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BOVINE LACTOFERRIN PROTECTS RSC96 SCHWANN CELLS FROM TUMOR NECROSIS FACTOR-α–INDUCED GROWTH ARREST VIA EXTRACELLULAR-SIGNAL-REGULATED KINASE 1/2

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Abstract—Bovine lactoferrin (bLF) is a member of the transferrin protein family that is abundant in bovine milk. Recent studies have shown that bLF plays roles in the regulation of cell growth. However, the biological function of bLF in the peripheral nervous system is still unclear. In this study, the immortalized rat Schwann cells (RSC96) were exposed to bLF at concentrations ranging from 10 to 800 μg/ml for 48 h and compared with control group. The bromodeoxyuridine BrdU cell viability assay was used to examine the effect of bLF on cell viability of RSC96 Schwann cells. Cell-counting test was used to assay the growth rate of RSC96 cells after exposure to bLF, and immunoblot analysis was used to test the signaling pathway controlled by bLF in the RSC96 cells. It was found that the viability of the RSC96 cells was increased by more than 25% when treated with 50 μg/ml bLF and the cell number of RSC96 cells was increased by more than threefold when treated with 800 μg/ml bLF. Our results showed that bLF could significantly improve viability and number of RSC96 Schwann cells. Also, bLF could significantly increase the phosphorylation state of extracellular-signal regulated kinase 1/2 (ERK1/2) that could be specifically inhibited by PD98059. Furthermore, bLF could not only protect RSC96 cells from tumor necrosis factor-α (TNF-α) –induced growth arrest but could also restore proliferation rate in TNF-α-treated RSC96 cells. In conclusion, bLF plays a crucial role in the proliferation of RSC96 Schwann cells and the protection of RSC96 Schwann cells from TNF-α-induced growth arrest via ERK1/2 protein. **© 2008 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: bovine lactoferrin, Schwann cell, TNF-α, ERK1/2, cell proliferation, neuroprotection.

Bovine lactoferrin (bLF) is an 80 kDa iron-binding glycoprotein that belongs to the transferrin family of proteins (Lindmark-Mansson and Akesson, 2000). In bovine milk, the concentration of bLF is present within the ranges of 0.02–0.2 mg/ml (Tsuda et al., 2000). Åbrink et al. (2000) have shown that the average physiological concentrations of lactoferrin in humans (hLF) are 1.0 μg/ml in plasma and 2.8 μg/ml in serum, respectively, and the highest levels of hLF in humans that can be detected are 51 μg/ml in saliva and 2.2 mg/ml in tears. Several functions of bLF have been demonstrated, including iron-adsorption, anti-microbial effects, stimulation of the immune response, and animal cell growth promotion (Arnold et al., 1980; Azuma et al., 1989). Recent studies have showed that hLF is a potent regulator of bone cell activity and can increase bone formation *in vivo* (Naot et al., 2005). Thus, hLF is a multifunctional protein with potential use as a therapeutic agent. In our previous study, we also demonstrated that hLF plays a crucial role in the regulation of apoptosis and anti-apoptosis in PC12 neuronal cells (Lin et al., 2005). However, it is not known whether bLF can regulate the survival of other neural tissue cells or not.

Schwann cells are the main supportive cells of the peripheral nervous system (PNS) and are responsible for the myelination of axons in the PNS. The myelin sheath that allows rapid neuronal conduction is formed from Schwann cells that tightly wrap around the axon during development (Garbay et al., 2000). Schwann cell proliferation is important for the development and repair of the PNS (Bunge, 1994). The RSC96 cell line is a spontaneously transformed rat Schwann cell line derived from long-term culture of rat primary Schwann cells (Hai et al., 2002). The RSC96 cell line is often used in the studies of myelination in the PNS.

Cytokine–Schwann cell interaction is important in the development, repair, and disorders of the PNS (Lisak et al., 1997). Tumor necrosis factor-α (TNF-α), a cytokine of the peripheral inflammatory response, is up-regulated in Schwann cells after peripheral nerve injury (Wagner and Myers, 1996; Botchkina et al., 1997). While TNF-α is toxic to many cell lines, TNF-α does not hurt cultured Schwann cells (Mithen et al., 1990). However, TNF-α could inhibit the proliferation of Schwann cells in a dose-dependent manner without altering the survival of Schwann cells (Chandross et al., 1996).

TNF-α is a primary mediator of inflammatory response and is primarily synthesized and released in the nervous system following injury to the PNS. TNF-α may be a signal that terminates both proliferation as well as junctional communication among Schwann cells after nerve injury (Chandross et al., 1996). Recently, erythropoietin has been demonstrated to directly counteract Schwann cell death induced by exogenously added TNF-α *in vitro* (Campana et al., 2006).

The physiological function and biological role of bLF in the Schwann cells are still unclear. Since bovine milk is

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Abbreviations: bLF, bovine lactoferrin; BrdU, bromodeoxyuridine; ERK1/2, extracellular-signal-regulated kinase 1/2; hLF, human lactoferrin; MAPK, mitogen-activated protein kinase; pERK1/2, phosphorylated extracellular-signal-regulated kinase 1/2; PNS, peripheral nervous system; TBST, Tris-buffered saline Tween 20; TNF-α, tumor necrosis factor-α.

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consumed by many people around the world, we hypothesized that bLF can have important effects on the proliferation, growth and protection of neural cells in the PNS, such as Schwann cells. Thus, the aim of this study was to investigate the effect of bLF on the proliferation of RSC96 Schwann cells and the prevention of RSC96 Schwann cells from TNF-α-induced growth arrest.

EXPERIMENTAL PROCEDURES

Cell culture

RSC96 cells are a spontaneously immortalized rat Schwann cell line (Badache and De Vries, 1998) which were obtained from Food Industry Research and Development Institute, Hsinchu, Taiwan, and were grown in Dulbecco's Modified Eagle's Medium culture medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), and 100 U/ml penicillin/streptomycin (Gibco-BRL) at 37 °C humidified environment containing 10% CO_2. To determine the effect of bLF on RSC96 cells, no serum was used in the culture of media to avoid any ill-defined effects from the mixture of growth factors in the serum.

Growth curve assay

RSC96 cells (5×10^4) were seeded in six-well plates overnight. Different doses of bLF or TNF-α were added to the cultured media, and the cells were incubated for 48 h. At the end of incubation, Trypan Blue staining was used to distinguish dead cells from live TNF-α-treated cells, and then the number of non-stained cells was counted to estimate the number of live Schwann cells. The growth curve of RSC96 cells was obtained by plotting the cell number of RSC96 cells against the incubation time in triplicate.

Cell proliferation assay (bromodeoxyuridine (BrdU) assay)

The RSC96 cells were seeded on 96-well plates and the BrdU assay (Roche, Penzberg, Germany) (Zwergel et al., 1998) was performed for cell proliferation. RSC96 cells were incubated with 100 μM BrdU labeling solution for 4 h at 37 °C. After removing the culture media, the cells were fixed and the DNA was denatured by FixDenat solution. The anti-BrdU-POD working solution and substrate solution were then added, and the absorbance of the samples was measured by an ELISA reader (SpectraMax 250, Molecular Devices, Sunnyvale, CA, USA) at 370 nm (reference wavelength: 492 nm).

Immunoblot analysis

After the treatment with bLF, the RSC96 cell lysates were collected for immunoblot analysis (Burnette, 1981). The concentration of proteins 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 polyvinylidene difluoride membrane for immunoblotting. The membrane was blocked by incubation in non-fat milk at room temperature for 2 h, incubated with anti-phospho-extracellular-signal-regulated kinase 1/2 (anti-pERK1/2) (p185/187) antibody (BioSource International, Camarillo, CA, USA), anti-ERK1/2 antibody (Upstate Biotechnology, Waltham, MA, USA), and anti-β-actin antibody (Chemicon International, Temecula, CA, USA) for 2 h at room temperature. The membrane was subsequently washed five times with Tris-buffered saline Tween 20 (TBST), and then incubated further at room temperature with horseradish peroxidase–conjugated secondary antibody (Chemicon International) for 2 h. Finally, the membrane was washed six times with TBST, and specific bands were made visible by chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The density of specific bands was then measured by a laser densitometer with ImageQuant software (version 5.2, Molecular Dynamics).

Statistical analysis

Statistical analysis was performed by using Mann-Whitney rank sum test (SigmaStat, SPSS, Chicago, IL, USA). The results were reported as mean±S.D. A P value <0.05 was considered statistically significant.

RESULTS

bLF enhanced RSC96 cell proliferation

The RSC96 cells were exposed to different predetermined concentrations of bLF for 48 h. As shown in Fig. 1A, when the RSC96 cells were treated with different concentrations (0, 10, 50, 200, 400, and 800 μg/ml) bLF for 48 h, the viability of RSC96 cells was increased to more than 25% when the concentration of bLF was 50 μg/ml or higher, and by about 100% when the concentration of bLF was 800 μg/ml (Fig. 1A). The number of RSC96 cells incubated with 10 μg/ml and 50 μg/ml bLF for 48 h was increased by more than twofold when compared with the control group (no bLF). When the RSC96 cells were treated with 800 μg/ml bLF for 48 h, the number of RSC96 cells was raised to over threefold when compared with the control group (Fig. 1B).

At the same time, we also examined the morphological changes of RSC96 cells after stimulation by bLF for 48 h. After treatment with various concentrations bLF for 48 h, the RSC96 cells showed no changes in morphology, but the number of RSC96 cells was increased in a dose-dependent manner (Fig. 1C–1H). These data suggested that bLF could increase the number of RSC96 cells dose-dependently.

bLF increased phosphorylation of ERK1/2 in RSC96 cells

Immunoblotting experiments revealed that 10–800 μg/ml bLF could gradually increase the phosphorylation of extracellular-signal-regulated kinase 1/2 (pERK1/2) in RSC96 cells: 10–200 μg/ml bLF could increase the pERK1/2 1.5- to twofold and 400–800 μg/ml bLF could increase the pERK1/2 three- to fivefold in RSC96 cells (Fig. 2A). As the concentration of bLF increased, there was a concomitant increase in pERK1/2 (Fig. 2A). Moreover, the expression of pERK1/2 in RSC96 cells treated by ERK1/2-specific inhibitor PD980059 was significantly decreased and could be restored by 50 μg/ml bLF for 48 h (Fig. 2B). In contrast, the levels of ERK1/2 and β-actin remained unchanged after treatment with various concentrations bLF with and without PD980059 in RSC96 cells (Fig. 2A, 2B).

bLF enhanced proliferation of TNF-α-treated RSC96 cells

After treatment with 10 and 40 ng/ml TNF-α, the number of RSC96 cells decreased to less than 50% as compared with the control group (Fig. 3A–C, 3G). These data dem-

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