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Human lactoferrin exerts bi-directional actions on PC12 cell survival via ERK1/2 pathway

Tsung-Yao Lin [a,1], Shih-Hwa Chiou [b,c,1], Mei Chen [b], Cheng-Deng Kuo [a,d,*]

[a] Laboratory of Biophysics, Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan
[b] Laboratory of Stem Cell Research, Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan
[c] Institute of Clinical Medicine, National Yang-Ming University School of Medicine, Taipei, Taiwan
[d] Institute of Emergency and Critical Care Medicine, National Yang-Ming University School of Medicine, Taipei, Taiwan

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Abstract

Human lactoferrin (hLF) is a member of the transferrin family and is found in most body fluids of human. Recent study showed that hLF played some roles in the regulation of cell growth. However, the biological function of hLF in the central nervous system and neuronal cells is still unclear. The MTT was used to assay cell viability, ELISA tests were used to assay caspase activities, and TUNEL staining was used to test the cytotoxicity of hLF to the cells. Our result showed that 700 μg/ml hLF significantly reduced the cell viability and increased the caspase 3 and 8 activities in PC12 neuronal cells. TUNEL staining further showed that 700 μg/ml hLF was cytotoxic to the PC12 through apoptosis-mediated pathway. In addition, 700 μg/ml hLF significantly decreased the protein expressions of phosphorylated extracellular-signal-regulated kinase 1/2 (ERK1/2) and Bcl-2 in PC12 cells, whereas 50 μg/ml hLF significantly increased the phosphorylation of ERK1/2 which could be specifically inhibited by PD98059. Furthermore, 50 μg/ml hLF could not only up-regulate the Bcl-2 expression but also protect PC12 cells from FasL-induced apoptosis. In conclusion, hLF plays a crucial role in the regulation of apoptosis and anti-apoptosis in PC12 neuronal cells via ERK1/2 phosphorylation pathway.

Keywords: Human lactoferrin; Apoptosis; ERK1/2; Bcl-2

Human lactoferrin (hLF) is an 80 kDa iron-binding glycoprotein that belongs to the transferrin family of proteins [1]. The hLF is present in most biological fluids of mammals, such as human milk, secondary granules of neutrophils, and plasma. The hLF has two binding sites for ferric ions and may reduce the conversion of hydrogen peroxide to hydroxyl radical via Fenton reaction [1]. Indeed, hLF plays a central role in the immune system. Physiological concentrations of lactoferrin can inhibit the growth of a wide spectrum of infectious organisms, including bacteria, viruses, parasites, and fungi, and also act as an antioxidant lipid protector [2].

PC12 cell line, derived from rat pheochromocytoma cells, is one of the most widely used neuronal cell lines for researches on the mechanisms of neuronal differentiation, neuronal repair, neuroprotection, and neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), etc., [3,4]. PC12 cells constitutively express the Fas/FasL system and Fas (CD95) receptor on cell membrane, providing a physiological model for the study of Fas-dependent apoptosis [5].

Recent study has showed that lactoferrin is a potent regulator of bone cell activity and can increase bone formation in vivo [6]. In addition, Kawamata et al. [7] reported that hLF expression was increased with age and is strongly associated with diffuse and consolidated amyloid deposits and extracellular neurofibrillary tangles in affected AD tissue. However, the physiological function and biological

* Corresponding author. Fax: +886 2 2871 0773.
E-mail address: cdkuo@vghtpe.gov.tw (C.-D. Kuo).
[1] These authors contributed equally to this work.

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role of hLF in the neuron cells are still unclear. Therefore, the aim of this study was to investigate the biological effect and cell viability effect of hLF on PC12 cells, and the associated mechanism.

Experimental procedures

Cell culture. PC12 cells derived from pheochromocytoma cells were obtained from Food Industry Research and Development Institute, Hsinchu, Taiwan, and were grown in RPMI-1640 culture medium (Gibco-BRL, New York, USA) supplemented with 10% horse serum (Gibco-BRL), 5% fetal bovine serum (Gibco-BRL), and 100 U/ml penicillin/streptomycin (Gibco-BRL) at 37 °C humidified environment containing 10% CO_2. When the cells became 70–80% confluent, they were harvested by detaching them from the dish surfaces by using a flow of medium from a pipette and scattering the cells through a 22-gauge needle.

Cell viability assay. The PC12 cells were seeded on 24-well plates at a density of 2×10^4 cells/well in RPMI-1640 medium, and methyl thiazol tetrazolium assay (MTT assay; Sigma–Aldrich, St. Louis, MO, USA) was performed for cell viability. PC12 cells were incubated with 0.25 mg/ml MTT for 4 h at 37 °C and the reaction was terminated by the addition of 100% iso-propanol. The amount of MTT fromazon product was determined by using a microplate reader and the absorbance was measured at 560 nm (SpectraMax 250, Molecular Devices, Sunnyvale, CA, USA).

ELISA. The activities of caspase 8 and 3 were determined by ELISA kit (Medical and Biological Laboratories, Nagoya, Japan) and quantified by reading at A_{490nm} (MRX; Dynatech Laboratories, Chantilly, VA). Each individual sample was analyzed in triplicate.

Immunoblot analysis. After the treatment with hLF, the PC12 cell lysates were collected and the concentration of protein was determined by using Protein Assay kit (Bio-Rad, Hercules, CA, USA). Cell extracts with sample buffer were placed in boiling water for 5 min and then separated by 10% SDS–PAGE gel. After electrophoresis, the gel was transferred onto a PVDF membrane for immunoblotting. The membrane was blocked by incubation in non-fat milk at room temperature for 2 h, incubated with anti-phospho-ERK1/2 (p185/187) antibody (BioSource International, Camarillo, CA, USA), anti-ERK1/2 and anti-Bcl-2 antibody (Upstate Biotechnology, Waltham, MA, USA), and anti-β-actin (Chemicon International, Temecula, CA, USA) for 2 h at room temperature, washed for five times by Tris-buffered saline Tween 20 (TBST), and then incubated further at room temperature with horseradish peroxidase-conjugated secondary antibody for 2 h. The membrane was washed six times by TBST and specific bands were made visible by chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

TUNEL assay. Apoptotic cells in tissue sections were identified by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method (In Situ Cell Death Detection Kit, POD, Roche Boehringer–Mannheim, IN, USA) as described previously [8]. Briefly, the cells with coverslips were washed with 1× PBS, fixed with 4% of paraformaldehyde for 10 min, permeabilized with 0.1% of Triton X-100 for 5 min, and incubated with the TUNEL reagent provided for 1 h. Chromogenic development was then applied with 3-amino-9-ethylcarbazole and slides were counterstained with H&E stain. Positive staining was identified under the light microscope as red granules.

Statistical analysis. Statistical analysis was performed by using Mann–Whitney rank sum test (SigmaStat, SPSS, Chicago, IL, USA). The results were reported as means ± SD. A value of $P < 0.05$ was considered statistically significant.

Results

hLF modulated PC12 cell viability

The cell viability of PC12 cells was analyzed by using MTT assay. The PC12 cells were exposed to predetermined concentrations of hLF for 24 and 48 h, respectively. As shown in Fig. 1A, when the PC12 cells were treated with hLF at different concentrations (0, 10, 50, 200, 400, and 700 μg/ml) for 24 h, the cell viability was not significantly affected if the concentration of hLF was lower than 200 μg/ml (Fig. 1A). When the concentration of hLF was increased to 400 or 700 μg/ml, the viability of PC12 cells was reduced by about 10% in 24 h. After exposure to hLF for 48 h, the viability of PC12 cells was reduced by 20–30% when the concentration of hLF was 200 μg/ml or higher, and by about 35% when the concentration of hLF was 700 μg/ml (Fig. 1B).

Prior to the observation of the effects of hLF on the morphological changes by microscope, the PC12 cells were treated with hLF for 24 and 48 h. After 24 h, the PC12 cells treated by 50 μg/ml hLF showed no changes in morphology but the number of PC12 cells were increased (Fig. 1C, panel a). The cell number of PC12 cells did not rise when treated with 700 μg/ml hLF for 24 h (Fig. 1C, panel c). After 48 h, PC12 cells treated with 50 μg/ml hLF proliferate obviously when compared with the seeding condition (Fig. 1C, panel b). PC12 cells treated with 700 μg/ml hLF for 48 h did not proliferate and the PC12 cells become cluster form and detach from culture dish (Fig. 1C, panel d). The growth rate of PC12 cells incubated with 50 μg/ml hLF for 48 h slowed down slightly when compared with the control group. The PC12 cells treated with 700 μg/ml hLF for 48 h stopped to proliferate (Fig. 1D).

hLF-induced apoptosis in PC12 cells

To further evaluate the apoptotic activity of PC12 cells in the different concentrations of hLF, the activities of caspases 8 and 3 were examined. PC12 cells were treated with 0, 10, 50, 200, 400, and 700 μg/ml hLF for 24 and 48 h. Then the cell lysates of hLF-treated PC12 cells were collected. As shown in Fig. 2A, when hLF was added to the medium of PC12 cells, the caspase 8 activity was clearly induced (compare black bar to white bar) by 200, 400, and 700 μg/ml hLF but not 10 and 50 μg/ml hLF (Fig. 2A). Consistent with the results for caspase 8, 200, 400, and 700 μg/ml hLF-treated PC12 cells also significantly increased caspase 3 activity but 10 and 50 μg/ml hLF did not (Fig. 2B). Moreover, the results of TUNEL staining further confirmed that the 200, 400, and 700 μg/ml hLF caused the cytotoxicity of PC12 through apoptosis-mediated pathway (Fig. 2C). In contrast, the no obvious apoptotic effects were detected by TUNEL staining in 10 μg/ml and 50 μg/ml hLF-treated PC12 cells (Fig. 2C). These results were consistently observed when caspase 3 and 8 were assayed.

Fifty micrograms per milliliters of hLF protected PC12 cells from FasL-induced apoptosis

To investigate whether different concentration hLF could act against or facilitate the Fas-dependent

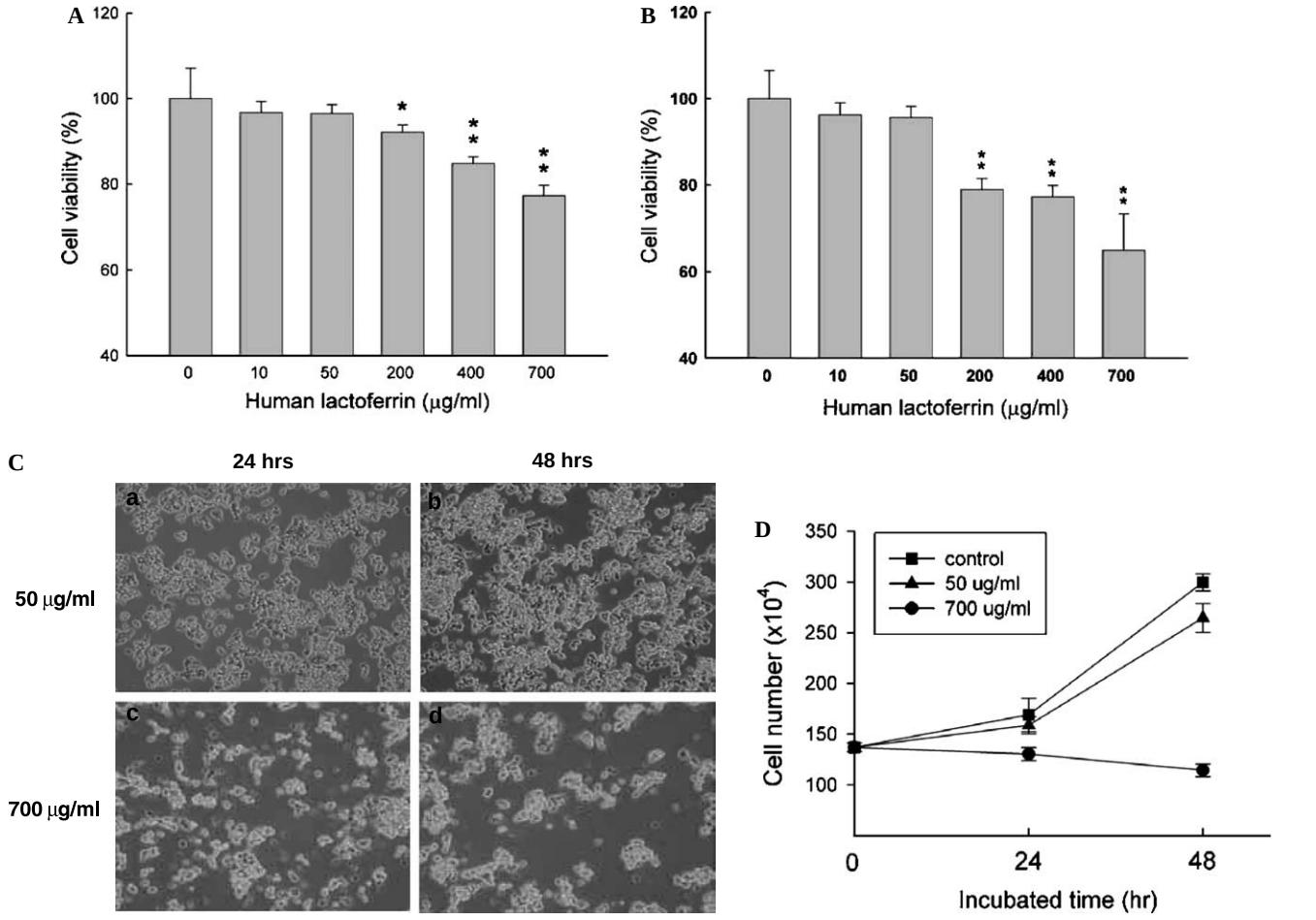

Fig. 1. Effect of different concentration hLF on the viability and growth rate of PC12 cells. The PC12 cells were treated with different concentration hLF for 24 h (A) and 48 h (B). Data (means ± SD of six separate experiments) are expressed as percentages of the control value (no hLF). $*P < 0.05$, $**P < 0.001$, as compared to the control. (C) Effect of hLF on the morphology of PC12 cells. The PC12 cells were treated with 50 μg/ml (panels a and b) and 700 μg/ml (panels c and d) hLF for 24 h (panels a and c) and 48 h (panels b and d). After incubation, the morphology of PC12 cells was examined under phase-contrast microscope of 100× power. (D) Growth curve of PC12 cells. 1.4×10^6 PC12 cells were plated on 6 cm culture dishes with and without hLF. After treating with 50 and 700 μg/ml hLF for 24 and 48 h, the number of PC12 cells was counted by hemocytometer, three counts for each point.

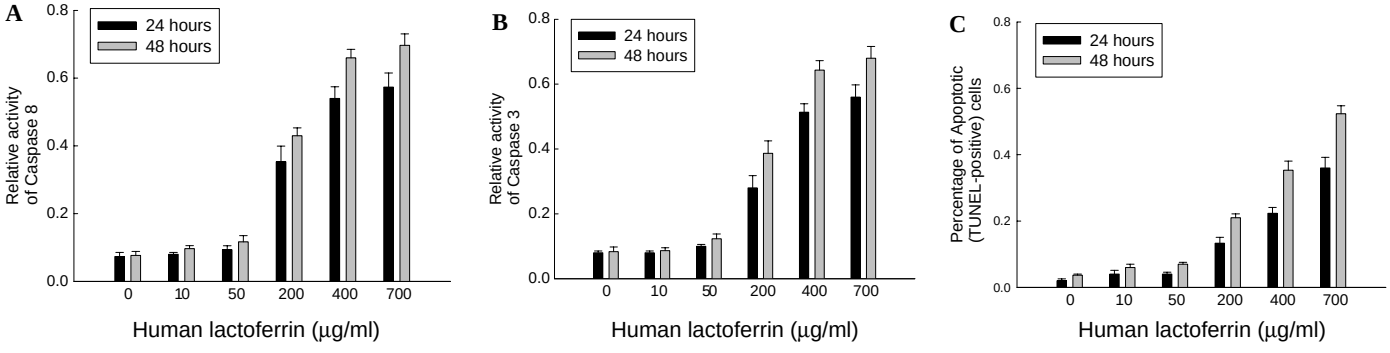

Fig. 2. High concentration hLF up-regulated the activity of caspases 3 and 8 in PC12 cells. The apoptotic activity of PC12 cells due to different concentration hLF was evaluated by using caspase 3 and 8 ELISA and TUNEL assay. The activities of caspase 8 (A) and caspase 3 (B) were determined in PC12 cells treated with 0, 10, 50, 200, 400, and 700 μg/ml hLF for 24 and 48 h. (C) By using TUNEL assay, the apoptotic activity of PC12 cells due to 0, 10, 50, 200, 400, and 700 μg/ml hLF was evaluated. Data shown here are means ± SD of three experiments.

apoptosis in PC12 cells, the apoptosis induced by 200 ng/ml recombinant protein of FasL (Upstate Biotechnology, NY, USA) and blocking assay using 500 ng/ml anti-Fas mAB (clone ZB4, MBL International, Watertown, MA, USA) for specifically blocking Fas-dependent apoptosis were conducted by culturing PC12 cells with 0, 50, or

700 μg/ml hLF for 48 h. By using caspase 3 and 8 ELSIA test, we found that 0 (control) and 700 μg/ml hLF-treated groups in which PC12 cells were exposed to FasL recombinant protein could increase the apoptotic activities as compared to those exposed to mock (Figs. 3A and B). The functional blocking of anti-Fas mAB further prevented the FasL-inducing apoptosis in 0 (control) and 700 μg/ml hLF-treated PC12 cell groups. Interestingly, whether added anti-Fas mAB was blocked or not, we found that the apoptotic activities of caspases 3 and 8 induced by FasL protein were inhibited in 50 μg/ml hLF-treated PC12 cells. Consistent with the caspase 3 and 8 data, the results of TUNEL assay further confirmed that 50 μg/ml hLF-treated group could protect FasL-induced apoptosis in PC12 cells (Fig. 3C, F, and G), but not in 0 (Fig. 3C–E) and 700 μg/ml hLF-treated PC12 cell groups (Fig. 3C, H, and I).

50 μg/ml hLF-activated phosphorylation of ERK1/2 in PC12 cells

To further determine the dose effects of hLF on the activation of ERK1/2, the PC12 cell lysates treated by the different hLF concentrations for 48 h were collected and estimated by

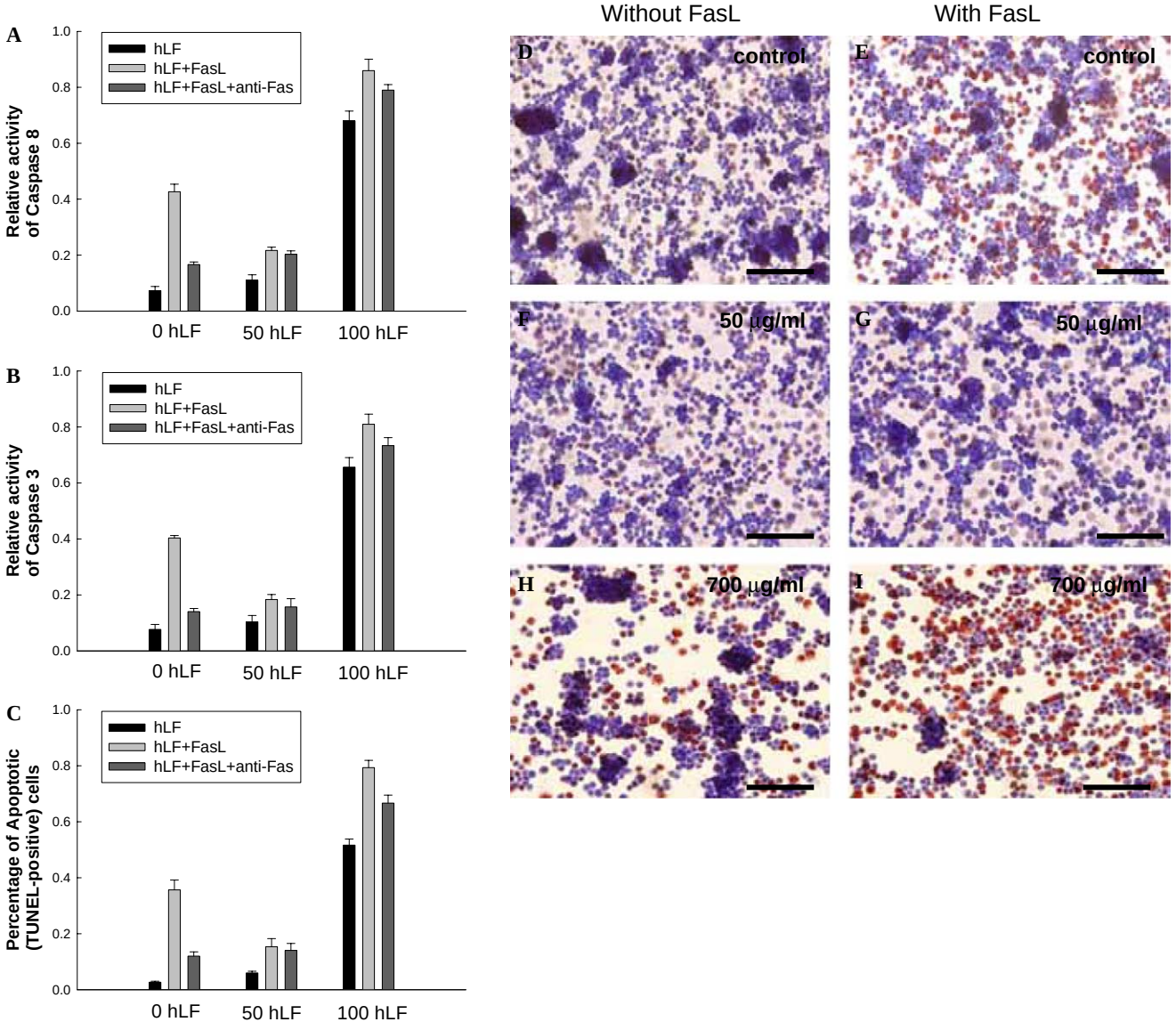

Fig. 3. hLF protected PC12 cells from FasL-induced apoptosis. FasL-induced apoptotic activity in hLF-treated PC12 cells was detected by using caspase 3 and 8 ELISA and TUNEL assays. The activity of FasL-induced apoptosis in PC12 cells due to 0, 50, and 700 μg/ml hLF was determined by caspase 8 (A) and caspase 3 (B) ELISA. The apoptotic activity of PC12 cells due to 0, 50, and 700 μg/ml hLF was evaluated by TUNEL assay (C). The activity of FasL-induced apoptosis in control (E) and 700 μg/ml hLF-treated (I) PC12 cells was significantly elevated, but not in 50 μg/ml hLF-treated PC12 cells (G). (D, F, and H) The PC12 cells treated with 0, 50, and 700 μg/ml hLF without adding the recombinant FasL protein. Blocking assay was conducted by the anti-Fas mAB (500 ng/ml; clone ZB4; MBL) in 0, 50, and 700 μg/ml hLF-treated PC12 cells (A–C) to further examine whether observed apoptosis is due to Fas–FasL interaction. Data shown here are means ± SD of three experiments. Bar = 100 μm.

immunoblotting assay. The phosphorylated protein of ERK1/2 in 50 μg/ml hLF-treated PC12 cells was significantly higher than those of control and 10 μg/ml hLF-treated PC12 cells (Fig. 4, upper lane in panels A and B). However, incubation of PC12 cells with higher than 200 μg/ml hLF led to reduced phosphorylation of ERK1/2. When the concentration of hLF reached 700 μg/ml, the phosphorylation level of ERK1/2 became less than that of the control (Figs. 4A and B). Moreover, the expressions of pERK1/2 in PC12 cells treated by 0, 10, 50, and even 700 μg/ml hLF were significantly decreased and blocked by ERK1/2-specific inhibitor PD980059 (Fig. 4, upper lane in panel B). In contrast, the levels of ERK1/2 and β-actin remained unchanged with or without PD980059 in 0, 10, 50, and 700 μg/ml hLF-treated PC12 cells (Fig. 4, lower two lanes in panel B).

50 μg/ml hLF up-regulated protein expression of Bcl-2 in PC12 cells

Treatment of PC12 cells with 50 and 700 μg/ml hLF affected the levels of Bcl-2 protein. As shown in Fig. 5A, low dose hLF (50 μg/ml) enhanced the level of Bcl-2 in PC12 cells after incubation for 48 h. However, treating with high dose hLF (700 μg/ml) for 48 h decreased the expression of Bcl-2 (Fig. 5, upper lane in panel A). In addition, 50 μg/ml and 700μg/ml hLF-treated PC12 cells for 48 h had no obvious change of Bax protein (Fig. 5, middle lane in panel A). The expressions of Bcl-2 in 0, 50 μg/ml hLF were also specifically decreased by PD98059 (Fig. 5, upper lane in panel A). Moreover, by using dual-immunofluorescent assay, the protein expression of Bcl-2 (cytosol, red color, Fig. 5B) was detected in the same PC12 cell in which the protein of phosphorylated ERK1/2 (nuclei, green color, Fig. 5B) was translocated in the cell nuclei.

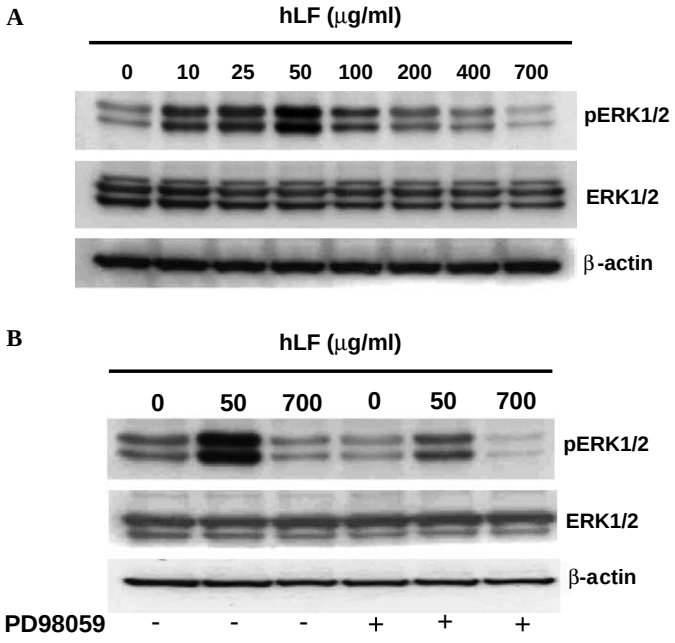

Fig. 4. Phosphorylation levels of ERK1/2 in PC12 cells treated by different concentration hLF. Low concentration hLF could increase the activation of ERK1/2. (A) PC12 cells were treated with different concentration hLF for 48 h and harvested to examine the dose effect of hLF on the phosphorylation level of ERK1/2. Un-phosphorylated forms of ERK1/2 and β-actin were measured as phosphorylated type. (B) The effect of ERK inhibitor PD98059 and hLF on the phosphorylation level of ERK1/2 in PC12 cells. PC12 cells were treated by 50 and 700 μg/ml hLF with or without 50 μM PD98059 for 48 h. Cell lysates were prepared and immunoblotted to measure the phosphorylation levels of ERK1/2 and β-actin. Un-phosphorylated forms of ERK1/2 were measured as phosphorylated type.

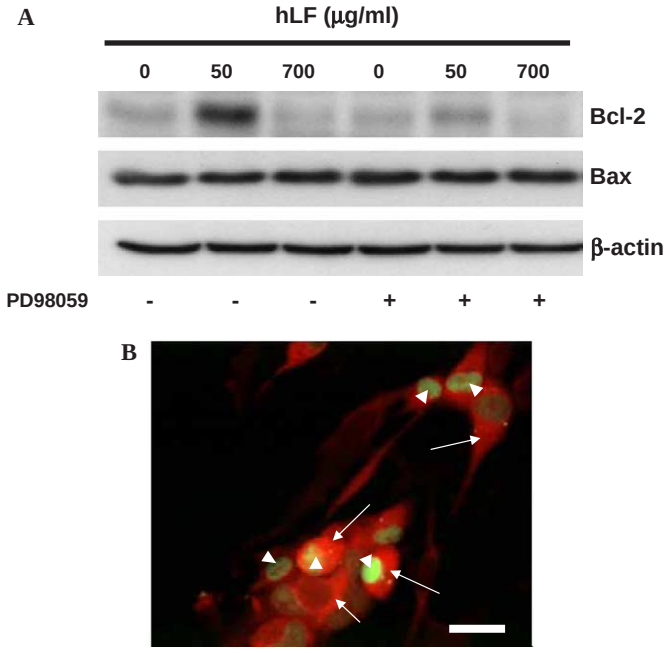

Fig. 5. The protein expression of Bcl-2 in the different concentration hLF-treated PC12 cells. The expression of Bcl-2 could be increased by low concentration hLF. (A) PC12 cells were treated by 0, 50, and 700 μg/ml hLF with or without 50 μM PD98059 for 48 h. Western blot analysis was then performed to assess the levels of Bcl-2, Bax, and β-actin. (B) The protein expression of Bcl-2 (cytosol; red color; arrows) and phosphorylated ERK (nuclei; green color; arrowheads) was both detected in the same cells treated with 50 μg/ml hLF under immunofluorescent microscope (FV300, Olympus). Bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Discussion

hLF is constitutively expressed in the milk secreted by the mammary gland and also in the blood derived from the neutrophils. In general, the physiological concentrations of hLF can be found in a wide range of body fluids, such as milk (up to 7 mg/ml), tear fluid (2.2 mg/ml), seminal plasma (0.4–1.9 mg/ml), synovial fluid (10–80 mg/ml), and plasma serum [9]. Naot et al. [6] demonstrated that 1000 μg/ml hLF stimulated the in vitro proliferation and differentiation of osteoblast-like cells. In contrast, we found that 50 μg/ml hLF could maintain the cell viability of PC12 neuronal cells, but higher than 200 μg/ml concentration of hLF would decrease cell viability of PC12 neuronal cells. Furthermore, severe apoptotic effects in PC12 neuronal

cells were noted when hLF concentration was higher than 700 μg/ml. These findings suggested that the concentrations of hLF could exert distinct effects on different types of organ-specific cells.

Apoptosis plays a key role in neuronal development, and the defects in apoptosis may cause various neurodegenerative disorders [10]. The elegant study conducted by Xia et al. [11] demonstrated that the dynamic balance between growth factor-activated EKR1/2 and stress-activated JNK-p38 pathways may be important in determining whether a cell survives or undergoes apoptosis. Indeed, ERK1/2 is an important regulator of neuronal function. Previous studies had revealed that activated ERK1/2 is essential for synaptic plasticity, learning, and memory [12]. ERK1/2 protein can be activated by neuronal growth factor (NGF) and can protect the neuron cells from apoptosis [13]. Recent studies showed that a cell-survival program involving phospho-activation of MEK1/2 and ERK1/2 in hypoxic neurons might help regulate neuronal fate following hypoxic-ischemic injury [14]. Martin et al. [4] further revealed that ERK1/2 activity in neuron cells might also control both synaptic plasticity and neuronal survival. In our study, we found that 10, 25, and 50 μg/ml hLF could maintain the cell viabilities and up-regulate the activation of ERK1/2 in hLF-treated PC12 cells dose-dependently. Importantly, by using blocking assay we further found that PD98059 not only specifically decreased the ERK1/2 activation but also declined the protein expression of Bcl-2. On the contrary, high concentration of 200, 400, and 700 μg/ml hLF could significantly decrease cell viabilities and down-regulate the ERK1/2 phosphorylation. In accordance with Xia et al. [11] and Le-Niculescu et al. [15], our results suggested that the neuronal survival signaling pathway involved in PC12 cells is through the activation of ERK1/2 but not p38 and/or JNK pathway. Moreover, our data implicated that the different dose effects of hLF in the regulation of signaling ERK1/2 pathway also play a crucial role in neuronal cell survival.

Bcl-2, an anti-apoptotic gene, has recently been identified as a neuronal cell death repressor [16] and plays an important role in the protection of neural cell death due to chemical damage and hypoxia [17]. Previous studies revealed that inhibition of ERK1/2 activities causes a downregulation of anti-apoptotic homologues such as Bcl-2, Mcl-1, and Bcl-XL, and that activation of the pathway functions to protect cells from apoptosis. Indeed, Bcl-2 is also a downstream protein of ERK1/2 and ERK1/2 as the responsible kinase for the phosphorylation of Bcl-2 [18]. A recent study further showed that the activation of ERK1/2 could provide a protective effect against gastric epithelial cell apoptosis through maintenance of Bcl-2 gene expression [19]. In line with these studies, our data further showed that hLF (50 μg/ml) improved the expression of Bcl-2 in PC12 cells through ERK1/2 phosphorylation pathway. Moreover, the high concentration (beyond 200 μg/ml) hLF not only significantly down-regulated the ERK1/2 phosphorylation but also dramatically decreased the Bcl-2 expression (Figs. 4A and 5A). The results of the caspase 3 and 8 and TUNEL assays further confirmed that 50 μg/ml hFL-treated group could protect FasL-induced apoptosis in PC12 cells, but not in 700 μg/ml. Taken together, our findings suggested that hLF (50 μg/ml) may provide Bcl-2-mediated anti-apoptotic effect and act against FasL-induced apoptosis in PC12 cells via ERK phosphorylation pathway.

In conclusion, the concentration of hLF plays a critical role in the regulation of apoptosis and anti-apoptosis in the neuronal cells through ERK-Bcl-2 pathway. A high level (700 μg/ml) of hLF is cytotoxic to the PC12 cells through the apoptosis-mediated pathway, whereas a low level (50 μg/ml) of hLF can not only up-regulate the protein expression of Bcl-2 in PC12-treated cells but also protect FasL-induced apoptosis in hLF-treated PC12 cells. Further studies are needed to explore the details of the mechanism of Bcl-2 posttranscriptional modulation of neuroprotection and neuroplasticity by which the ERK1/2 pathway affects the treatment effect of hLF in the CNS.

Acknowledgments

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