INCREASED EXPRESSION OF HEAT SHOCK PROTEIN (HSP)72 IN A HUMAN PROXIMAL TUBULAR CELL LINE (HK-2) WITH GENTAMICIN-INDUCED INJURY

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ABSTRACT — Gentamicin (GM) has been widely used as an antibiotic and its nephrotoxicity has been recognized. However, the alternation of heat shock protein (HSP) 72 as an inductive protein in proximal tubular cells treated with GM is still unclear. In this study, GM cytotoxicity and its effect on the expression of HSP72 in human kidney proximal tubular (HK-2) cells were measured. HK-2 cells were incubated for 24 hr, 48 hr, 72 hr, and 96 hr with GM only and GM plus MnCl₂, respectively. Cytotoxicity was determined by the release of lactate dehydrogenase (LDH). Activity of N-acetyl-β-D-glucosaminidase (NAG) and effects of GM on oxidation in HK-2 cells were investigated by measurements of malondialdehyde (MDA) content and superoxide dismutase (SOD) activity, and the ability of viable cells to reduce a tetrazolium-based compound (MTT). The expression of HSP72 was measured by immunocytochemistry, Western blotting and RT-PCR. Cells were exposed to GM at a concentration of 100 µg/ml. After 24 hr MTT uptake decreased significantly and then gradually until 96 hr. LDH release increased time-dependently from 24 hr to 72 hr, but decreased at 96 hr compared with the data at 72 hr when cells were treated with GM only. Both results of NAG and SOD activities and results of MDA content were similar to that of the LDH release. The amount of HSP72 positive cells increased at 24 hr after exposure to GM up to 72 hr. HSP72 expression increased significantly from 24 hr, and reached its peak at 72 hr when cells were treated with GM only. Furthermore, the change of the HSP72 gene transcription was similar to the expression of HSP72.

These results demonstrated that GM treatment could induce damage to HK-2 cells and that the expression of HSP72 increased when cells were injured by GM.

KEY WORDS: Gentamicin, Cytotoxicity, Nephrotoxicity, Heat shock protein

INTRODUCTION

Due to its merits, gentamicin (GM), an aminoglycoside antibiotic, is widely used in clinical practice for the treatment of life-threatening gram-negative infections (Eisenberg et al., 1987; Bennet et al., 1982). However, acute renal failure (ARF) is a major complication of GM treatment that largely limits its use, complicating and increasing the price of treatment (Ali et al., 2002). It has been shown that oxidative stress is involved in GM-induced renal damage (Baliga et al., 1999). GM is also shown to enhance the generation of superoxide anion and hydrogen peroxide by renal cortical mitochondria (Walker et al., 1999). It is assumed that GM inhibits the activity of lysosomal phospholipase in proximal tubular epithelial cells (Karl et al., 1982). Moreover, GM simultaneously induces the proliferation and apoptosis of mesangial cells in vitro and glomerular mesangial cells in vivo (Martinez-Salgado et al., 2004).

Manganese (Mn²⁺) is an important cofactor of the mitochondrial superoxide dismutase, an antioxidant enzyme which scavenges oxygen-free radicals. Furthermore, it is known that Mn²⁺ inhibits microsomal lipid peroxidation (LPO) and demonstrates antioxidant properties in an in vitro system generating oxygen-free

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radicals (Casalino et al., 2002). This suggests that the effect of Mn$^{2+}$ on the oxidant system depends on the quantity of administration (Zheng et al., 1998). Mn$^{2+}$ at a low dose may prevent GM treatment from inducing nephrotoxicity.

Heat shock proteins (HSPs), also called molecular chaperones, are highly conserved proteins expressed, and they fulfill their functions under normal cellular conditions. HSP could be rapidly induced in cells in response to abrupt and adverse changes in their environment (Lindquist et al., 1980; Itoh et al., 1991; Freeman et al., 1996). Mammalian HSPs are classified into four families according to their approximate molecular masses and degrees of homology (Lindquist et al., 1980; Itoh et al., 1991).

The cytosolic 70-kDa molecular chaperones (HSP70s) are present in cells as two different gene products, but are closely related to each other: a stress-inducible form, HSP72 (known as HSP70), and a constitutively expressed form, HSP73 (known as 70-kDa heat shock cognate protein, HSC70) (Lindquist et al., 1980). The induction of heat shock proteins (HSPs) is thought to play a protective role in ischemic acute renal failure (ARF). In a previous study, HSP73 molecular chaperones were rapidly induced in rat kidneys with GM-induced acute tubular injury (Komatsuda et al., 1993). GM preferentially binded to the peptide binding domain of HSP73, and when protected by HSP73 in the lysosomes in proximal tubules may cause nephrotoxicity by GM (Toshio et al., 2004). Some findings suggest that HSP72 could attenuate cisplatin (CDDP)-induced nephrotoxicity. The protective effects of HSP72 are associated with an increased Bcl-2/Bax ratio and decreased apoptosis (Hua et al., 2003).

Whether or not HSP72, the other member of the HSP70s family, is one of the reactive elements caused by GM treatment in kidney proximal tubular cells is still unknown. In the present study, we exposed HK-2 cells, a human proximal tubular cell line, to GM to duplicate a kidney injury model in vitro and to detect the expression of HSP72. We discussed the relationship between the expression of HSP72 and GM-induced kidney injury.

**Materials and Methods**

GM and MTT were supplied by Sigma Co. (St. Louis, MO). HSP72 mouse monoclonal antibody was provided by NeoMarkers. All other reagents were obtained from commercial suppliers and used without further purification.

**Cell culture and treatments**

All experiments were performed using HK-2 cells, an immortalized proximal tubular cell line derived from a normal adult human kidney. Cells were cultured in DMEM medium with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL Grand Island, NY). The cultured cells were stored in a humidified incubator (95% air with 5% CO2) at 37°C. These were then separated into three groups which were incubated with medium only, a medium containing GM at a concentration of 100 µg/ml, and a medium containing GM (100 µg/ml) + MnCl₂ (2 µg/ml), respectively. MTT assay, LDH release, NAG and SOD activities were detected at various time points (24 hr, 48 hr, 72 hr, and 96 hr).

**MTT assay**

This assay for cell viability is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase in viable cells to produce a purple formazan product. Each experiment was performed according to the reported method (Ryan et al., 1994).

**Measurement of lactate dehydrogenase (LDH) release**

HK-2 cells were planted into 24-well plates (1×10⁶ cell/ml) and allowed to grow for 1 day. Cell viability after incubation was estimated by determining the release of LDH from the cells. LDH release was measured by determining LDH activity (measured spectrophotometrically by NADH oxidation at 440 nm) in media.

**N-acetyl-β-D-glucosaminidase (NAG) activity assay**

NAG activity was determined using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as a substrate from which NAG liberates 4-methylumbelliferone at pH 4.5. One unit of NAG activity was defined as the amount of NAG catalyzing the liberation of 1 µmol of 4-methylumbelliferone per minute at 37°C. Protein was measured (at 400 nm) by a modified method of Bradford using Bio-Rad Protein Assay (Bio-Rad, Richmond, CA).

**Malondialdehyde (MDA) content assay**

Malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) assay, as described by Draper and Hadley, 1990.
Superoxide dismutase (SOD) activity assay
Superoxide dismutase (SOD) activity in cell plasma was determined by spectrophotometrically monitoring (at 550 nm) the rate of inhibition of formazan production consequent to the reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride (INT) by superoxide anions generated by a xanthine-xanthine oxidase system. Human erythrocyte SOD was used as a standard. One SOD unit was defined as the amount of enzyme that inhibited formazan production by 50% at 37°C.

Immunocytochemistry
Cells grown on sections were fixed and immunocytochemistry procedure followed description (Nadia, et al., 2002). Finally, the slides were developed in 0.05% freshly prepared diaminobenzidine solution (DAB) for 2 min, counterstained with hematoxylin, and then dehydrated, air-dried, and mounted. PBS was used to substitute the primary antibody as a negative control. Photomicrographs were prepared with a Spot 2 digital camera and software (Diagnostic Instruments, Inc., Sterling Heights, MI).

Western blotting analysis
Western blot analysis of the HSP72 level was carried out according to a previously published procedure (Li, et al., 1995). Briefly, after total protein in HK-2 cells was extracted, aliquots (10 µg) of proteins were separated by SDS−PAGE on 6% acrylamide Laemmli minigels and transferred onto nitrocellulose membranes overnight. Membranes were blocked for 60 min with 5% nonfat milk in PBS containing 0.5% Tween 20 and incubated for 2 hr at room temperature with HSP72 mouse monoclonal antibody (200-fold dilution) at 4°C overnight. After incubation with peroxidase-conjugated secondary antibody for 60 min at room temperature, blots were developed using the ECL detection system.

Statistical analysis
The values were presented as means±S.E.M. The differences between group means were analyzed using Dunnet’s test. Results were considered statistically significant when p<0.05.

RESULTS

Cytotoxicity of GM in HK-2 cells
Cytotoxicity of GM in HK-2 cells was assessed by MTT reduction, LDH release, NAG and SOD activities. After exposure to GM at 100 µg/ml for 24 hr, 48 hr, 72 hr, and 96 hr, MTT reduction was observed (Fig. 1). After 24 hr, MTT uptake decreased significantly and then gradually until 96 hr. On the other hand, cells treated with GM+MnCl2 did not show reduction at 24 hr. From 48 hr to 96 hr, MTT reduction was highly evident at this group, but less significant than with GM-treated cells.

LDH release was used as a second marker of cytotoxicity. The release of LDH increased time-dependently from 24 hr to 72 hr (Fig. 2). However, the release of LDH decreased at 96 hr compared with the data at 72 hr. At the same time, cells treated with GM+MnCl2 did not show reduction at 24 hr. From 48 hr to 96 hr, MTT reduction was highly evident at this group, but less significant than with GM-treated cells.

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NAG activity assay was used as a third marker of cytotoxicity. This condition is similar to that of the LDH release detection. NAG activity increased time-dependently from 24 hr to 72 hr (Fig. 3). However, NAG activity decreased at 96 hr compared with the
Fig. 1. Cytotoxicity of gentamicin in HK-2 cells. HK-2 cells were exposed to gentamicin in 100 µg/ml for 24 hr, 48 hr, 72 hr, 96 hr and gentamicin+MnCl₂ for 24 hr, 48 hr, 72 hr, 96 hr, MTT reduction was determined. Values are mean ± SD of measurements from nine separate HK-2 cell cultures. Number of experiments (n) = 12. *p < 0.01 vs control, #p < 0.01 vs gentamicin+MnCl₂ group.

Fig. 2. Cytotoxicity of gentamicin in HK-2 cells. HK-2 cells were exposed to gentamicin in 100 µg/ml for 24 hr, 48 hr, 72 hr, 96 hr and gentamicin+MnCl₂ for 24 hr, 48 hr, 72 hr, 96 hr, LDH release was determined. Values are mean ± SD of measurements from nine separate HK-2 cell cultures. Number of experiments (n) = 6. *p < 0.01 vs control, #p < 0.01 vs gentamicin+MnCl₂ group.
Gentamicin treatment could induce damage to HK-2 cells, and HSP72 expression increased when cells were injured by Gentamicin.

data at 72 hr. On the other hand, cells treated with GM+MnCl2 did not show significant increase at 24 hr. Although the increase in NAG activity was observed from 24 hr to 96 hr, it was lower than that of GM-treated cells from 24 hr to 72 hr. However, NAG activity is higher when exposed to GM+MnCl2 at 96 hr than when exposed to GM only at 96 hr.

Effects of GM on oxidation in HK-2 cells
Evaluation of MDA levels was performed to estimate the degree of lipid peroxidation in cell culture. Fig. 4 shows that GM caused concentration-dependent and time-dependent increases in MDA levels. Meanwhile, Mn2+ slightly inhibited the increase in MDA content in the GM+MnCl2 group. SOD activity was used to detect cellular scavenging superoxide anion capability. Total SOD activity diminished in the GM group compared to the control group from 24 hr to 96 hr exposure to GM (Fig. 5). Mn2+ partially prevented the decrease in total SOD activity in the GM+MnCl2 group. This was similar to that of the MTT reduction assay.

Heat shock protein (HSP) 72 expression when exposed to GM
The rate of HSP72 positive cells increased at 24 hr up to 72 hr when treated by GM at a concentration of 100 µg/ml (Fig. 6). It was higher than the control group from 48 hr after cells were exposed to GM+MnCl2, but lower than the cells exposed only to GM. Quantitative densitometric measurements of HSP72 bands showed that the expression increased markedly from 24 hr after exposure to GM (Fig. 7). It increased continually and reached its peak at 72 hr with treatment by GM. After that, this abundance was reduced at 96 hr, but still higher than that of the control group. Proteins extracted from cells exposed to GM at a concentration of 100 µg/ml and Mn2+ showed that the expression of HSP72 increased gradually from 48 hr to 96 hr. Furthermore, the expression of HSP72 at each time point was less than the expression for cells exposed only to GM. From the results of RT-PCR, the change of HSP72 gene transcription is similar to the expression of HSP72 (Fig. 8). An increased production of HSP72 is associated to a parallel rise of HSP72 protein content.

DISCUSSION

The clinical use of GM is limited due to its nephrotoxicity. GM-induced nephrotoxicity is characterized by direct tubular necrosis, which is localized mainly to
Fig. 4. Effects of GM on oxidation in HK-2 cells. HK-2 cells were exposed to gentamicin in 100 µg/ml for 24 hr, 48 hr, 72 hr, 96 hr and gentamicin+MnCl₂ for 24 hr, 48 hr, 72 hr, 96 hr, MDA content was determined. Values are mean ± SD of measurements from nine separate HK-2 cell cultures. Number of experiments (n) = 6. *p < 0.01 vs control, #p < 0.01 vs gentamicin+MnCl₂ group.

Fig. 5. Effects of GM on oxidation in HK-2 cells. HK-2 cells were exposed to gentamicin in 100 µg/ml for 24 hr, 48 hr, 72 hr, 96 hr and gentamicin+MnCl₂ for 24 hr, 48 hr, 72 hr, 96 hr, SOD activity was determined. Values are mean ± SD of measurements from nine separate HK-2 cell cultures. Number of experiments (n) = 6. *p < 0.01 vs control, #p < 0.01 vs gentamicin+MnCl₂ group.
Gentamicin treatment could induce damage to HK-2 cells, and HSP72 expression increased when cells were injured by Gentamicin.

![Fig. 6. Expression of HSP72 in HK-2 cells. Immunocytochemistry analysis of HSP72 expression in HK-2 cells exposed to gentamicin in 100 µg/ml for 24 hr, 48 hr, 72 hr, 96 hr and gentamicin+MnCl2 for 24 hr, 48 hr, 72 hr, 96 hr. Values are mean ± SD of measurements from nine separate HK-2 cell cultures. Number of experiments (n) = 6. *p < 0.01 vs control, #p < 0.01 vs gentamicin+MnCl2 group.]

![Fig. 7. Expression of HSP72 in HK-2 cells. Western-blot analysis of HSP72 and GAPDH expression in HK-2 cells exposed to gentamicin+MnCl2 for 24 hr (1), 48 hr (2), 72 hr (3), 96 hr (4) and gentamicin in 100 µg/ml for 24 hr (5), 48 hr (6), 72 hr (7), 96 hr (8). (B) Quantification of HSP72 expression by densitometer. Values are mean ± SD of measurements from nine separate HK-2 cell cultures. Number of experiments (n) = 6. *p < 0.01 vs control, #p < 0.01 vs gentamicin+MnCl2 group at the same time point.]
the proximal tubule. It is known that GM inhibits the activity of lysosomal phospholipases in proximal tubular epithelial cells. Both in vitro and in vivo studies showed that GM has enhanced the generation of reactive oxygen metabolites (ROS) (Baliga et al., 1999). GM treatment induces simultaneous mesangial proliferation and apoptosis in rats.

In our present study, GM inhibited the proliferation of human kidney tubular cells and furthermore increased MDA content and SOD activity in vitro. This indicates that GM is effective in inducing the oxidative process and scavenging O$_2^-$, LDH and NAG in the cell culture medium are indicative of cell membrane damage. LDH release and NAG activity increased after HK-2 cells were treated by GM. After the peak at 72 hr, LDH release and NAG activity decreased gradually at 100 µg/ml concentration due to the low cell viability at this concentration after exposure for 72 hr. These results suggest that the cytotoxicity concentration of GM in HK-2 cells was 100 µg/ml and was time-dependent.

70-kDa molecular chaperones (HSP70s) are a family of highly conserved proteins which contribute to cell survival after various pathological and physiological stresses. A number of studies have demonstrated that HSP70s are inducible in tubular epithelial cells following renal ischemia/reperfusion (Emami et al., 1991; Morita et al., 1995) and toxic injury (Komatsuda et al., 1993; Satoh et al., 1994). They play an essential role in protein metabolism under both

![Fig. 8](image)

**Fig. 8.** Expression of HSP72mRNA in HK-2 cells. RT-PCR analysis of HSP72 and GAPDH expression in HK-2 cells exposed to gentamicin+MnCl$_2$ for 24 hr (1), 48 hr (2), 72 hr (3), 96 hr (4) and gentamicin in 100 µg/ml for 24 hr (5), 48 hr (6), 72 hr (7), 96 hr (8). (B) Quantification of HSP72 expression by densitometer. Values are mean ± SD of measurements from nine separate HK-2 cell cultures. Number of experiments (n) = 6. *p < 0.01 vs control. #p < 0.01 vs gentamicin+MnCl$_2$ group at the same time point.
Gentamicin treatment could induce damage to HK-2 cells, and HSP72 expression increased when cells were injured by Gentamicin.

stress and normal conditions. Recent studies reported that HSP70s may play a protective role in ischemic acute renal failure (ARF).

HSP72 in one of the members of the HSP family. Classified as a readily inducible protein, HSP72 can protect a variety of cells, including renal tubule cells, from thermal, toxic and ischemic injuries in vitro (Turman et al., 1999). A number of studies have shown the rapid induction of HSP72 in renal tubules in rat kidneys after transient ischemia (Van Why et al., 1992). Transfection of LLC-PK1 cells with HSP72 offers protection against cisplatin (CDDP)-induced kidney tubular damage (Komatsuda et al., 1999) and increased levels of intracellular HSP72 correlate with resistance to CDDP in tumor cell lines (Abe et al., 1999).

In our present study, HSP72 expression increased parallel with the degree of HK-2 cell injury for 72 hr. However, the expression decreased at 96 h due to survival of only a few cells in such a serious injury. On the other hand, to prevent injury by GM, cells were treated with Mn\(^{2+}\), which showed a gradual increase in the expression of HSP72 from 48 hr to 96 hr. Furthermore, the expression of HSP72 at each time point was less than that of the cells exposed only to GM. HSP72 expression was induced markedly after HK-2 cells were seriously damaged by GM. In addition, pretreatment with MnCl\(_2\) attenuated the GM-induced HK-2 cell damage. The expression of HSP72 was less than in HK-2 cells treated by GM only. This finding suggests that HSP72, as a group of protective proteins, is involved, at least in part, in the GM-induced HK-2 cell injury.

The induction of a heat shock response prevents subsequent H\(_2\)O\(_2\)-induced cell death in the human premonocytic line U937 (Donati et al., 1990). It has been shown that mitochondria represent the primary intracellular target for heat shock-induced protection against oxidative injury in U937 cells (Polla et al., 1996). Since protection correlated best with the expression of HSP72, they suggested its specific role in mitochondrial protection against oxidative injury. GM-inhibited SOD activity was shown in our study. It seems that it effectively harmed the cellular oxidant defense system, eventually leading to mitochondrial damage. The accumulation of HSP72 which was induced by GM might protect cells from oxidative and other injuries.

In conclusion, our studies show that GM treatment could induce human kidney tubular cell line (HK-2) damage. The expression of heat shock protein 72 increased when cells were injured by GM. This increased expression may protect cells from the damage induced by GM. In clinical applications, some measures which are used to induce the expression of HSP72 can be taken to attenuate injury by GM in the course of treatment. Finally, further experimentation should be done before clinical application.

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