Biometals (2009) 22:511–519
DOI 10.1007/s10534-009-9208-5

Gadolinium promoted proliferation and enhanced survival in human cervical carcinoma cells

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Received: 14 June 2008 / Accepted: 14 January 2009 / Published online: 29 January 2009

Abstract The effect of gadolinium chloride (Gd) on the proliferation of HeLa cells was investigated at lower concentration. The results obtained by MTT and cell cycle analysis showed that Gd promoted proliferation by inducing *S* phase entry in HeLa cells at the concentration less than 100 μM. It was further evidenced by both an increase in the levels of phosphorylation of retinoblastoma protein (pRb) and a remarkable increase in cyclin E expression. Moreover, the survival of cells, exposed to Gd up to 3–5 days, was increased compared with control. The attenuation of the serum deprivation-induced cell loss by Gd was associated with the sustained activation of FAK (PY^{397}) and the delayed activation of JNKs pathway. Besides, it appeared that Gd promoted cell proliferation and survival in HeLa cells was not contributed to the ROS generation. Based on the present results, both positive and negative effects of the lanthanides as potential drugs or diagnostic agents are discussed.

Keywords Gadolinium · Proliferation · Cell cycle · Survival

Abbreviations

BSA	Bovine serum albumin
DCFH-DA	2, 7-Dichlorofluorescein diacetate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EGTA	Ethyleneglycol bis (2-aminoethyl ether) tetraacetic acid
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Gd	Gadolinium chloride
HE	Dihydroethidium
HRP	Horseradish peroxidase
MTT	3-(4, 5-dimethylthiazoyl-2-yl) 2,5-diphenyltetrazolium bromide
p38 MAP kinase	p38 mitogen-activated protein kinases
PI	Propidium iodide
PMSF	Phenyl methyl sulfonyl fluoride
pRb	Retinoblastoma protein

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PVDF	Polyvinylidene difluoride
RNase	Ribonuclease
ROS	Reactive oxygen species
SAPK/JNK	Stress activated protein kinase/ c-jun N-terminal kinase
SDS	Sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis

Introduction

Recent advances and successful therapeutic applications of the lanthanides aroused growing interests in the study on the mechanisms of their biological properties. However, more concerns for the safety of lanthanides-based drugs are indicated. The controversy about the safety and efficacy in the lanthanum carbonate used as a non-calcium-containing phosphate binder has not been completely settled yet (Lizon and Fritsch 1999; Brancaccio and Cozzolino 2007; Cozzolino 2007; Lacour et al. 2007). Aime et al. (2007) called for caution in the treatment of uremic patients with lanthanum carbonate. In addition, as is known, organic chelates of lanthanide have been widely used in clinical practice as contrasting agents in MRI and adverse reactions were not commonly reported. But a recent survey found that the nephrogenic systemic fibrosis (NSF) observed in patients was possibly related to the administration of gadolinium-based contrast agent for MRI (Thomsen 2004). Moreover, a wide variety of physiological changes brought about by gadolinium salts were reported, as reviewed by Adding et al. (2001). Actually, all these effects are more or less related to their effects on cell proliferation and apoptosis. Among most of the previous studies, the following three aspects should be noted.

Firstly, lanthanides affect a biological event from two opposite sides. The dual effects of lanthanides were discussed on the basis of bi-directional dose dependence (Wang et al. 1999). The proapoptosis effect frequently appeared at the concentration higher up to millimolar levels (Mizgerd et al. 1996; Sato et al. 1998; Greisberg et al. 2001; Dai et al. 2002; Shi and Huang 2005), while proliferative effect often occured at micromolar concentration (Yu et al. 2005, 2006; Fu et al. 2008).

Secondly, for lanthanides as the potential drug candidates or diagnostic agents, they are always in stable chelate forms (Fricker 2006; Hashemy et al. 2006). So the concentrations of lanthanides ions, expected to exert biological effects will be at a very low level.

Thirdly, many previous studies investigated the cytotoxic effects of lanthanides on cancer cells (Mizgerd et al. 1996; Sato et al. 1998; Greisberg et al. 2001; Dai et al. 2002; Shi and Huang 2005). These studies usually used species such as chlorides, nitrates or acetates of lanthanides (III). They have shown that only at higher concentrations usually up to millimolar concentrations, lanthanides can exhibit inhibitory effects. It prompted us to ask the question: what will happen if we use lower concentrations of lanthanide ions?

Thus, the aim of this study was to determine whether at lower concentrations the lanthanides salts themselves can promote cell proliferation or trigger apoptosis of a tumor cell line, HeLa, and the underlying mechanisms were discussed.

Materials and methods

Materials

Gadolinium oxide (purity $> 99.99\%$) was obtained from Beijing Chemical Company. Fetal bovine serum (FBS) was obtained from Hyclone. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco. 3-(4,5-dimethylthiazoyl-2-yl), 5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and ribonuclease (RNase) were obtained from Sigma–Aldrich. Hydroethidine (HE) and 2,7-dichlorofluorescein diacetate (DCFH-DA) were from Molecular Probes. Anti-cyclin A, anti-cyclin D, anti-cyclin E antibodies, and HRP-labeled rabbit anti-mouse IgG were obtained from Santa Cruz Biotechnology. Antibodies against β-actin, p38 MAP kinase, phospho-p38 MAP kinase (Thr 180/Tyr 182), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185) and phosphorylated pRb at Ser780 antibodies were from Cell Signaling Technology. Anti-FAK [pY397] polyclonal antibody was purchased from Biosource. Other reagents were of analytical grade.

DCFH-DA and HE solutions were both prepared by dissolving in DMSO and were diluted with PBS to final concentration of 5 μM, respectively.

Gadolinium chloride ($GdCl_3$) solution was prepared by dissolving gadolinium oxide in HCl. The solution was evaporated to remove the excess HCl and redissolved in 1 mM HCl and the $GdCl_3$ stocking solution was diluted to 50 mM.

Methods

Cell culture

HeLa cells, a human cervix epithelial carcinoma cell line, were obtained from Cell Culture Center of Peking Union Medical College. The cells were maintained in DMEM, supplemented with 10% (v/v) FBS and 100 units penicillin 100 μg streptomycin per ml at 37°C in a humidified incubator with 5% CO_2. Depending on experimental design, cells were subcultured at a ratio between 1:2 and 1:3. The logarithmically growing cells were used for all experiments.

Serum-starvation and the treatment of the cells

Unless otherwise stated, cells in all the experiments were driven into quiescence by serum deprivation, then they were exposed to DMEM containing 0.5% FBS with or without Gd.

MTT assay

The cells in exponential growth were seeded equivalently in 96-well microplates in growth medium and then incubated at 37°C in a humidified incubator with 5% CO_2. After 24 h, the medium was removed and replaced with DMEM medium containing 0.5% FBS with or without various concentrations of $GdCl_3$ (0.1, 1, 10, 50, 100 μM) for 24–48 h. Then each well was treated with 100 μl MTT in DMEM medium containing 0.5% FBS to reach a final concentration of 0.5 mg/ml and kept for 4 h. Finally the medium with MTT was removed and 100 μl of DMSO was added to dissolve the formazan crystals formed. After 20 min incubation, the cell viability was assayed by measuring the absorbance of each well at 570 nm using a microplate reader (TECAN SUNRISE, Switzerland). At least triplicate cultures were established for each treatment. The cell viability (%) was calculated according to the following equation:

$$\text{Cell viability } (\%) = [A_{570}\ (\text{sample})/A_{570}(\text{control})] \times 100\%,$$

where *A*sample represented the reading from the wells treated with Gd and *A*control from those treated with medium only.

Cell cycle distribution analysis

The cells were harvested by trypsinization in the presence of 100 μM $GdCl_3$. Then they were fixed in the ice-cold 70% ethanol and left overnight at −20°C. The fixed cells were washed with PBS and resuspended in PI (7.5 μM/ml) and RNase (100 μg/ml) solution. The suspension of cells was incubated at 37°C for 30 min in the dark. The percentage of cells in the different phases of the cell cycle was measured with a FASCAN flow cytometer (FACS-calibur, Beckton Dickenson, USA). A minimum of 15,000 gated events was collected. The data were analyzed using the Modfit software (Beckton Dickenson).

Western blotting

The cells were washed with ice-cold PBS and collected by scraping with a rubber policeman after the treatment of 100 μM Gd. Then the cells were lysed in cold TSE buffer (10 mM Tris–HCl, 250 mM sucrose, 1 mM EGTA, pH7.4) containing a variety of protease and phosphatase inhibitors (5 mg/ml aprotinin, 5 mg/ml leupeptin, 100 mM PMSF, 1 mM Na_3VO_4, 50 mM NaF, and 1 μM/ml DTT) by sonication. The lysates were clarified by centrifugation (12,000 rpm, 20 min) at 4°C and the supernatant was collected.

The protein concentration was determined using the Bradford assay. The protein lysates were boiled in SDS gel loading buffer for 5 min. Then 20 μg of protein from each sample was loaded and separated by SDS–PAGE, using 7.5% separating gels. Then the protein was electrophoretically transferred to a PVDF membrane and blocked with 5% BSA in TBS buffer (20 mM Tris base, 1.37 mM NaCl, pH 7.6) for 2 h. Afterwards, it was incubated with appropriate primary antibodies in TBS/T (TBS containing 0.05% Tween 20) containing 5% BSA at 4°C overnight. After three washes with TBS/T, the membrane was incubated with HRP-conjugated secondary

antibody in TBS/T containing 5% BSA at 1:5,000 dilution for 1 h. The blots were then processed with an enhanced chemiluminescence (ECL) plus kit (Amersham Corp, USA) with Kodak X-ray films according to the protocol provided by the manufacturer. Band densities were measured and analyzed with the BandLeader^{TM} V3.00 software.

ROS detection

DCFH-DA was used to evaluate the intracellular ROS level in the form of cellular peroxides. It is a cell-permeant dye, once inside the cell, is cleaved by intracellular esterase into its nonfluorescent form DCFH and then rapidly oxidized to green-fluorescing DCF by cellular peroxides, mainly hydroperoxide. For determination of intracellular ROS in superoxide radical form, the fluorescent dye hydroethidine (HE) was used. It can be oxidized to a fluorescent product, which binds to DNA and leads to enhancement of red fluorescence (Doudican et al. 2005; Dai et al. 2006). The cells were seeded on Petri dish and cultured until 80–90% confluent, then treated with DMEM medium with or without 100 μM $GdCl_3$ for 4 or 12 h. DCFH-DA (5 μM) or HE (5 μM) was applied to the cells after two washes with PBS and incubated in the dark for 20 min at 37°C. The samples were observed using a laser scanning confocal microscope (Leica TCS NT, Germany).

Data statistical analysis

Data were represented as means ± SD of at least three independent experiments. Differences between values were tested using the Student's *t*-test, and $P < 0.05$ was considered as statistical significance.

Results

Gd promoted cell proliferation by promoting *S* phase entry in HeLa cells

The cell proliferation was evaluated by MTT assay as described in the experimental section. As seen in Fig. 1, the increase of cell viability was observed at concentrations of 50 and 100 μM Gd at both 24 and 48 h.

To clarify the mechanism of Gd-promoted cell growth described above, the cell cycle distribution of HeLa cells was analyzed by flow cytometry. The results showed that Gd promoted G_1/S cell cycle progression. As shown in Fig. 2b, the percentage of *S* phase was increased up to 34% after the treatment of Gd for 24 h compared with 25% in *S* phase for the control group. It was further evidenced by the expressions of cell-cycle-associated proteins involved in G_1/S phase transition. The results given in Fig. 3 demonstrated a sustained increase up to 12 h in the levels of p-pRb (Ser^{780}) in presence of Gd, while the control cells exposed to 0.5% FBS showed an increase only for 1 h. Additionally, the phosphorylated levels (Ser^{780}) of Rb protein was obviously higher than that in control cells at 4, 8, 12 and 16 h, respectively.

Concomitantly, the protein expression of cyclin E was remarkably increased after the treatment of Gd at 4 h and sustained up to 16 h. Nevertheless, the expression levels of cyclin D and cyclin A remained relatively unchanged.

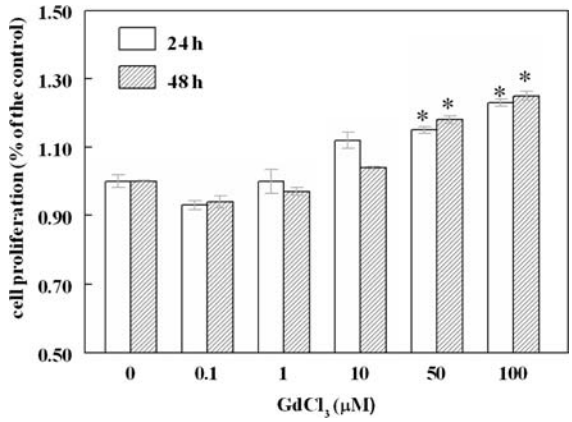

Fig. 1 Effects of Gd on cell proliferation by MTT test. Various concentrations of Gd were added to the medium and incubated for 24 and 48 h, respectively. Data were presented as means ± SD ($n = 3$; $^*P < 0.05$)

Gd attenuated the serum deprivation-induced cell loss

Next we extended the treatment time period of Gd in serum deprivation state. As can be seen in Fig. 4, the percentage of cell death was significantly decreased in the presence of 100 μM Gd on 3–5 days, respectively. It showed that Gd attenuated the serum starvation-induced cell loss, indicative of a cytoprotective effect of Gd.

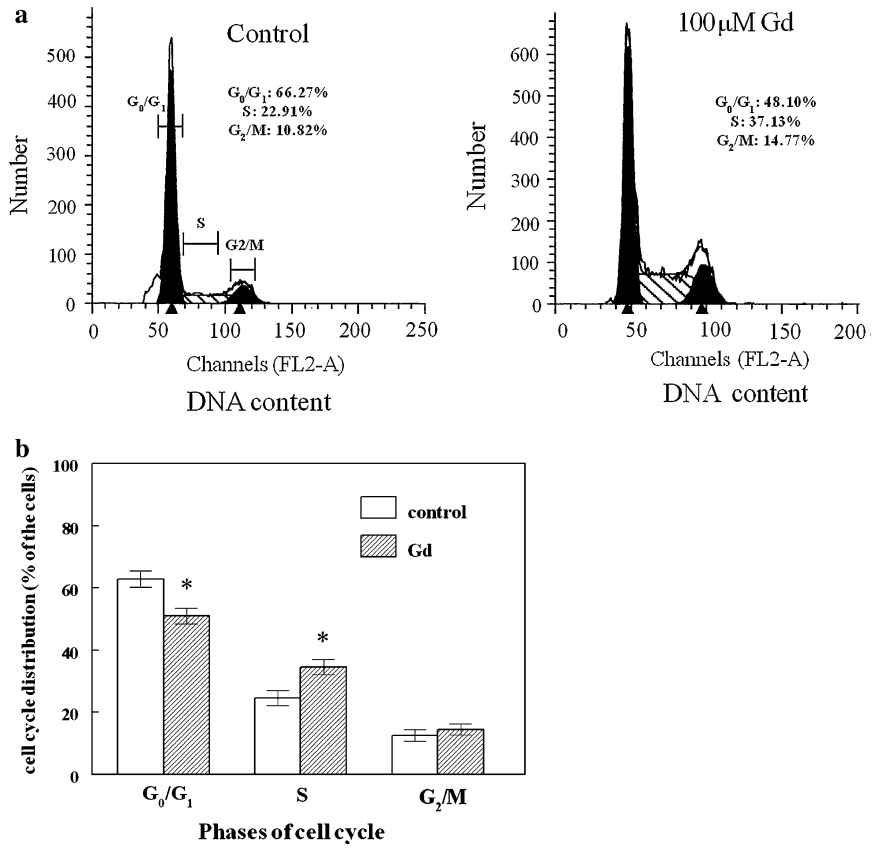

Fig. 2 Gd promoted *S* phase entry in HeLa cells. Cells were treated with or without 100 μM Gd for 24 h. **a** Percentages of cells in G_0/G_1, *S*, or G_2/M phases were indicated. The *hatched areas* represented *S* phase. Data shown were the representative of three independent experiments. **b** Statistical analysis of the distribution of the cell cycle after the treatment with Gd ($n = 3$; $^*P < 0.05$, compared with control)

Sustained activation of FAK was related to Gd-attenuated serum deprivation-induced cell loss

As to the related mechanism, it was noticed that morphology changes in HeLa cells were observed at 48 h by Gd treatment, which provided evidence to interpret its protective effect. The shapes of the control cells exposed to 0.5% FBS were mostly round in shape after 48 h whereas those exposed to Gd kept in flattening shapes (data not shown), demonstrating better cell attachment may help cell to survive. Since it is well established that focal adhesion kinase (FAK) plays an important role in cell-extracellular matrix interaction (Schlaepfer et al. 1999; Mitra and Schlaepfer 2006; van Nimwegen and van de Water 2007). Thus, the activation of FAK was examined. As shown in Fig. 5a, the levels of phosphorylated FAK (pY-397) were remarkably higher when exposed to Gd compared with that in the control cells at 8 and 12 h, although decreased levels of phosphorylated-FAK were observed in both control and Gd-treated cells after 4 h.

Delayed activation of JNKs was associated with Gd-attenuated serum deprivation-induced cell loss

P38- and JNKs MAPKs are the two independent stress kinase signaling pathways (Cuenda and Rousseau 2007; Weston and Davis 2007). Therefore, the two pathways were assessed and the results were shown in Fig. 5b. The JNKs activations were observed clearly at 72 h after the treatment of Gd. Comparing with this, it was remarkably activated earlier at 48 h for control cells. It revealed that the delayed JNK activation may be another factor causing the attenuation of cell loss by Gd. However, there was not much difference for the activation of p38 pathway, though the level of phosphorylated p38 was evidently increased after 24 h exposure of Gd.

ROS did not involve in the proliferation-promotion and cytoprotective effects of Gd

Some studies indicated ROS was involved in La^{3+}, Gd^{3+} or Yb^{3+}-induced cellular apoptosis or stress

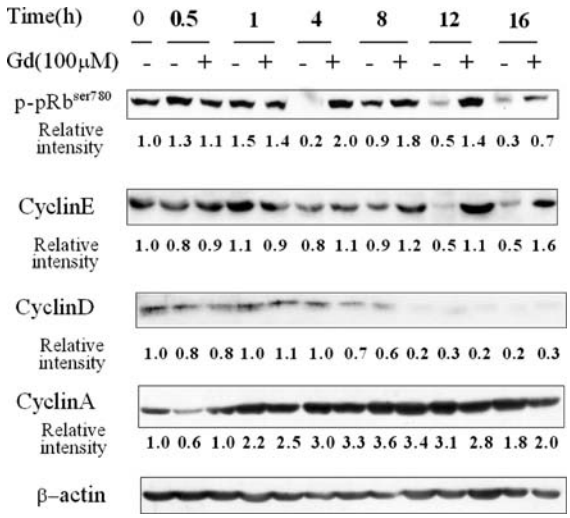

Fig. 3 The levels of phosphorylation of pRb and expressions of cyclins upon treatment with 100 μM Gd. The cells were seeded equivalently in Petri dishes in the growth medium for 24 h. Then it was replaced with DMEM medium for 24 h starvation. The DMEM containing 0.5% FBS with or without 100 μM Gd was added, respectively, for certain time periods. The cells were lysed and the extracts were electrophoresed and detected as described in experimental section. Data shown were the representative of three independent experiments. The *numbers* below the blots depicted values of densitometric evaluation (ratio of p-pRb (Ser-780)/*β*-actin and Cyclins/*β*-actin, normalized to 0.5% FBS control)

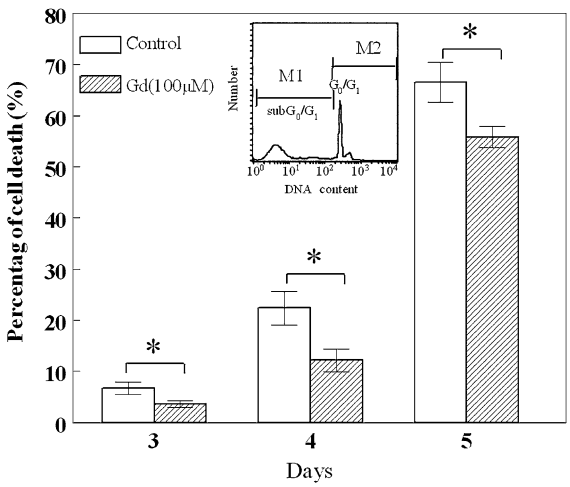

Fig. 4 Gd attenuated the serum deprivation-induced cell loss. Cells was treated with DMEM medium containing 0.5% FBS with or without 100 μM Gd^{3+} for 3, 4 or 5 days were collected for cell cycle analysis. The *inserted frame* showed the way to quantify the dead cells. Death fraction was recognized as sub-G_0/G_1 population of cell cycle measured by flow cytometry. *M1* represented the percentage of the dead cells. *M2* represented the percentage of the survival cells ($n = 5$; $^*P < 0.05$, compared with control)

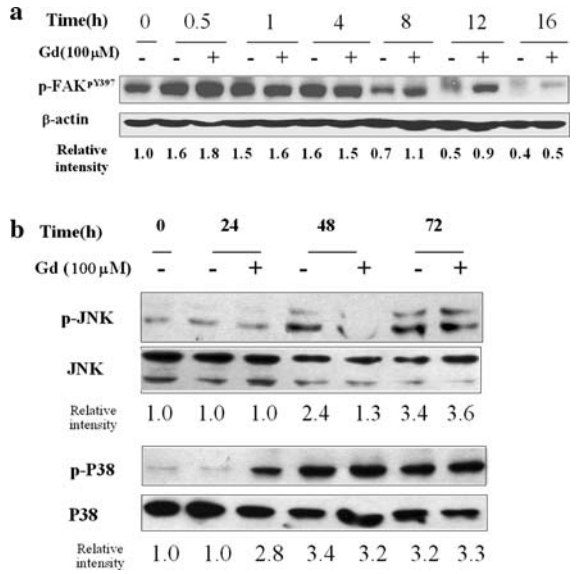

Fig. 5 Immunoblot analysis of the phosphorylation states of protein FAK/JNK/p38 in HeLa cells. The samples were treated as described in Fig. 3. The *numbers* below the blots depicted values of densitometric evaluation (ratio of p-FAK (PY-397)/*β*-actin, p-JNK/JNK or p-p38/p38, respectively, normalized to 0.5% FBS control). **a** The effect of Gd on the level of phosphorylation of FAK. **b** The effect of Gd on the level of phosphorylation of JNK and p38

such as swelling of mitochondria (Liu et al. 2003) and unfolded protein response in human hepatoblastoma HepG2 cells (Shen et al. 2008). However, it was also reported that lanthanum chloride inhibited H_2O_2-induced elevation in ROS level in rat calcifying vascular cells and thus may suppressed H_2O_2-enhanced osteoblastic differentiation and apoptosis (Shi et al. 2008). Then, if Gd promoted proliferation and enhanced survival of serum deprivation-caused cell death, considering the complicated roles of ROS in Ln-induced cellular responses, it prompted us to determine whether ROS was related to this process.

The effect of Gd on intracellular ROS level in HeLa cells was monitored by fluorescent staining of intracellular $\cdot O_2^-$ and H_2O_2 with HE (specific for $\cdot O_2^-$) and DCFH-DA (specific for H_2O_2), respectively as described in "Materials and methods". However, neither the $\cdot O_2^-$ nor the H_2O_2 level was observed to increase after the treatment of Gd either for 4 or 12 h (data not shown). Hence ROS may not be related to the proliferative and cytoprotective effects of Gd.

Discussion

In the present study, we focused on the effects of the gadolinium ions at lower concentration. As the results showed, Gd promoted proliferation of HeLa cells at the concentration even less than 100 μM. An increase in HeLa cell proliferation by Gd treatment (Fig. 1) was consistent with the results shown in cell cycle distribution (Fig. 2a, b). In addition, Gd caused an increase in phosphorylation of pRb, a regulator of G_1 to *S* phase transition. As is well known, the inactivation of pRb by phosphorylation leads to the release of E2F family of transcription factors, which can activate transcription of various genes to promote cell cycle progression entry into *S* phase (Dyson 1998; Nevins 1998). The results showed a remarkable increase in the level of cyclin E expression after exposure to Gd (Fig. 3). Cyclin E is the prominent target of E2F1/2/3 after pRb is inactivated, which drives cells to pass through the restrict point, entering into cell cycle (Geng et al. 1996). Furthermore, the prolonged exposure to Gd led to a decrease in cell death due to serum starvation, pointing to a cytoprotective effect of Gd.

The proliferative and cytoprotective effect exerted by Gd may be seen as a double-edged sword. On the one hand, it can protect cells from damage by external stimuli. In vivo tests showed that $GdCl_3$ could prevent and/or reduce liver damage when the rats was injected 24 h before $Pb(NO_3)_2$ injection, which was known to induce liver hyperplasia followed by apoptosis (Pagliara et al. 2003). This is in agreement with our results in HeLa cells. Besides, it was demonstrated that $GdCl_3$ treatment of rats increased hepatocyte replication fivefold in 24 h and threefold in 48 h (Rose et al. 2001). But this effect was contributed to tumor necrosis factor α released from Kupffer cells at early time points. However, based on the current results, the possibility that a direct mitogenic action of $GdCl_3$ on hepatocytes can not be excluded. On the other hand, the proliferative and cytoprotective effect by Gd may cause negative effects. Gadolinium texaphyrin complex (MGd) is currently subjected to phase III clinical trials for the treatment of brain metastases of non-small cell lung cancer (Fricker 2006; Hashemy et al. 2006). MGd has been regarded as an proapoptotic agent by forming superoxide and other ROS by redox cycling on account of texaphyrin ligand. However, it was found that MGd resulted in growth suppression but not apoptosis in Burkitt's lymphoma-derived cell line (Ramos et al. 2006) and thus weakened the activity of MGd. The above study contributed it to the oxidative stress-regulated phosphorylation status of Akt/protein kinase B. But it cannot completely exclude the possible cytoprotective effect by Gd ion itself. In the present study, the morphology changes induced by Gd provided an additional evidence to interpret its protective effect. The enhanced cell attachment because of cell flattening rather than rounding may help cell to survive. Similar phenomenon was described previously in Gd^{3+} or Tb^{3+}-treated B16F10 melanoma cells (Cox et al. 2002). Ahmad et al. (1999) also showed that during acute endotoxemia in rats treatment with $GdCl_3$ alone caused a marked increase in expression of intercellular adhesion molecule-1 on endothelial cells and hepatocytes, and of $\beta 2$ integrins on macrophages and endothelial cells. As we know, the attachment of cells to basal substrates or to other cells can regulate their progression through specific stages of the cell cycle. Therefore the sustained tyrosine phosphorylation of FAK at Tyr-397 may be related to the enhanced survival by Gd treatment. These data indicated that the activity of MGd may be improved by combining it with agents that inhibit FAK phosphorylation besides inhibiting Akt phosphorylation.

In summary, it is reasonable to conclude that at the concentration less than 100 μM, the gadolinium chloride did not induce apoptosis but rather promote proliferation and enhance survival in HeLa cells, which may be related to the sustained activation of FAK and delayed activation of JNKs.

Acknowledgments This work was supported by the National Natural Science Foundation of China (No. 20637010).

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