Journal of Cellular Biochemistry 94:508–519 (2005)

La^{3+}-Promoted Proliferation Is Interconnected With Apoptosis in NIH 3T3 Cells

Siwang Yu,[1] Lan Yuan,[1] Xiaoda Yang,[1,2]* Kui Wang,[1,2] Ya Ke,[1,3] and Zhong Ming Qian[1,3]*

[1]Department of Chemical Biology and Joint Laboratory of Peking University & The Hong Kong Polytechnic University, School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing 100083, People's Republic of China
[2]National Research Laboratories of Natural and Biomimetic Drugs, Peking University Health Science Center, Beijing 100083, People's Republic of China
[3]Department of Applied Biology & Chemical Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong, People's Republic of China

Abstract Lanthanum ion (La^{3+}) has been reported to affect proliferation or apoptosis of different cells. In the present study, La^{3+} was confirmed to promote both proliferation and apoptosis of NIH 3T3 cells at the same concentrations. La^{3+} was shown to promote proliferation by helping the cells to pass through the G1/S restriction point and enter S phase, however, the proliferating cells induced by incubation with La^{3+} eventually underwent apoptosis. The proliferation and apoptosis of NIH 3T3 cells induced by La^{3+} were well correlated with cell cycle alterations. La^{3+} caused the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2; while inhibition of ERK phosphorylation by 2′-amino-3′-methoxyflavone (PD98059) suppressed both proliferation and apoptosis induced by La^{3+}. Based on the above experimental results, we postulated that La^{3+}-promoted proliferation of NIH 3T3 cells could be interconnected with the cell apoptosis, possibly through cell cycle machinery. Our results thus support the recent hypothesis that proliferation and apoptosis of cell are intrinsically coordinated. J. Cell. Biochem. 94: 508–519, 2005. © 2004 Wiley-Liss, Inc.

Key words: La^{3+}; proliferation; apoptosis; cell cycle; ERK

Lanthanides are a group of metals, increasingly used in industry. Lanthanide ions exhibit similar chemical properties and biological effects. Although their physiological functions as xenobiotics are still unknown, they were noted for their diversified biological effects with potential medical applications [Wang et al., 1999], and have been widely used as diagnosis tools [Dobrynina et al., 1997]. In addition, lanthanides compounds have been used to increase the production of crops and to promote the growth of livestock in China for many years [He and Rambeck, 2000]. These applications increased the chances of accidental exposure, both acute and chronic [Qiang et al., 1994] and therefore studies on their biological effects, especially on cell proliferation and apoptosis, are of increasing interest for researchers.

Among the members of lanthanides, La^{3+} is of considerable research interest because of its pharmacologic properties [Das et al., 1988]. It is used extensively as a calcium channel blocker or antagonist in cellular or other biological systems [Miledi, 1971; Block et al., 1998]. A variety of other biological activities have also been noted including interaction with biological membranes [Fiskin et al., 1980; Anghileri et al., 1987] and modulation of neurotransmitter receptor response [Barila et al., 2001].

However, apparently contradictory results have been observed on the effects of La^{3+} on

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 20101001, 20331020; Grant sponsor: Joint-Research Program of Hong Kong Polytechnic University and Peking University.

*Correspondence to: Xiaoda Yang, PhD, and Zhong Ming Qian, MD, PhD, Department of Chemical Biology and Joint Laboratory of Peking University & The Hong Kong Polytechnic University, School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing 100083, P.R. China.
E-mail: xyang@bjmu.edu.cn; bczmqian@polyu.edu.hk

Received 4 May 2004; Accepted 9 August 2004
DOI 10.1002/jcb.20303

© 2004 Wiley-Liss, Inc.

cell proliferation or apoptosis. It has been shown to inhibit cell proliferation and tumor growth [Anghileri et al., 1987; Dai et al., 2002] as well as to induce cell death [Palmer et al., 1987]. But in some other reports, it exhibits proliferative effects [Smith and Smith, 1984; Praeger and Gilchrest, 1989] and functions to inhibit apoptosis.

Such contradictory results were also observed in the cellular effects of other inorganic ions such as cadmium [Misra et al., 2002; Shih et al., 2004], arsenic [Simeonova et al., 2000; Liu et al., 2003b], and nickel [Lisby et al., 1999; Kim et al., 2002]. These paradoxical results described above have often been ascribed to the differences between the cell lines or the concentrations of chemicals. Nevertheless, little attention was paid to the interconnection between cell proliferation and apoptosis.

Proliferation and apoptosis are essential yet opposing cellular processes. However, in recent years it was postulated that cell proliferation and apoptosis might be intrinsically coordinated and the balance between them should be stringently controlled [Vermeulen et al., 2003]. One possible mechanism for maintaining the balance is via factor-dependent signaling from the environment for cell survival and proliferation [Jones and Kazlauskas, 2001]. The cross-talk between the signaling pathways, which control proliferation and apoptosis, is more important. It was suggested that proliferation and apoptosis might be coordinated through the direct coupling of cell cycle progression and programmed cell death [Evan et al., 1995]. This postulation was supported by the fact that inappropriate signals to proliferate were often found to promote apoptosis through this coordination [Guo and Hay, 1999]. For instants, a number of oncogene products, which drive the cell cycle progression, could also result in apoptosis [Packham et al., 1996; Latella et al., 2000; Greene et al., 2004].

It was proposed that the coordination described above should be crucial for normal development and homeostasis in metazoans [Evan and Vousden, 2001] and might play important role in cellular response to xenobiotics [Schulte-Hermann et al., 1999]. However, little research has been done on these issues for xenobiotics.

In present study, we report for the first time our investigation on the effects of La^{3+} on proliferation associated with apoptosis in NIH 3T3 cells. La^{3+} was found to promote both proliferation and apoptosis of NIH 3T3 cells at the same concentration, and the processes were shown to correlate with cell cycle alterations. La^{3+} caused a transient phosphorylation of ERK, while inhibition of ERK phosphorylation by the MEK inhibitor PD98059 eliminated the La^{3+}-promoted proliferation and apoptosis. Based on the above observations, we postulated that there could be a link between La^{3+}-induced cell proliferation and apoptosis, possibly through cell cycle machinery. And this might account for the dual effects of La^{3+} on cells.

MATERIALS AND METHODS

Materials

Dulbeccol's Modified Eagle's Medium (DMEM) was obtained from GibcoBRL; fetal bovine serum (FBS) was purchased from Hyclone; trypsin, 5-bromo-2′-deoxyuridine (BrdU), propidium iodide (PI), 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT), Hoechst33258, and the MEK inhibitor 2′-amino-3′-methoxyflavone (PD98059) were purchased from Sigma. Antibodies against BrdU, extra-cellular signal-regulated kinase (ERK), phosphorylated ERK (pERK), and the corresponding secondary antibodies were obtained from Santa Cruz. Other reagents were of analytical grade. La^{3+} (lanthanum chloride) solution was prepared from lanthanum oxide (purity $>99.9\%$).

Cell Culture

NIH 3T3, a murine embryo fibroblast cell line, was obtained from Peking University Health Science Center. The cells were cultured in DMEM supplemented with 10% FBS and 100 U penicillin-100 μg streptomycin per ml at 37°C in a humidified 5% CO_2 atmosphere. All experiments were performed using cells within 30 passages.

Cell Proliferation and DNA Synthesis Assay

Half-confluent NIH 3T3 cells in exponential growth were incubated at 37°C with various concentrations of La^{3+} in serum-free DMEM medium for different time intervals. In other experiments, cells were incubated with La^{3+} in presence of 10% FBS. MTT was dissolved in PBS solution (5 mg/ml) and added to the culture medium to reach a final concentration of 0.5 mg/ml. After the cells were left at 37°C for 4 h, the

medium was aspirated and 200 μl of DMSO was added to dissolve the formazan crystals formed. The absorbance of the samples was determined at 570 nm with a reference at 650 nm on a microplate reader (TECAN SUNRISE, Switzerland). Another cell culture, after incubation with La^{3+} as described above, was trypsinized and resuspended in PBS solution, and the number of cells was counted with a hemocytometer.

DNA synthesis of NIH 3T3 cells was assessed by BrdU incorporation as described previously [Zatterstrom et al., 1992]. Briefly, cells were incubated with various concentrations of La^{3+} in serum-free DMEM medium for 24 h. The BrdU solution in PBS (3 mg/ml) was added to the culture medium to achieve a final concentration of 10 μg/ml and left for 1 h. Cells were then collected by trypsinizing and cellular DNA was denatured with a 2 M HCl solution. The BrdU incorporated was stained with a mouse anti-BrdU monoclonal antibody followed by a FITC-conjugated goat-anti-mouse IgG. The fluorescence associated with BrdU was measured by Fluorescence-Activated Cell Sorting (FACS; excitation, 490 nm; emission, 535 nm; FACScan, BD Bioscience, San Jose, CA). The percentage of BrdU-positive cells was calculated with the CELLQuest software.

Hoechst33258 Staining

NIH 3T3 cells were grown on slides and incubated with 100 μM La^{3+} in serum-free DMEM medium at 37°C for 48 h. The medium was removed and cells were fixed with 4% formaldehyde in PBS solution at 4°C for 30 min. After three washes with PBS, the cells were stained with 5 μg/ml of Hoechst33258 in PBS solution and the nuclear morphology was observed on a laser-scan confocal fluorescence microscope (excitation, 363 nm; emission, 440 ± 20 nm band pass. Leica, TCS SP2, Germany).

DNA Fragmentation Assay

La^{3+}-induced cellular DNA fragmentation of NIH 3T3 cells was analyzed as described previously [Walker et al., 1999]. Briefly, half-confluent cells in exponential growth were incubated with various concentrations of La^{3+} in serum-free DMEM medium for 60 h. The cells were harvested by trypsin digestion and dissolved with a lysis buffer (20 mM EDTA, 50 mM Tris-HCl, 0.5% SDS, pH 8.0). The cell lysate was treated with proteinase K and DNase-free RNase A, and DNA was extracted by phenol-extraction and ethanol-precipitation. The DNA samples were then analyzed using agarose gel electrophoresis.

Tracing of Cells' Fate Upon Incubation With La^{3+}

NIH 3T3 cells were grown on slides and incubated with 100 μM La^{3+} in serum-free DMEM medium for 24 h. BrdU solution in PBS (3 mg/ml) was added into the culture medium to achieve a final concentration of 10 μg/ml and left for 1 h. Then the medium was replaced with fresh one containing the same concentration of La^{3+} and incubated for up to 24 h. Control cells were grown in either medium supplemented with 10% FBS or serum-free medium, pulse labeled with BrdU as described above, and then cultured in the same medium for an additional 24 h. The cells were fixed with 4% formaldehyde in PBS and stained with anti-BrdU antibodies as described above. Then the samples were observed on a laser-scanning confocal microscope (excitation, 488 nm; emission, 540 ± 20 nm band pass. Leica, TCS SP2, Germany). Transmission gray-scale graphs were also obtained for observation of cellular morphology.

FACS Analysis of Cell Cycle Distribution and Apoptosis

NIH 3T3 cells were incubated at 37°C with various concentrations of La^{3+} in serum-free DMEM medium for different time intervals. The cells were collected by trypsinizing and fixed with 70% ethanol at 4°C overnight, then treated with DNase-free RNase and stained with 10 μg/ml PI in PBS for 0.5 h. The cellular DNA content was analyzed on a Beckton Dickinson FACScan cytometer. The cell cycle distribution was calculated using the ModFit3.0 software, and the subdiploid population was marked as apoptotic.

Western Blotting of ERK Phosphorylation

NIH 3T3 cells were growth in 10-cm plates. The cells were serum-starved for 12 h and then incubated with various concentrations of La^{3+}. Thirty minutes later, the cells were lysed in cold TGH buffer (1% Triton X-100, 10% Glycerol, 50 mM HEPES, pH 7.2) containing 100 mM NaCl and a variety of protease and phosphatase inhibitors (5 μg/ml aprotinin, 5 μg/ml leupeptin, 100 mM PMSF, 1 mM Na_3VO_4, 50 mM NaF, and

1 mM β-phosphaglycerol). The lysate were clarified by centrifugation (13,000*g*, 10 min), and aliquots (80 μg in total protein) were subject to SDS–PAGE. The protein was electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 10% non-fat dry milk in Tris balanced saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 2 h. The staining of phosphorylated ERK protein was carried out by incubating with mouse monoclonal anti-pERK antibodies in TBS containing 5% BSA and 0.02% Tween 20 overnight at 4°C followed by a HRP-conjugated rabbit-anti-mouse IgG for 1 h. The amounts of pERK were determined using the enhanced chemiluminescence kit (ECL, Santa Cruz) with Kodak X-ray films according to the protocol provided by the manufacturer.

The amounts of ERK were also determined by Western blotting using a rabbit polyclonal anti-ERK antibody and following a similar procedure as above.

Effects of MEK1 Inhibitor 2′-Amino-3′-Methoxyflavone (PD98059)

Half-confluent NIH 3T3 cells in exponential growth were incubated with 30 μM of PD98059 for 30 min. La^{3+} was added into cultures to obtain a final concentration of 100 μM and the cells were left at 37°C for different time intervals. Then analyses on cell viability (at 24 h, by MTT assay), cell cycle distribution, apoptotic proportion (at 48 h, by FACS), and ERK phosphorylation (at 30 min, by Western blot) were carried out as described above.

Data Statistical Analysis

Data are represented as means ± SEM. Differences between values were tested using the Student's *t*-test, $P < 0.05$ was considered as statistical significant.

RESULTS

Effect of La^{3+} on Proliferation of NIH 3T3 Cells

The effects of La^{3+} on cell numbers, mitochondrial enzyme activity (measured by MTT assays), and DNA synthesis (measured by BrdU incorporation) are shown in Figure 1A–C. The three characters in the process of cell proliferation were shown to increase upon incubation with La^{3+} in a similar concentration-dependent manner, which clearly indicates that La^{3+} could promote NIH 3T3 cell proliferation. As shown in Figure 1D, incubation of 3T3 cells with La^{3+} resulted in a time-dependent cell proliferation within 36 h; but prolonged treatment of cells with La^{3+} led to a turning of the cell viability curve to sharp decline, suggesting change of the action pattern of lanthanide. In addition, addition of 10% FBS completely inhibited the effects of La^{3+} (data not shown).

Effect of La^{3+} on Apoptosis of NIH 3T3 Cells

As shown in Figure 2A, 48 h of incubation of 3T3 cells with 100 μM of La^{3+} resulted in perinuclear chromatin condensation and fragmentation of cell nucleus, which are typical apoptotic characteristics. Agarose gel electrophoresis (Fig. 2B) showed that the cellular DNA of La^{3+}-treated cells exhibited typical apoptotic DNA ladder accompanied with increased electrophoretic mobility of high molecular weight DNA, indicating that both inter-nucleosomal DNA cleavage and high molecular weight DNA degradation had occurred. By contrast, the addition of 10% FBS completely inhibited the DNA fragmentation induced by La^{3+}, and control cells grown in serum-free medium showed only a little but visible DNA fragmentation.

The fate of proliferating cells induced by La^{3+} was traced by pulse BrdU labeling as described in the Materials and Methods. The results were shown in Figure 3, and the statistical analysis of data was in Table I. In the control samples, NIH 3T3 cells grown in medium supplemented with 10% FBS exhibited a high rate of BrdU labeling (~66.3%) and few apoptotic cells (~2.6%) were observed (Fig. 3A). Few of cells grown in serum-free medium were labeled by BrdU (~3.7%) or observed in apoptotic morphology (~13.2%) characterized by chromatin condensation, cell shrinkage, and detachment from the slides, etc. [Saraste and Pulkki, 2000] (Fig. 3B). In contrast, cells incubated with La^{3+} for 36 h gave a high rate of BrdU labeling (~15.2%) (Fig. 3C), and almost all BrdU-labeled cells (~93.0%) exhibited apoptotic morphology after 48 h of incubation with La^{3+} (Fig. 3D), suggesting high correlation between La^{3+}-induced proliferation and apoptosis of 3T3 cells.

Effects of La^{3+} on Cell Cycle and Apoptosis

As shown in Figure 4A, the DNA synthesis (S) phase population of control cells decreased quickly after deprivation of serum, whereas the S phase population of cells incubated with

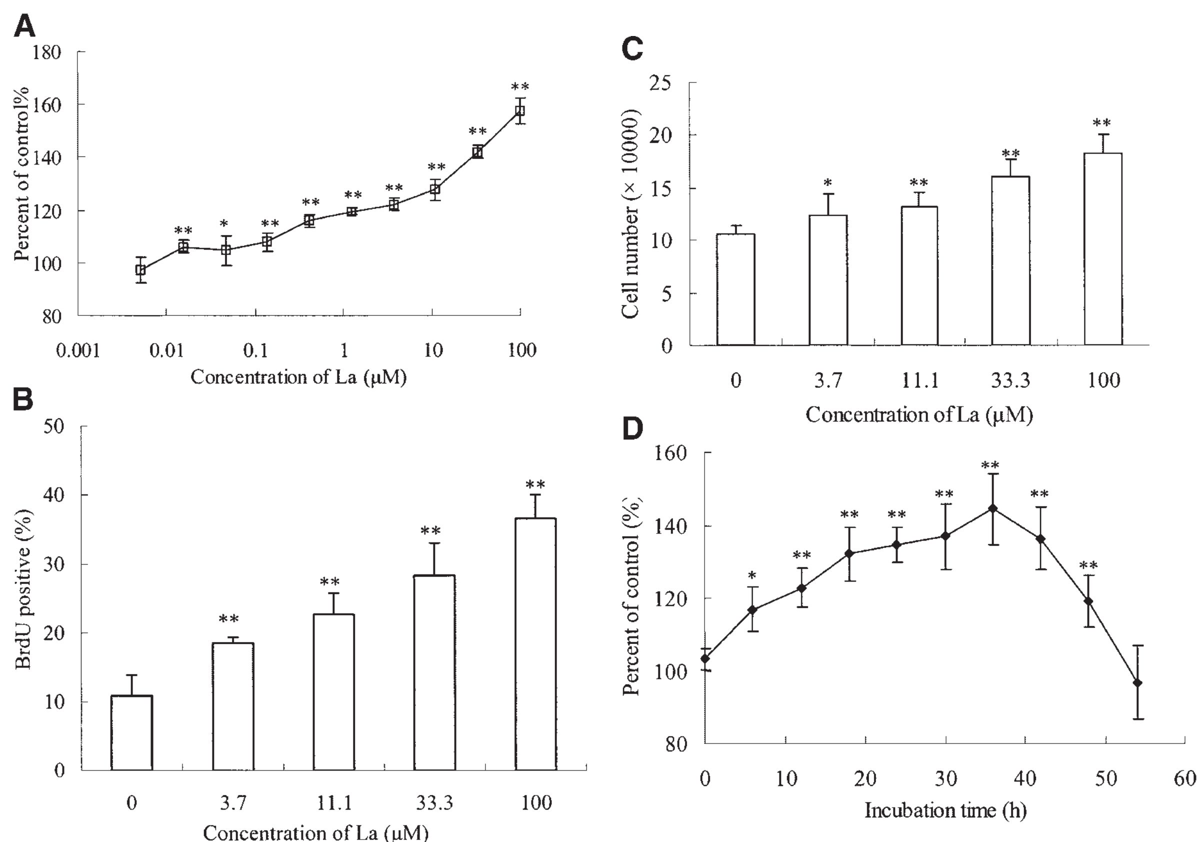

Fig. 1. Effects of La^{3+} on the proliferation of NIH 3T3 cells. Cells in exponential growth were treated with various concentrations of La^{3+} for 24 h, and then analyzed by MTT assay (**A**), BrdU incorporation assay (**B**), and cell counting (**C**). The time-lapse change of cell viability upon incubation with 100 μM of La^{3+} is determined by MTT assay and the result is shown in (**D**). Data shown are the means ± SEM of quadruplicate experiments, $*P < 0.05$, $**P < 0.01$.

La^{3+} remained at the same level within 12 h and kept in a higher level compared with the control samples. The changes of S phase population were mainly compensated by the changes of G0-G1 phase population. In addition, a time-dependent increase of apoptotic population was observed (Fig. 4B). These changes of cell cycle distribution and apoptotic proportion were concentration-dependent. Upon incubating with La^{3+} for 24 h, S phase population increased along with the rising of La^{3+} concentration (Fig. 4C); meanwhile, cell population in the G0-G1 phase decreased. It was also observed that both the apoptotic (subdiploid) population and the S phase population increased in a similar concentration-dependent manner upon incubation of cells with La^{3+} for 48 h (Fig. 4D). For cells incubated with La^{3+} in the presence of 10% FBS, no changes were observed in the population in S phase or the apoptotic state (Table II).

La^{3+} Induces ERK Phosphorylation and Inhibition of ERK Phosphorylation Inhibits Both La^{3+}-Promoted Proliferation and Apoptosis

As ERK cascade is extensively participate in cellular responses to a variety of extracellular stimulations, the involvement of ERK activation in the La^{3+}-induced proliferation and apoptosis is investigated and the results were shown in Figure 5. La^{3+} induced a fast increase of phosphorylated ERK level within 30 min in a La^{3+} concentration-dependent manner (Fig. 5A), while the level of ERK showed no obvious change. La^{3+}-induced phosphorylation of ERK was inhibited upon pretreatment with PD98059 (Fig. 5B). Meanwhile, PD98059 also suppressed La^{3+}-promoted cell proliferation (Fig. 5C). Interestingly, whereas PD98059 itself induced a moderate increase of cell apoptosis, it significantly suppressed both La^{3+}-induced increase of S phase and apoptotic population (Fig. 5D).

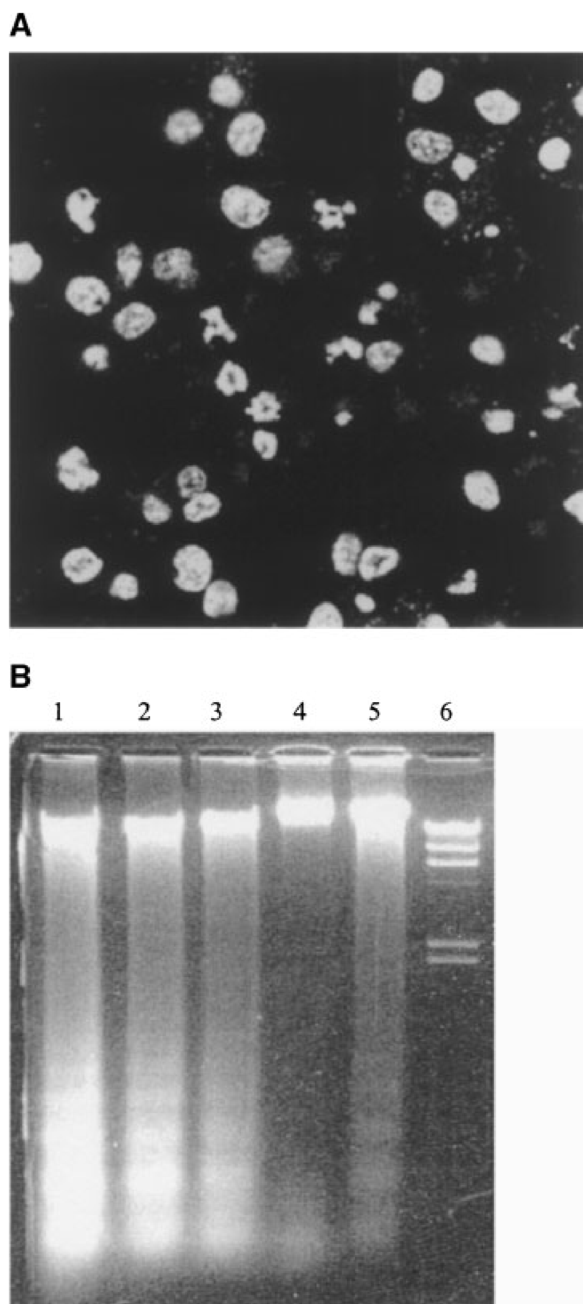

Fig. 2. Effects of La^{3+} on NIH 3T3 cell apoptosis. **A**: Nuclear morphology of cells treated with 100 μM La^{3+} for 48 h. **B**: Agarose gel electrophoresis of cellular DNA upon treatment of cells with La^{3+} for 60 h. **Lane 1–3**: Samples of cells treated with 100, 33.3, 11.1 μM of La^{3+}, respectively; **lane 4**, sample of cells treated with 100 μM La^{3+} in the presence of 10% FBS; **lane 5**, cells grown in serum-free medium; **lane 6**, molecular weight marker. Data shown are the representative of three independent experiments.

DISCUSSION

The proliferative effect of La^{3+} on cells has been reported by several authors [Smith and Smith, 1984; Praeger and Gilchrest, 1989]. On the other hand, it was also reported that La^{3+} could inhibit cell proliferation and promote apoptosis [Yamage and Evans, 1989; Ji et al., 2000; Liu et al., 2003a]. Those contradictory results were obtained with different cells and La^{3+} concentrations. However, there emerged a possible interconnection between La^{3+}-induced proliferation and apoptosis that might be more important for the duality of the effect of La^{3+}. In the present study, the effects of La^{3+} on cell proliferation and apoptosis and their interconnection were investigated employing a murine embryo fibroblast cell line, NIH 3T3 cells.

The proliferative effect of La^{3+} on NIH 3T3 cells was confirmed by various methods, which measure different aspects of cell proliferation, including cell numbers, mitochondrial enzyme activity, and DNA synthesis (Fig. 1A–C). The experimental results all demonstrated that La^{3+} promoted NIH 3T3 cell proliferation in a concentration-dependent manner.

However, prolonged incubation of NIH 3T3 cells with La^{3+} over 36 h resulted in significant loss of cell viability (Fig. 1D). The observed nuclear fragmentation and chromatin condensation (Fig. 2A) of cells incubated with La^{3+} for 48 h suggest that those cells underwent an apoptotic process. This was ascertained by the occurrence of apoptotic degradation of the cellular DNA of cells incubated with La^{3+} for 60 h, shown by agarose gel electrophoresis analysis (Fig. 2B). The temporal change of cell viability suggests that La^{3+}-induced cell proliferation might be rapidly followed by apoptosis.

To explore the possible relationship between La^{3+}-induced proliferation and apoptosis, the proliferating cells were pulse labeled with BrdU and their succeeding fate was monitored as shown in Figure 3 and Table I. The experimental results showed that La^{3+} increased the number of proliferating (BrdU-positive) cells, but almost all La^{3+}-induced proliferating cells were observed to commit apoptosis later. In contrast, fewer cells, which not treated with La^{3+}, were BrdU-positive or underwent apoptosis; and many cells grown in medium supplemented with 10% FBS were BrdU-positive but very few of them underwent apoptosis, indicating that La^{3+}-induced apoptosis was not the result of BrdU incorporation. This complete overlap of apoptotic cells with ever proliferating ones suggested that proliferation promoted by La^{3+} could be intrinsically linked with the later

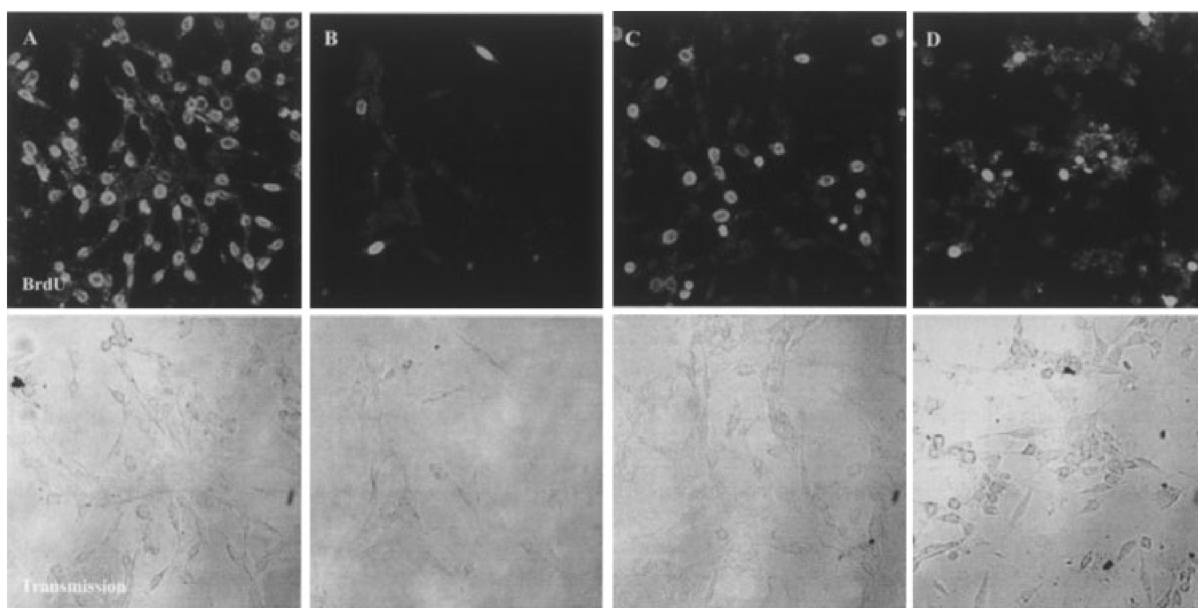

Fig. 3. BrdU tracing of cells upon incubation with 100 μM of La^{3+}. Cells were incubated with 100 μM of La^{3+} for 24 h, pulse-labeled with BrdU, and incubated with fresh media containing the same concentration of La^{3+} for another 12 (**C**) or 24 h (**D**). Control cells were grown in either medium supplemented with 10% FBS (**A**) or serum-free medium (**B**). Data shown are the representative of three independent experiments.

apoptosis. Moreover, the fact that serum-starvation inhibited both the proliferative and proapoptotic effects of La^{3+} also supports the above postulation.

The cell cycle is the innate mechanism by which eukaryotic cells proliferate [Tapon et al., 2001]; it was also proposed that cell cycle machinery was involved in apoptosis. A hypothesized linkage between proliferation and apoptosis is through the direct coupling of cell cycle progression and programmed cell death [Guo and Hay, 1999]. Therefore, the effects of La^{3+} on the cell cycle were analyzed to explore the mechanism of La^{3+}-promoted cell proliferation and the possible relation with La^{3+}-induced proliferation and apoptosis.

Based on the ways La^{3+} affects the cell cycle, it can be deduced that La^{3+} could promote the 3T3 cells to pass through G1/S restriction point, thus promoting more cells to enter into DNA synthesis (S phase). The experimental results supporting this interpretation include:

(i) Rising concentrations of La^{3+} increased the S phase population and decreased the G0-G1 phase population of NIH 3T3 cells (Fig. 4C), which is in good agreement with increased BrdU incorporation (Fig. 1B).

TABLE I. Statistic Analysis of BrdU Tracing of Cells

Sample[a,b]	Percentage of BrdU labeled cell	Percentage of apoptotic cell[c]	Ratio of BPA cell to total BrdU labeled cell[d]
SEM	3.7 ± 0.4	13.2 ± 2.6	Not detected
FBS	$66.3 \pm 1.5^{**}$	$2.6 \pm 0.3^{*}$	0.7 ± 0.2
La	$15.2 \pm 5.7^{**}$	$32.1 \pm 7.4^{**}$	$93.0 \pm 3.8^{**}$

[a]Cells were incubated with 100 μM of La^{3+} for 24 h, pulse-labeled with BrdU, and incubated with fresh media containing the same concentration of La^{3+} for another 24 h (La). Control cells were grown in either medium supplemented with 10% fetal bovine serum (FBS) or serum-free DMEM medium (SFM).
[b]Data are average of two independent experiments. The total cell number counted was 295, 429, and 366 for samples SFM, FBS, and La, respectively. $^{*}P < 0.05$. $^{**}P < 0.01$.
[c]The apoptotic cells were identified by their morphological features, including chromatin condensation, cell shrinkage, detachment from the slides, etc.
[d]The BPA cell (both proliferating and apoptotic-featured cell) refers to the cells that showed apoptotic features as well as labeled by BrdU in the mean time.

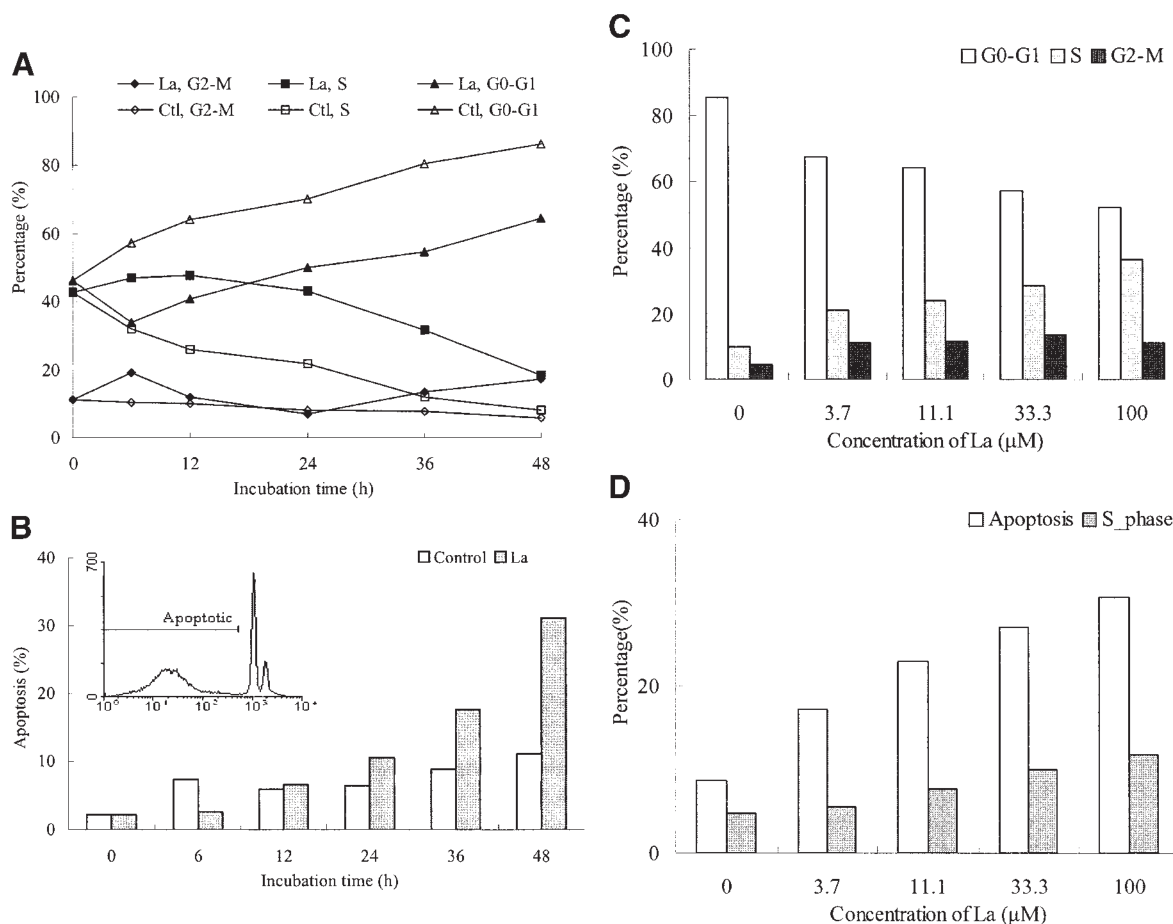

Fig. 4. Effects of La^{3+} on cell cycle distribution and apoptosis of NIH 3T3 cells. **A**: Time-lapse analysis of change in cell population upon incubation with 100 μM of La^{3+}. "Ctl" refers to the control samples and "La" refers to the samples treated with La^{3+}. **B**: Time-lapse analysis of change in the apoptotic population of cells upon incubation with 100 μM of La^{3+}. Insert shows La^{3+}-induced apoptotic (subdiploid) peak revealed by FACS. The control samples in (A) and (B) were grown in serum-free DMEM. **C**: Concentration dependency of cell population upon incubation with 100 μM of La^{3+} for 24 h. **D**: Concentration dependencies of apoptotic and S phase population upon incubation with 100 μM of La^{3+} for 48 h. Data shown are the representative of three independent experiments.

These results indicate that La^{3+} promoted the DNA synthesis activity of NIH 3T3 cells.

(ii) The S phase population of control cells decreased quickly after serum was removed, but addition of La^{3+} maintained the S phase population for at least 12 h (Fig. 4A). It was known that fibroblasts require continuous exposure to growth factors such as serum for continuous cycling and proliferating. Upon deprivation of growth factor, only the cells which have passed the G1/S restriction point continue to cycle until completing mitosis, while the other cells will exit into G0 phase [Jones and Kazlauskas, 2001]. Therefore, the above results suggest that La^{3+} increased cellular DNA synthesis by promoting G1 to S phase transition.

(iii) La^{3+} could induce a fast increase of ERK1/2 phosphorylation in a concentration-dependent manner (Fig. 5A). It has been

TABLE II. Effects of La^{3+} on NIH 3T3 Cells in the Presence of 10% FBS*

Concentration of La^{3+} (μM)	Apoptosis (%)	S phase (%)
0	2.21	33.12
3.7	1.44	30.77
11.1	2.67	33.62
33.3	1.5	29.39
100	3.94	31.81

*Data shown are from one representative experiment of three independent experiments.

known that activation of ERK by phosphorylation is required for fibroblasts to pass the G1/S restriction point [Weber et al., 1997]. ERK activation has also been shown to participate in cellular response to xenobiotics [Kyriakis, 1999]. In addition, inhibition of ERK phosphorylation by PD98059, a specific MEK inhibitor [Hotokezaka et al., 2002], also stopped the La^{3+}-induced increase of S phase population and cell proliferation (Fig. 5B–D).

The effects of La^{3+} on the cell cycle also indicated that La^{3+}-promoted proliferation of NIH 3T3 cells could be correlated with La^{3+}-induced apoptosis through cell cycle machinery. The following experimental results supported this viewpoint:

(i) Fluorescence activated-cell sorting (FACS) analysis of cells incubated with La^{3+} revealed a time-dependent increase of apoptotic (subdiploid) population (Fig. 4B), suggesting that the apoptotic process was initiated along with the beginning of cell proliferation. Upon incubated with La^{3+} for 48 h, the cells exhibited a remarkable apoptotic population, and the apoptotic population was found to increase in parallel with increase of the S phase population (Fig. 4D).

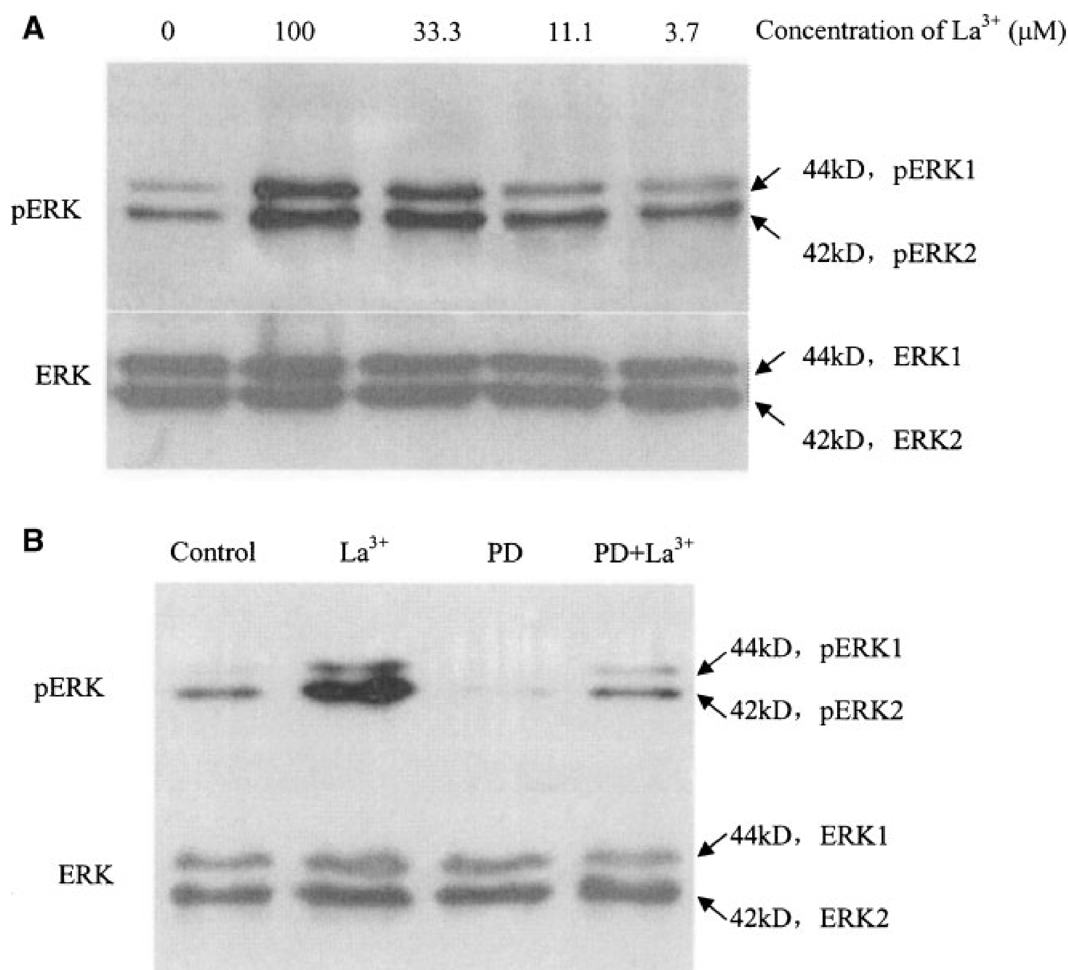

Fig. 5. La^{3+} induces ERK phosphorylation and its role in both La^{3+}-promoted proliferation and apoptosis. **A**: The concentration-dependency of the levels of ERK and pERK upon stimulation of quiescent cells with La^{3+} for 30 min. **B**: The inhibitory effect of PD98059 (30 μM) on La^{3+}-induced ERK phosphorylation. Data shown are the representative of three independent experiments. **C**: The inhibitory effect of PD98059 (30 μM) on La^{3+}-promoted cell proliferation. The proliferative activity was measured by MTT assay after incubated with La^{3+} for 24 h. **D**: The inhibitory effect of PD98059 (30 μM) on La^{3+}-induced increase of apoptotic and S phase population measured by FACS after incubated with La^{3+} for 48 h. Data shown are the means ± SEM of three independent experiments, $*P < 0.05$, $**P < 0.01$.

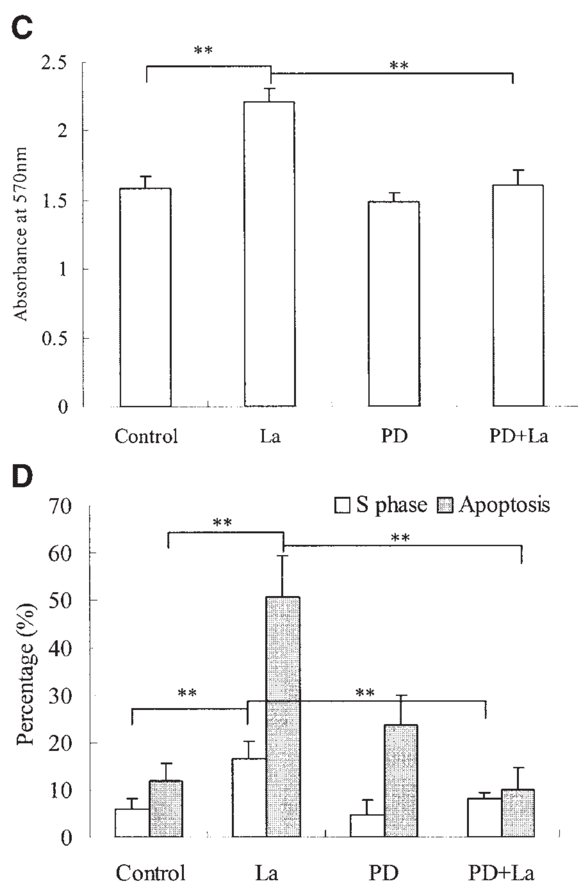

Fig. 5. *(Continued)*

(ii) The time course of cell viability change (Fig. 1D) was in good agreement with changes of S phase and apoptotic cell population (Fig. 4A,B): upon incubation of NIH 3T3 cells with La^{3+}, the S phase population was larger than apoptotic population within 36 h, while the cell viability kept increasing. After this period, apoptotic population increased quickly and surpassed the S phase population; consequently, the cell viability decreased sharply. These results suggest the existence of a balance between apoptosis and proliferation in the observed cell populations; this equilibrium shifts to apoptosis upon prolonged incubation of cells with La^{3+}.

(iii) PD98059 not only inhibited the La^{3+}-induced ERK phosphorylation, but also eliminated the increase of both S phase and apoptotic population of 3T3 cells upon incubating with La^{3+} (Fig. 5D).

Our results suggest that La^{3+}-induced cell apoptosis is due to the inappropriate signaling of cell growth initiated by La^{3+}. This is in consistent with our previous observation that La^{3+} induced apoptosis of hepatic cells via the mitochondrial pathways [Liu et al., 2003a]. Nevertheless, we cannot rule out the possibility of involvement of cell damage in the process of apoptosis by the direct binding of La^{3+} ions to biological macromolecules [Cheng et al., 1999; Lizon and Fritsch, 1999; Franklin, 2001; Komiyama, 2001].

The interconnection for La^{3+}-induced proliferation and apoptosis could provide hints in resolving the problem of contradictory results observed in the effects of La^{3+} on cell growth. From these results, it is found that (i) La^{3+} tended to promote apoptosis in tumor cells, but exhibited proliferative effect on embryo fibroblast cells, and (ii) the concentration of La^{3+} to promote cell apoptosis was normally higher than that for promoting cell proliferation. We speculated that La^{3+} could initiate proliferative as well as apoptotic signals in the same time. However, the differences in the sensitivity to apoptotic or proliferative signals of different cells might result in shifts in the balance between proliferation and apoptosis. In addition, the cell damage caused by direct binding of La^{3+} will increase with the increase of La^{3+} concentration, which should drive the balance toward apoptosis.

The dual effect of La^{3+} on cell proliferation and apoptosis apparently exhibit some similarities to that of some other toxic metals such as cadmium [Misra et al., 2002; Shih et al., 2004], arsenic [Simeonova et al., 2000; Liu et al., 2003b], and nickel [Lisby et al., 1999; Kim et al., 2002] described above. It is worthwhile to note that La^{3+} at high concentration induce cell apoptosis by elevating cellular ROS level [Liu et al., 2003a], which might be a common effect of biology of heavy other metals [Liu and Shi, 2001] especially in the overload state. Also, it might be possible that these metal ions could function by acting as analogs of essential ions such as iron, which play crucial role in regulating cell growth [Laskey et al., 1988]. Nonetheless, it is too early so far to interpret whether other metal ions share some mechanisms similar to that of La^{3+} in inducing cell proliferation or apoptosis.

In summary, the effects of La^{3+} on proliferation and apoptosis of NIH 3T3 cells were

investigated. La^{3+} was shown to promote proliferation by helping the cells to pass the G1/S restriction point and enter S phase. At the same time, incubation of cells with La^{3+} resulted in apoptosis, which was well correlated with cell proliferation process. La^{3+} was also shown to induce phosphorylation of ERK, while the inhibition of the ERK phosphorylation by PD98059 suppressed both La^{3+}-induced proliferation and apoptosis. Based on above results, we postulate that La^{3+}-promoted proliferation of NIH 3T3 cells could be interconnected with the cell apoptosis, possibly through the cell cycle machinery. Our results thus also support the recent hypothesis that proliferation and apoptosis of cell are intrinsically coordinated.

REFERENCES

Anghileri LJ, Crone-Escanye MC, Robert J. 1987. Antitumor activity of gallium and lanthanum: Role of cation-cell membrane interaction. Anticancer Res 7:1205–1207.

Barila B, Cupello A, Robello M. 2001. Modulation by lanthanum ions of gamma-aminobutyric acid (A) receptors of rat cerebellum granule cells in culture: Clues on their subunit composition. Neurosci Lett 298:13–16.

Block BM, Stacey WC, Jones SW. 1998. Surface charge and lanthanum block of calcium current in bullfrog sympathetic neurons. Biophys J 74:2278–2284.

Cheng Y, Liu M, Li R, Wang C, Bai C, Wang K. 1999. Gadolinium induces domain and pore formation of human erythrocyte membrane: An atomic force microscopic study. Biochim Biophys Acta 1421:249–260.

Dai Y, Li J, Yu I, Dai G, Hu A, Yuan L, Wen Z. 2002. Effects of rare earth compounds on growth and apoptosis of leukemic cell lines. In Vitro Cell Dev Biol Anim 38:373–375.

Das T, Sharma A, Talukder G. 1988. Effects of lanthanum in cellular systems. Biol Trace Elem Res 18:201–228.

Dobrynina N, Feofanova M, Gorelov I. 1997. Mixed lanthanide complexes in biology and medicine. J Inorg Biochem 67:168.

Evan GI, Vousden KH. 2001. Proliferation, cell cycle and apoptosis in cancer. Nature 411:342–348.

Evan GI, Brown L, Whyte M, Harrington E. 1995. Apoptosis and the cell cycle. Curr Opin Cell Biol 7:825–834.

Fiskin AM, Melnykovych G, Peterson G. 1980. Transient expression of lanthanum binding sites over surfaces of HeLa cells. Eur J Cell Biol 21:151–159.

Franklin SJ. 2001. Lanthanide-mediated DNA hydrolysis. Curr Opin Chem Biol 5:201–208.

Greene LA, Biswas SC, Liu DX. 2004. Cell cycle molecules and vertebrate neuron death: E2F at the hub. Cell Death Differ 11:49–60.

Guo M, Hay BA. 1999. Cell proliferation and apoptosis. Curr Opin Cell Biol 11:745–752.

He ML, Rambeck WA. 2000. Rare earth elements—A new generation of growth promoters for pigs? Arch Tierernahr 53:323–334.

Hotokezaka H, Sakai E, Kanaoka K, Saito K, Matsuo K, Kitaura H, Yoshida N, Nakayama K. 2002. U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells. J Biol Chem 277:47366–47372.

Ji YJ, Xiao B, Wang ZH, Cui MZ, Lu YY. 2000. The suppression effect of light rare earth elements on proliferation of two cancer cell lines. Biomed Environ Sci 13:287–292.

Jones SM, Kazlauskas A. 2001. Growth factor-dependent signaling and cell cycle progression. FEBS Lett 490:110–116.

Kim K, Lee SH, Seo YR, Perkins SN, Kasprzak KS. 2002. Nickel(II)-induced apoptosis in murine T cell hybridoma cells is associated with increased fas ligand expression. Toxicol Appl Pharmacol 185:41–47.

Komiyama M. 2001. Lanthanide ion-mediated peptide hydrolysis. Met Ions Biol Syst 38:25–41.

Kyriakis JM. 1999. Making the connection: Coupling of stress-activated ERK/MAPK (extracellular-signal-regulated kinase/mitogen-activated protein kinase) core signalling modules to extracellular stimuli and biological responses. Biochem Soc Symp 64:29–48.

Laskey J, Webb I, Schulman HM, Ponka P, Laskey JD, 1988. Evidence that transferrin supports cell proliferation by supplying iron for DNA synthesis. Control of heme synthesis during Friend cell differentiation: Role of iron and transferrin. Exp Cell Res 176:87–95.

Latella L, Sacchi A, Crescenzi M. 2000. Long-term fate of terminally differentiated skeletal muscle cells following E1A-initiated cell cycle reactivation. Cell Death Different 7:145–154.

Lisby S, Hansen LH, Menn T, Baadsgaard O. 1999. Nickel-induced proliferation of both memory and naive T cells in patch test-negative individuals. Clin Exp Immunol 117:217–222.

Liu KJ, Shi X. 2001. In vivo reduction of chromium (VI) and its related free radical generation. Mol Cell Biochem 222:41–47.

Liu HX, Yuan L, Yang X, Wang K. 2003a. La3+, Gd3+, and Yb3+ induced changes in mitochondrial structure, membrane permeability, cytochrome c release and intracellular ROS level. Chemico-Biol Interact 146:27–37.

Liu L, Trimarchi JR, Navarro P, Blasco MA, Keefe DL. 2003b. Oxidative stress contributes to arsenic-induced telomere attrition, chromosome instability, and apoptosis. J Biol Chem 278:31998–32004.

Lizon C, Fritsch P. 1999. Chemical toxicity of some actinides and lanthanides towards alveolar macrophages: An in vitro study. Int J Radiat Biol 75:1459–1471.

Miledi R. 1971. Lanthanum ions abolish the "calcium response" of nerve terminals. Nature 229:410–411.

Misra UK, Gawdi G, Akabani G, Pizzo SV, Fujiwara Y, Watanabe S, Kaji T, Kastelan M, Gerencer M, Kastelan A, Gamulin S. 2002. Cadmium-induced DNA synthesis and cell proliferation in macrophages: The role of intracellular calcium and signal transduction mechanisms. Cell Signal 14:327–340.

Packham G, Porter CW, Cleveland JL. 1996. c-Myc induces apoptosis and cell cycle progression by separable, yet overlapping, pathways. Oncogene 13:461–469.

Palmer RJ, Butenhoff JL, Stevens JB. 1987. Cytotoxicity of the rare earth metals cerium, lanthanum, and neodymium in vitro: Comparisons with cadmium in a pulmon-

ary macrophage primary culture system. Environ Res 43:142–156.

Praeger FC, Gilchrest BA. 1989. Calcium, lanthanum, pyrophosphate, and hydroxyapatite: A comparative study in fibroblast mitogenicity. Proc Soc Exp Biol Med 190:28–34.

Qiang T, Xiao-Rong W, Li-Qing T, Le Mei D. 1994. Bioaccumulation of the rare earth elements lanthanum, gadolinium and yttrium in carp (Cyprinus carpio). Environ Pollut 85:345–350.

Saraste A, Pulkki K. 2000. Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Res 45:528–537.

Schulte-Hermann R, Bursch W, Marian B, Grasl-Kraupp B. 1999. Active cell death (apoptosis) and cellular proliferation as indicators of exposure to carcinogens. IARC Sci Publ 146:273–285.

Shih CM, Ko WC, Wu JS, Wei YH, Wang LF, Chang EE, Lo TY, Cheng HH, Chen CT. 2004. Mediating of caspase-independent apoptosis by cadmium through the mitochondria-ROS pathway in MRC-5 fibroblasts. J Cell Biochem 91:384–397.

Simeonova PP, Wang S, Toriuma W, Kommineni V, Matheson J, Unimye N, Kayama F, Harki D, Ding M, Vallyathan V, Luster MI. 2000. Arsenic mediates cell proliferation and gene expression in the bladder epithelium: Association with activating protein-1 transactivation. Cancer Res 60:3445–3453.

Smith JB, Smith L. 1984. Initiation of DNA synthesis in quiescent Swiss 3T3 and 3T6 cells by lanthanum. Biosci Rep 4:777–782.

Tapon N, Moberg KH, Hariharan IK. 2001. The coupling of cell growth to the cell cycle. Curr Opin Cell Biol 13:731–737.

Vermeulen K, Berneman ZN, Van Bockstaele DR. 2003. Cell cycle and apoptosis. Cell Prolif 36:165–175.

Walker PR, Leblanc J, Smith B, Pandey S, Sikorska M. 1999. Detection of DNA fragmentation and endonucleases in apoptosis. Methods 17:329–338.

Wang K, Li RC, Cheng Y, Zhu B. 1999. Lanthanides—The future drugs? Coordinat Chemist Rev 190–192:297–308.

Weber JD, Raben DM, Phillips PJ, Baldassare JJ. 1997. Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. Biochem J 326:61–68.

Yamage M, Evans CH. 1989. Suppression of mitogen- and antigen-induced lymphocyte proliferation by lanthanides. Experientia 45:1129–1131.

Zatterstrom UK, Johansson M, Kallen A, Baldetorp B, Oredsson S, Wennerberg J, Killander D. 1992. Comparison of BrdUrd and [3H]TdR incorporation to estimate cell proliferation, cell loss, and potential doubling time in tumor xenografts. Cytometry 13:872–879.