. *Occup Environ Med* 1995; 52: 181-184
2. Gubrelay U, Mehta A, Singh M, Flora SJ. Comparative hepatic and renal toxicity of cadmium in male and female rats. *J Environ Biol* 2004; 25: 65-73
3. Coutant A, Lebeau J, Bidon-Wagner N, Levalois C, Lecart D, Chevillard S. Cadmium-induced apoptosis in lymphoblastoid cell line: involvement of caspase-dependent and -independent pathways. *Biochimie* 2006; 88: 1815-1822
4. Trinche F, Rigo M, Filosa S, Volpe MG, Parisi E, Scudiero R. Cadmium distribution and metallothionein expression in lizard tissues following acute and chronic cadmium intoxication. *Comp Biochem Physiol C Toxicol Pharmacol* 2006; 144: 272-278
5. Amara S, Abdelmelek M, Garrel C, Guiraud P, Douki T, Sanjyo N, Kobayashi T, Kamata T, Mizusawa H, Kotzbauer PT, Milbrandt J, Lowenstein CJ, Vadrot N, Aoudjehane L, Conti F, Bringuier AF, Zou K, Sakaguchi N, Ikematsu S, Sakuma S, Gimenez MS. Induction of redox changes, apoptosis in vitro and non-alcoholic liver disease models.

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1. Nishijo M, Nakagawa H, Morikawa Y, Tabata M, Senma M, Miura K, Takahara H, Kawanami H, Nishi M, Mizukoshi K. Mortality of inhabitants in an area polluted by cadmium: 15 year follow up. *Occup Environ Med* 1995; 52: 181-184
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