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Report

Effect of GATA Transcription Factors on Cadmium Toxicity in Human Proximal Tubular Cells

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Cadmium (Cd) is an environmental toxic heavy metal that causes renal dysfunction. Cd triggers renal dysfunction through proximal tubular cell toxicity. Our previous study demonstrated that Cd changed the activities of various transcription factors in human proximal tubular HK-2 cells. Interestingly, several GATA family members were included in the transcription factors whose activities were decreased by Cd treatment. The GATA family has diverse roles in cell proliferation, tissue development, disease regulation, and carcinogenesis. In this study, we examined whether knockdown of GATA family members affected the viability of HK-2 cells. The single knockdown of *GATA1*, *GATA3* or *GATA6* using siRNA significantly decreased HK-2 cell viability. In particular, *GATA6* knockdown led to the greatest effect on HK-2 cells viability. Cd increased mRNA levels of *GATA3* and *GATA6* but did not affect that of *GATA1*. The GATA family may regulate the expression of downstream factors involved in common pathway. Therefore, the effect of combined knockdown of *GATA1/3/6* on the viability of HK-2 cells was examined. Our results indicated that the effect on HK-2 cell viability following knockdown of multiple GATA family members was comparable to the single knockdown of *GATA6*. These results suggest, therefore, that multiple GATA family members may be involved in modulating Cd renal toxicity through a common pathway.

Key words cadmium, GATA family, HK-2 cells, multiple knockdown

INTRODUCTION

Cadmium (Cd) is a toxic heavy metal that bioaccumulates in the human body. In humans the biological half-life of Cd is long (10–30 years), and Cd accumulates at high concentrations in the kidney.[1-3] Although Cd bound to the metal binding protein, metallothionein, does not show a toxic effect, a high concentration of the free form of Cd induces cytotoxicity.[4] Cd filtered by glomeruli is reabsorbed through proximal tubules, and as a consequence the proximal tubular cell is the target of Cd toxicity.[2] Our previous studies demonstrated that Cd alters levels of transcription factors in human proximal tubular HK-2 cells.[5,6] Furthermore, gene expression changes induced by Cd led to the alteration of transcription factor activity and affected cell viability.[5,6] Among the transcription factors whose activities were decreased by Cd treatment, there were four GATA family members.[6] The GATA family is composed of six members that regulate diverse processes in the development and differentiation of various tissues.[7,8] Based on their expression profiles, GATA1/2/3 are classified as hematopoietic transcription factors, and GATA4/5/6 are involved in the differentiation of endodermal tissues.[7,8] Recently, it has been reported that mutations in *GATA* genes are related to several diseases such as hypoparathyroidism, sensorineural deafness and renal insufficiency (HDR) syndrome, congenital heart disease (CHD) and chronic or acute myeloid leukemia.[8] In some cases, several GATA family members are involved in one common disease; for example, GATA1/2/3 are involved in leukemia and GATA 4/5/6 are involved in CHD.[8] In this study, we determined the effect of suppression of individual GATA family members and multiple knockdowns of several GATA family members on HK-2 cell viability.

MATERIALS AND METHODS

Cell Culture and Treatment HK-2 cells purchased from ATCC (Manassas, MA, USA) were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 25 µg/mL streptomycin (DS Pharm, Osaka, Japan), 25 U/mL penicillin (DS Pharm), 10 ng/mL EGF (epidermal growth factor; Sigma-Aldrich), 1% Insulin-Transferrin-Selenium-X (Gibco), and 5 ng/mL hydrocortisone at 37°C in a humidified incubator containing 5% CO_2.

siRNA Transfection The following Silencer Select Predesigned siRNAs were purchased from Ambion (Grand Island, NY, USA): s5594 and s5595 for human *GATA1*; s5599 and s5600 for human *GATA3*; and s5606 and s5607 for human *GATA6*. Control siRNA (Silencer Select Negative Control #1 siRNA) was also purchased from Ambion. siRNA transfection was performed using Lipofectamine RNAiMAX (Invitrogen,

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Grand Island, NY, USA). After the siRNA mixture was incubated for 15 min with Lipofectamine RNAiMAX and Opti-MEM I Reduced Serum Medium (Opti-MEM; Gibco), HK-2 cells were transfected with the siRNA mixture (1 nM siRNA/sequence, 0.2% Lipofectamine RNAiMAX, 10% Opti-MEM) for 24 h or 48 h.

RNA Extraction Cells were washed twice with ice-cold PBS(-) [phosphate-buffered saline(-)] and total RNA was extracted with the PureLink™ RNA Mini Kit (Ambion) following the manufacturer's instructions. RNA was quantified and purity was determined using the BioSpec-nano (Shimadzu, Kyoto, Japan).

Real Time RT-PCR Total RNA was incubated with the PrimeScript reverse transcription (RT) Reagent Kit (Perfect Real Time) (Takara Bio, Shiga, Japan) to generate cDNA. Real-time PCR was performed with SYBR Premix Ex TaqII (Perfect Real Time) (Takara Bio) and the Thermal Cycler Dice Real Time System (Takara Bio). PCR conditions were as follows: 10 s hot-start at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Gene expression was normalized to *GAPDH* mRNA levels. The oligonucleotide sequences of the primers were as follows: sense, 5′-CCAGCTTCCTGGAGACTTTG-3′, and antisense, 5′-CTATTGGGGACAGGGAGTGA-3′, for the human *GATA1* gene; sense, 5′-AAGGCAGGGAGTGTGTGAAC-3′, and antisense, 5′-CGGTTCTGTCCGTTCATTTT-3′, for the human *GATA3* gene; sense, 5′-GTGCCCAGACCACTTGCTAT-3′, and antisense, 5′-TGGAGTCATGGGAATGGAAT-3′, for the human *GATA6* gene; sense, 5′-GCACCGTCAAGGCTGAGAAC-3′, and antisense, 5′-TGGTGAAGACGCCAGTGGA-3′, for the human *GAPDH* gene.

Cell Viability HK-2 cells treated with siRNA mixture were grown in 96-well plates and cultured for 24 h and 48 h. After siRNA transfection, the medium was replaced with fresh 10% FBS-DMEM/F-12 containing MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; Dojindo Laboratories, Kumamoto, Japan] and incubated for another 4 h at 37°C. After removing the medium, 100 µL dimethyl sulfoxide (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) was added to MTT formazan. Absorbance at 570 nm was measured using an iMark Microplate Reader (Bio-Rad, Hercules, CA).

Cd Treatment HK-2 cells were grown in plates at a density of 250 cells/mm^2 and cultured for 48 h. The culture medium was discarded, and the cells were treated with Cd ($CdCl_2$; FUJIFILM Wako Pure Chemical Co.) in serum-free culture medium.

Statistical Analysis Statistical analyses were performed using one or two-way ANOVA. When the F value was significant ($P < 0.05$), Bonferroni's multiple *t*-test was performed for post-hoc comparison ($P < 0.05$).

RESULTS

Effect of *GATA* Single Knockdown on the Viability of HK-2 Cells Our previous study demonstrated that treatment with 40 µM Cd for 3 h decreased the transcriptional activity of four GATA family members: GATA1, 81%; GATA 3, 64%; GATA 2, 63%; and GATA6, 58%.[6] To elucidate the effect of the decrease in cellular GATA levels on HK-2 cell viability, we examined the effect of siRNA treatment against individual *GATA* genes on cell viability. The treatment of *GATA1* siRNA for 24 h decreased the level of *GATA1* mRNA by approximately 30% (Fig. 1A) and correlated with a significant decrease in cell viability (Fig. 1B). However, after 48 h treatment with siRNA there was no effect on viability (Fig. 1C). Treatment with siRNA against *GATA3* for 24 h decreased the level of *GATA3* mRNA by approximately 45% (Fig. 1D). *GATA3* siRNA treatment for 24 h and 48 h decreased cell viability by 10–15% (Figs. 1E and F). *GATA6* siRNA treatment for 24 h suppressed the expression of *GATA6* by more than 70% (Fig 1G). Treatment with siRNA against *GATA6* decreased cell viability by approximately 30% at 24 h and 15% at 48 h (Figs. 1H and I). *GATA2* siRNA treatment did not affect the cell viability (data not shown). These results suggest that the suppression of some GATA family members contributed to cellular toxicity in HK-2 cells.

Effect of Cd on the Expression of *GATA* Family Genes in HK-2 Cells Next, we examined the effect of Cd on *GATA* family gene expression in HK-2 cells. We have previously shown that treatment with 40 µM Cd for 3 h decreased the activities of GATA transcription factors in HK-2 cells. Therefore, the mRNA levels of individual *GATA* family members were examined in HK-2 cells treated with 40 µM Cd for 1–6 h. The mRNA level of *GATA1* was unchanged by Cd treatment; however, there was a significant increase in the mRNA levels of *GATA3* and *GATA6* following Cd treatment (Figs. 2A, B, C). These results suggest that the effect of Cd on GATA family transcriptional activity was not because of a decrease in mRNA levels and indicates that Cd may have a posttranslational effect on GATA transcription factors.

Effect of Multiple Knockdown of *GATA* Family Members on the Viability of HK-2 Cells Finally, we examined the effect of multiple knockdown of *GATA* family members on HK-2 cell viability. Simultaneous introduction of siRNAs targeting *GATA1*, *GATA3*, and *GATA6* resulted in the reduction of about 40% for *GATA1*, 70% for *GATA3*, and 90% for *GATA6* mRNA (Figs. 3A, B, C). In addition, treatment with multiple *GATA* siRNAs for 24 h resulted in 40% inhibition of cell viability, and treatment for 48 h suppressed cell viability by 10% (Figs. 3D and E).

DISCUSSION

The GATA family binds to the (G/A)GATA(G/A) motif on DNA, regulating expression of various genes.[9] Mouse embryos in which GATA family members, other than GATA5, are deficient leads to lethality.[8] In this study, among the genes encoding GATA proteins, *GATA1*, *GATA3* and *GATA6* were knocked down with siRNA and the effect on cell viability was examined. Single knockdown of three GATA family members resulted in a decrease in cell viability. This was particularly apparent after 24 h treatment with *GATA6* siRNA that suppressed cell viability by about 30%. Individual GATA family members may be attributed to specific diseases but may also collectively be involved in the same disease.[8] In this study therefore, we examined the viability of HK-2 cells treated with multiple *GATA* siRNAs. The combination of siRNAs against *GATA1*, *GATA3* and *GATA6* led to a 40% and 10% inhibition of cell survival after 24 h and 48 h respectively. The effect on survival with the multiple knockdown was similar to the single knockdown of *GATA6*. Therefore, it is conceivable that the three GATA family members studied here are involved in Cd toxicity in proximal tubular cells *via* the same pathway.

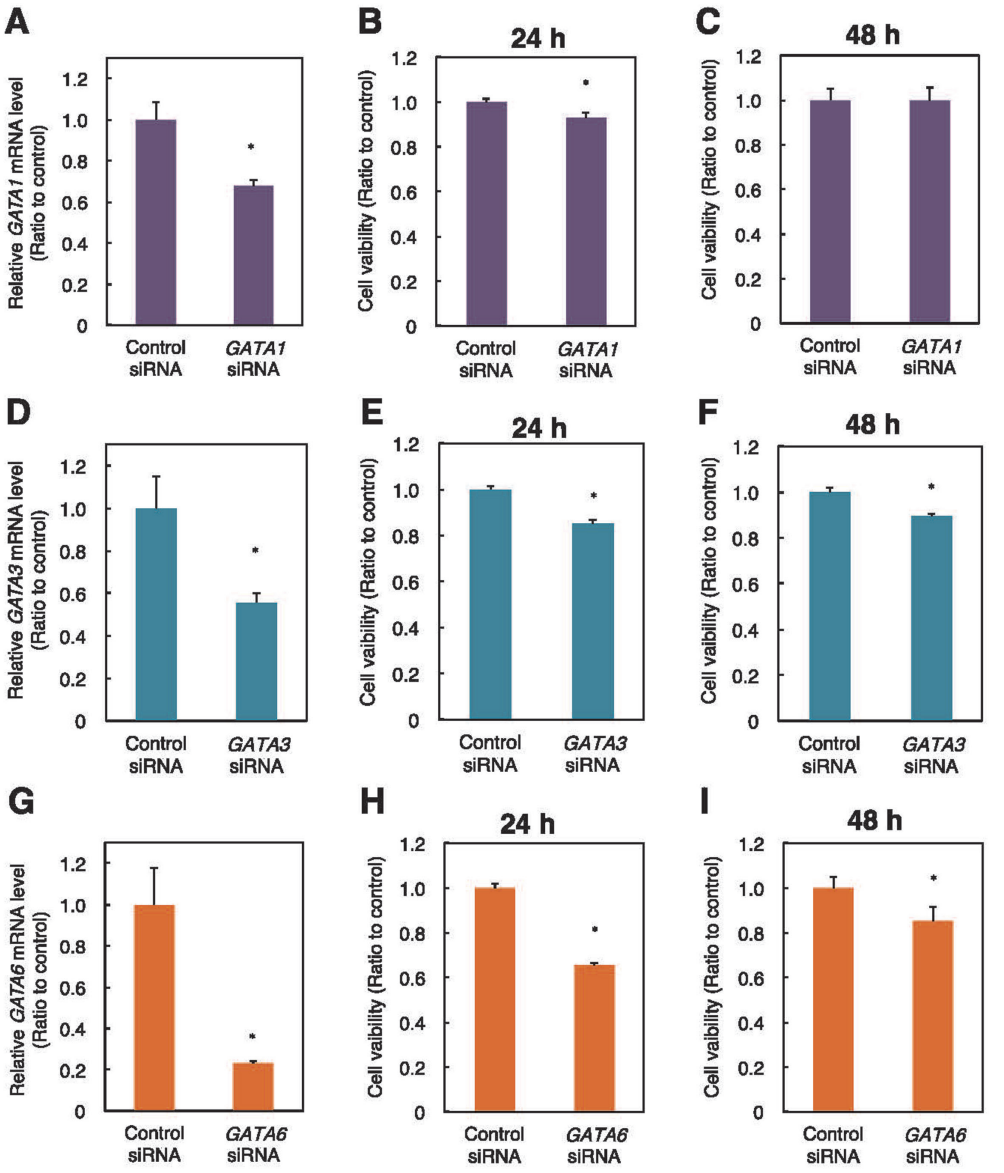

Fig. 1. Effect of Single *GATA* siRNA on the Viability of HK-2 Cells

HK-2 cells were treated with control siRNA, *GATA1*, *GATA3*, or *GATA6* siRNA for 24 h and 48 h. mRNA levels of *GATA1* in HK-2 cells treated with control siRNA or *GATA1* siRNA for 24 h (A). Cell viability of HK-2 cells treated with control siRNA or *GATA1* siRNA for 24 h (B) and 48 h (C). mRNA levels of *GATA3* in HK-2 cells treated with control siRNA or *GATA3* siRNA for 24 h (D). Cell viability of HK-2 cells treated with control siRNA or *GATA3* siRNA for 24 h (E) and 48 h (F). mRNA levels of *GATA6* in HK-2 cells treated with control siRNA or *GATA6* siRNA for 24 h (G). Cell viability of HK-2 cells treated with control siRNA or *GATA6* siRNA for 24 h (H) and 48 h (I). mRNA levels were normalized to *GAPDH*. Values are means ± S.D. (n=3). *Significantly different from the control group, $P < 0.05$.

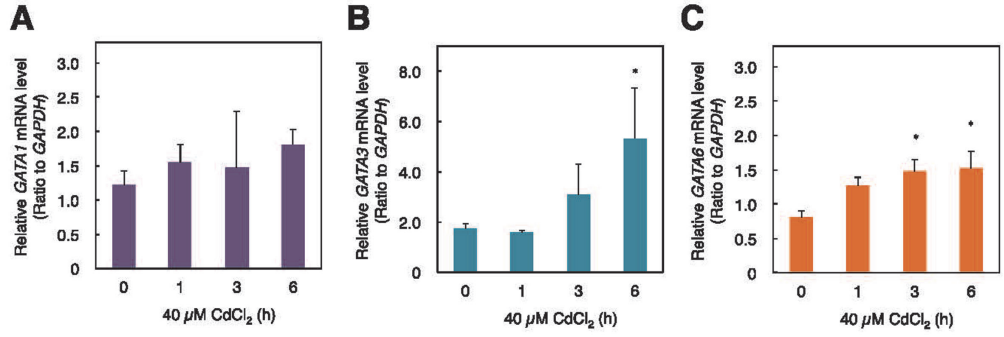

Fig. 2. Effect of Cd on mRNA Levels of *GATA* Family Members in HK-2 Cells

HK-2 cells were seeded at a density of 250 cells/mm^2 and cultured for 48 h. Cells were treated with 40 μM Cd ($CdCl_2$) in serum-free medium for indicated hours. *GATA* family gene mRNA levels were examined using real-time RT-PCR. (A) *GATA1*. (B) *GATA3*. (C) *GATA6*. mRNA levels were normalized to *GAPDH*. Values are means ± S.D. (n = 3). *Significantly different from the control group, $P < 0.05$.

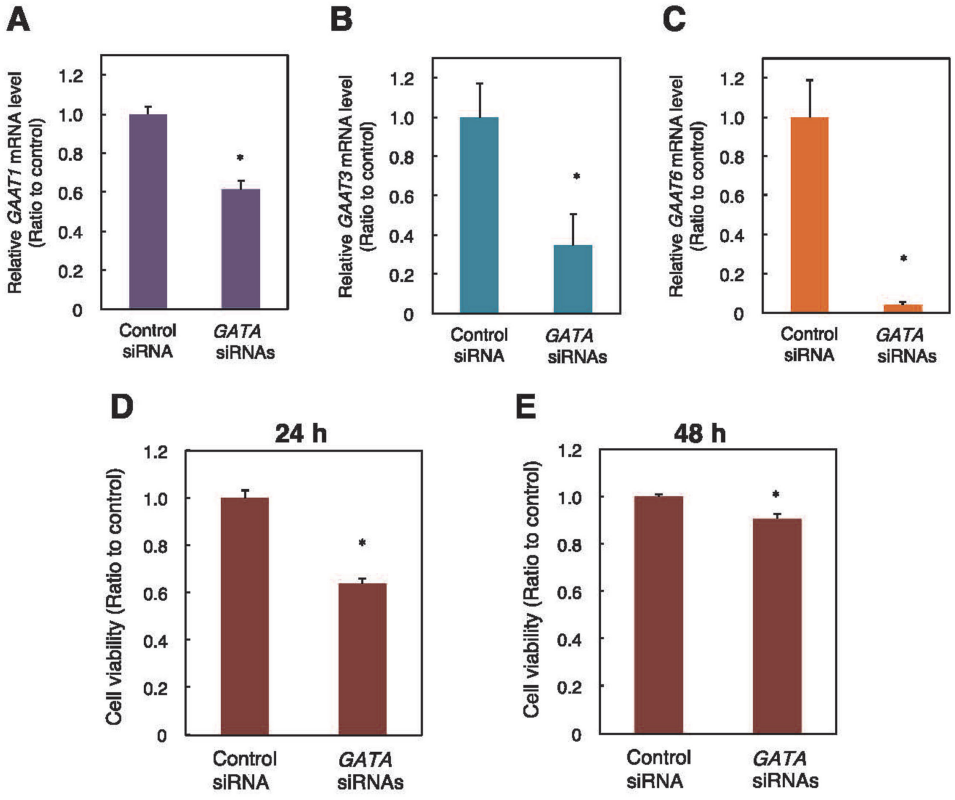

Fig. 3. Effect of Multi-Knockdown of *GATA* Family Members on the Viability of HK-2 Cells

HK-2 cells were treated with control siRNA or multiple siRNAs (*GATA1*, *GATA3*, and *GATA6* siRNA) for 24 h and 48 h. mRNA levels of *GATA1* (A), *GATA3* (B), and *GATA6* (C) in HK-2 cells treated with control siRNA or *GATA* siRNAs for 24 h. mRNA levels were normalized to *GAPDH*. Cell viability of HK-2 cells treated with control siRNA or *GATA* siRNAs for 24 h (D) and 48 h (E). Values are means ± S.D. (n=3). *Significantly different from the control group, $P < 0.05$.

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Conflict of interest The authors declare no conflict of interest.

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