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E2F1-directed activation of Bcl-2 is correlated with lactoferrin-induced apoptosis in Jurkat leukemia T lymphocytes

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Abstract Lactoferrin (Lf) has been shown to control the proliferation of a variety of mammalian cells. Recently, we reported that human Lf induces apoptosis via a c-Jun N-terminal kinases (JNK)-associated Bcl-2 pathway that stimulates programmed cell death. In order to gain insight into the mechanism underlying Lf-triggered apoptotic features, we attempted to determine the mechanisms whereby the Lf-induced Bcl-2 family proteins exert their pro- or anti-apoptotic effects in Jurkat leukemia T lymphocytes. Treatment of the cells with high concentrations of Lf resulted in a significant reduction in in vitro growth and cell viability. As the levels of Lf increased, greater quantities of CDK6 and hyper-phosphorylated retinoblastoma protein were produced, resulting in the induction of E2F1-dependent apoptosis. Simultaneously, PARP and caspases were efficiently cleaved during Lf-induced apoptosis. The E2F1-induced apoptotic process occurred preferentially in p53-deficient Jurkat leukemia cells. Therefore, we attempted to determine whether E2F1-regulated Bcl-2 family proteins involved in the apoptotic process were relevant to Lf-induced apoptosis. We found that Lf increased the interaction of Bcl-2 with the pro-apoptotic protein Bad, whereas the total protein levels did not change significantly. Our results, collectively, suggest that Lf exploits the control mechanism of E2F1-regulated target genes or Bcl-2 family gene networks involved in the apoptotic process in Jurkat human leukemia T lymphocytes.

Keywords Lactoferrin · Iron · Apoptosis · Jurkat · Bcl-2

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Introduction

Lactoferrin (Lf) is a multi-functional iron-binding glycoprotein that is found abundantly in the colostrum. It is also present in other body fluids secreted from the secondary granules of neutrophils (Ward et al. 2005). It has been reported that Lf is involved in mammalian host defense against infection and inflammation via the activation of immune cells, most notably natural killer cells and monocytes. Additionally, the results of some recent studies have shown that Lf regulates cell growth-related genes in certain tumor cell types (Xiao et al. 2004), thus suggesting that Lf may be involved in the control of cell growth, proliferation, and apoptosis.

Apoptosis is a complicated cell death process that is involved in the regulation of tissue homeostasis.

The signaling pathways resulting in apoptotic cell death are initiated by intracellular and extracellular stimuli, and are controlled by a variety of cellular factors including E2F1(Ginsberg 2002). The E2F family of transcription factors plays a critical role in the regulation of cell proliferation and apoptosis. E2F1-induced apoptosis is mediated either by a p53-dependent or by a p53-independent pathway. It has been demonstrated that E2Fs induces pro-apoptotic members of the Bcl-2 (B-cell lymphoma 2) family, such as Bim, Bad, Bak and Bid1 (Ma et al. 2002; Pediconi et al. 2003; Polager et al. 2002). Additionally, it was reported that E2F1 induces p53-independent apoptosis via its downstream mitochondrial apoptosis-inducing factors (Xie et al. 2006).

Cell death pathways triggered by a variety of signals can lead to the export of cytochrome c to the cytosol (Caroppi et al. 2009). This cytochrome c release process generally requires the activation not only of BH3-only proteins, such as Bim, Bad, Bid, Bik, Bmf, Puma, Noxa, and Hrk, but also of the pro-apoptotic proteins, Bax and Bak (Wang and Youle 2009). The pro-apoptotic proteins promote apoptosis by forming pores in the mitochondrial outer membrane and facilitating cytochrome c release. Bak and Bax are, therefore, important mediators of apoptotic pathways.

Genetically programmed cell death is required for the proper development of and cellular defense against threats to the integrity of the organism. However, a variety of cellular circumstances involve disruptions of apoptotic regulation, causing deregulations of growth or uncontrollable proliferation. Considering this, apoptosis clearly performs some crucial functions in the regulation of tumor progression. Previous studies have shown that Lf inhibits cell proliferation via cell cycle arrest or apoptosis (Lee et al. 2009a; Son et al. 2006). Lf concentration has also been implicated as crucial to the control of both cell survival and cell death. It is generally held that serum levels of Lf are predominantly neutrophil-derived and in normal subjects usually remain below 10 μg/ml. However, under certain physiological conditions, cellular levels of Lf exposure can be considerably higher than this, and these higher Lf levels are strongly associated with a range of diseases (Wong et al. 2009). In a previous study, a relatively high concentration of human Lf coupled with a natural iron saturation was shown to be sufficient to induce apoptosis in Jurkat leukemia T cells (Lee et al. 2009b). However, the precise mechanism underlying the regulation of Lf-mediated cell death has yet to be clearly elucidated.

In this study, in an effort to elucidate the mechanism of Lf-induced apoptosis, we investigated the Bcl-2-regulated apoptotic pathway in the Lf-induced apoptotic signaling pathway. We determined that the Lf-activated apoptotic machinery employed the Bcl-2 family proteins as effector molecules, thereby resulting in the cell death of leukemia T-lymphocytes.

Materials and methods

Cell culture and transient transfection

Jurkat cells were maintained in RPMI 1640 media (Cambrex, USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, USA) and 100 U/ml penicillin/streptomycin (Gibco-BRL) in humidified incubator at 37°C and 5% CO_2. Every 3 days, the rapidly growing cells were split with fresh medium. For the experiments, cells were seeded at 2×10^5 cells/ml in six-well plates, and treated with human Lf (L4894, Sigma-Aldrich, USA) at the indicated concentration for 24 h. The cells were grown up to 70% confluence and transiently transfected with pLf-V5 or a control plasmid by electroporation according to the manufacturer's instructions. In order to construct pLf-V5, a 2.1 Kb cDNA encoding Lf was isolated with *XbaI* from the plasmid pLf (He and Furmanski 1995). The fragment was inserted into the plasmid pcDNA6/V5-His A (Invitrogen).

Cell viability assay

The effect of Lf on the viability of Jurkat cells was determined by trypan blue dye exclusion and MTT assays. Briefly, the cells were plated at a density of 2×10^5 in six-well plates and in medium containing Lf or vehicle. After incubation for 24 h, an aliquot of cell suspension was mixed with an equal volume of trypan blue and then cells were counted under the microscope. For the MTT assay, cells were plated in 96-well microtiter plated and treated with Lf or vehicle alone. The cells were incubated for 24 h at 37°C in a humidified incubator at the end of which

MTT reagent (0.25 mg/ml in PBS) was added to each well and incubated for 4 h. MTT-formazan crystals were dissolved by DMSO and the absorbance was determined at 570 nm.

Annexin V-binding analysis

Jurkat cells were treated with a different type of Lf at the indicated concentration. Lf-treated Jurkat cells were collected via centrifugation, washed twice in phosphate-buffered saline (PBS), and then resuspended in 100 μl of Annexin V binding buffer. 5 μl of Annexin V-FITC (BD Biosciences, USA) was added to resuspended cells, followed by 15 min of incubation at room temperature in darkness. Apoptotic cells were analyzed by flow cytometry using Cell Quest-pro software (Becton–Dickinson, CA).

Luciferase assay

The cells were transfected with E2F1-luc. After 24 h, Lf was added at the indicated concentration for 24 h. Luciferase activity was determined using Luciferase Reporter Assay System according to manufacturer's instructions (Promega).

RT-PCR

After treatment of Jurkat cells with Lf for 24 h, total cellular RNAs were prepared with TRIzol (Invitrogen Life Technologies) according to the manufacturer's recommendations. Equal amounts of total RNA were reverse transcribed with oligo (dT) and reverse transcriptase (Promega). The gene transcripts were identified via PCR.

Co-immunoprecipitation

Jurkat cells were treated with Lf for 24 h. Cells were harvested and lysed in HNTG buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol) with the addition of protease inhibitors. After lysis at 4°C, lysates were pre-cleared using A/G beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C and incubated with anti-Bcl-2 antibody (Cell signaling technology). After 18 h, the agarose beads were added for 1 h and washed five times in cold lysis buffer, and the immunoprecipitates were subjected to SDS-PAGE.

Immunoblot analysis

Each immunoblot analysis was done as described (Son et al. 2006). Anti-Bcl-2, anti-cleaved caspase 7, anti-phospho-Bcl-2, anti-Bad, anti-PARP, anti-cleaved PARP, and anti-phospho-retinoblastoma protein (Rb) antibody were purchased from cell signaling Technology, and anti-actin, anti-Lf, anti-cyclin-dependent kinases (CDK) 6, anti-Rb antibody was purchased from Santa Cruz Biotechnology, Inc. The immunocomplex was visualized with the ECL system (Millipore, USA).

Results

Lf induces apoptosis in Jurkat cells

We have previously demonstrated that Lf might play a role in the regulation of cell proliferation and death (Lee et al. 2009b). We conducted further experiments to determine whether Lf could indeed regulate cell death, and consequently attenuate cell viability in tumor cells. To assess the effects of Lf on cell viability, human leukemic Jurkat cells were treated with 700 μg/ml of Lf in culture medium for 24 h, and then analyzed via trypan blue exclusion assay. Figures 1a and b show that Lf-treated cells evidenced significant reductions in both cell viability and growth, thereby suggesting that Lf exerts an anti-proliferative effect in malignant cells. We then attempted to determine whether the anti-proliferative effects of Lf in Jurkat cells involved the genetically programmed cell death process. According to the results of our Annexin-V assay (Fig. 1c), Lf treatment to the cells induced apoptosis. This observation indicates that 700 μg/ml of natural Lf with iron exerts a substantial apoptosis-promoting effect in Jurkat cells.

As is shown in Fig. 2a, an increase in the Lf concentration to 700 μg/ml resulted in a substantially greater cleavage of poly (ADP-ribose) polymerase (PARP), a protein involved in a number of cellular processes associated with DNA repair and programmed cell death. We also assessed whether Lf gene expression, rather than the simple administration of Lf protein to the cells, was also capable of inducing apoptosis (Fig. 2b). The expression of pEGFP-N1 may give evidence that over-expression

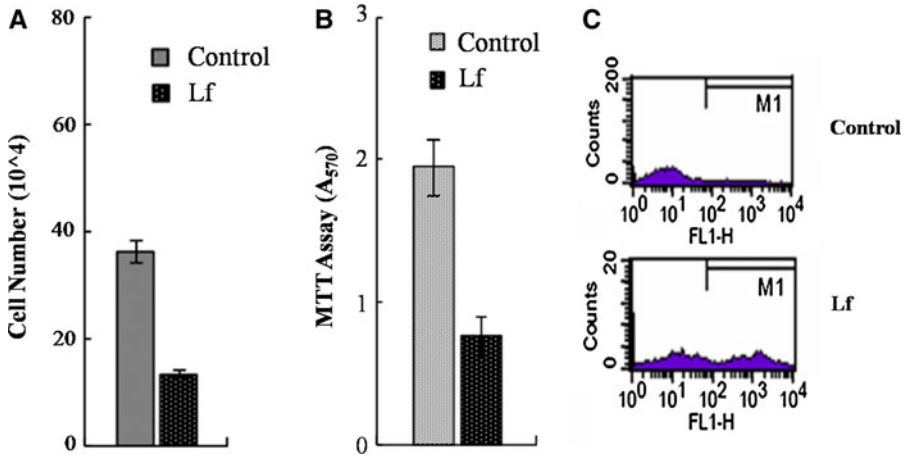

Fig. 1 Effect of Lf on Jurkat cell viability Jurkat cells were treated with 700 μg/ml of Lf for 24 h. Cell viability was assessed by Trypan Blue exclusion assay (**a**) and MTT assay (**b**) as described in the material and methods. Each bar represents the means ± SE of triplicate samples from three different experiments. (**c**) After treatment of Jurkat cells with 700 μg/ml of Lf for 24 h, the Annexin V-FITC positive cells were analyzed by flow cytometry

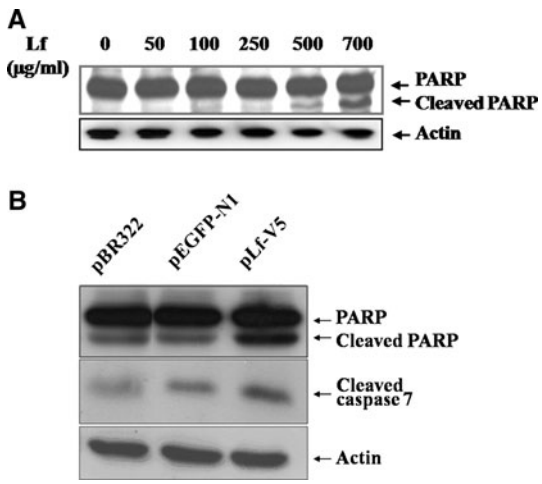

Fig. 2 Lf-induced apoptosis. **a** A dose-dependent manner of Lf-induced apoptosis was analyzed. Jurkat cells were incubated with the indicated concentration of Lf, and harvested for immunoblotting using antibody against PARP and actin. PARP and cleaved PARP were indicated by arrows. **b** 24 h after transient transfection of Jurkat cells with V5-tagged Lf expression vector (pLf-V5), pBR322 or pEGFP-N1 (Clontech Lab., Inc), the total protein extracts from cells were immunoblotted with anti-PARP, anti-cleaved caspase 7, and anti-actin antibody

of GFP as a control protein does not lead to cell apoptosis. This experimental data may provide a way to prove that the Lf-mediated apoptosis is not induced by any toxic or biologically active molecules. Cells transfected transiently with the Lf expression plasmid evidenced cleavages of both PARP and caspase 7.

Lf induces cell death via E2F1-dependent apoptosis pathway

Recent reports have shown that a variety of components of the cell-cycle machinery are activated in cells exposed to apoptotic stimuli. Lf has been believed to control the proliferation of a variety of mammalian cells (Ward et al. 2005). However, the downstream effectors that mediate apoptosis in response to Lf treatment remain to be clearly identified. Therefore, we have begun to assess which of these factors are regulatory molecules that are relevant to Lf-mediated cell survival and death. In response to Lf treatment, the levels of the cyclin-dependent kinase 6 (CDK6) and phosphorylated Rb at Ser^{795} increased (Fig. 3a). In order to investigate the role of phosphorylated Rb in Lf-induced apoptosis, we attempted to determine whether CDK-dependent Rb phosphorylation regulates the formation of Rb-E2F1 complex with E2F1 in response to Lf. Figure 3b demonstrates that the levels of the Rb-E2F1 complex were indeed reduced although Lf treatment did not influence significantly the protein level of E2F1, but did induce an increase in the level of phosphorylated Rb protein. To explore in more detail the mechanisms underlying Lf-induced apoptosis, we constructed a luciferase reporter driven by the E2F1 promoter. Transient transfection of Jurkat cells with this reporter demonstrated that the E2F1 promoter-driven luciferase activity increased

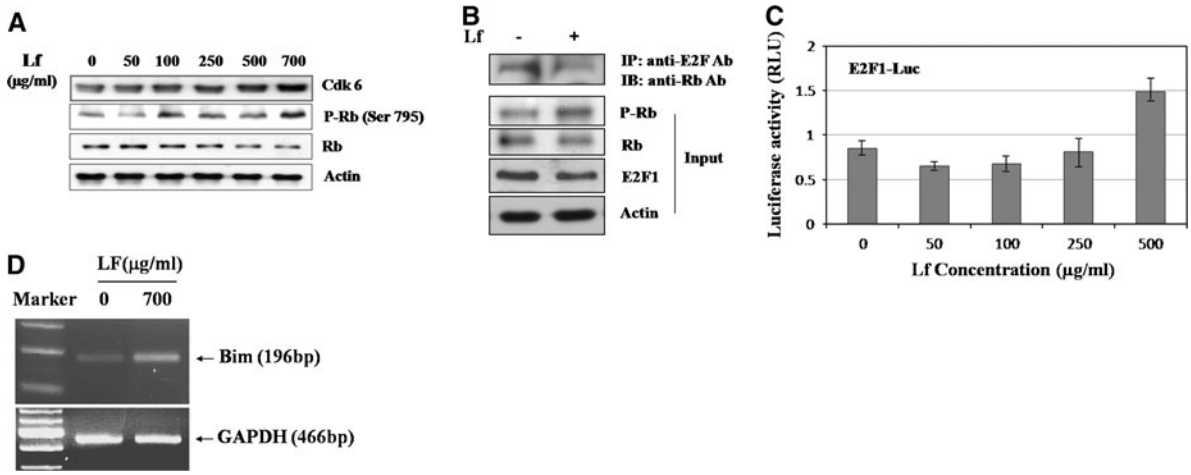

Fig. 3 Lf-induced apoptosis via E2F1-dependent apoptosis pathway. **a** Levels of Cdk6, Rb, phosphorylated Rb at Ser^{795} (P-Rb), and actin were determined by immunoblot analysis using each specific antibody. Jurkat cells were incubated with the indicated concentration of Lf for 24 h. **b** After treatment of Jurkat cells with 700 μg/ml of Lf, cell lysates were immunoprecipitated with E2F1 antibody, and immunoblotted with anti-Rb antibody. Input lysates were analyzed by immunoblotting with antibodies against Rb, phospho-Rb (P-Rb), and E2F1, and actin. **c** Jurkat cells transfected with plasmid pE2F1-Luc were incubated with the indicated concentration of Lf. Relative luciferase activities were analyzed with 100 μg of total lysates. All point bars represent the means ± SE of triplicate samples from three or four different experiments. **d** Total RNA was prepared after treatment with 500 μg/ml of Lf for 24 h. For RT-PCR experiments, cDNA was amplified with primers for Bim gene via PCR. GAPDH expression was used as an internal control

proportionally with increasing concentrations of Lf (Fig. 3c). This de-repression of E2F1 and consequent E2F1-responsive genes may be crucial to an understanding of Lf-induced Jurkat cell death (Pediconi et al. 2003).

As a result of Lf-mediated phosphorylation of Rb, the E2F1 transcription factor is consequently released, thereby triggering both proliferation and apoptosis via activation of its target genes. However, it has been reported that CDK6 targets the Rb protein, decreases cell growth in many types of cell lines, and that elevated levels of CDK6 in cells lead to apoptosis (Ojala et al. 2000). Several studies have demonstrated that E2F1 induces expression of the pro-apoptotic BH3 only protein genes, which are members of the Bcl-2 protein family (Adams and Cory 2007). Therefore, we attempted to determine whether Lf treatment effects any changes in the expression of E2F1 target genes involved in the apoptosis since the Cdk-dependent Rb phosphorylation enhances the availability of E2F1. Figure 3d demonstrated that Lf-mediated E2F1 activity resulted in higher expression of Bim gene, which could affect the neutralizing capacities of anti-apoptotic molecules. Additional results were obtained via immunoblot assays with antibodies against other E2F1-responsive Bcl-2 family proteins (data not shown). These results show that Lf-mediated cell death may occur via the pathway regulated by pro-apoptotic E2F1 target genes, including a variety of pro-apoptotic BH3 only protein genes.

Lf increases the level of interaction between Bcl-2 and Bad

The apoptotic pathway is generally mediated through inactivation of anti-apoptotic Bcl-2 via Bax/Bak and/or BH3-only proteins including Bad, Bim and Puma (Adams and Cory 2007; Danial 2007). We showed that Lf treatment induced apoptosis of Jurkat cells via Rb phosphorylation followed by activation of E2F1-responsive Bim gene. It is suggested that Lf controls the activities of Bcl-2 and BH3-only proteins, which may influence the permeability of the mitochondrial outer membrane via a multiple conformational changes in Bax and/or Bak (Chipuk et al. 2006). Therefore, it has been proposed that the Bcl-2 family proteins, which are recognized as the "gatekeepers" of the apoptotic machinery, might be a crucial

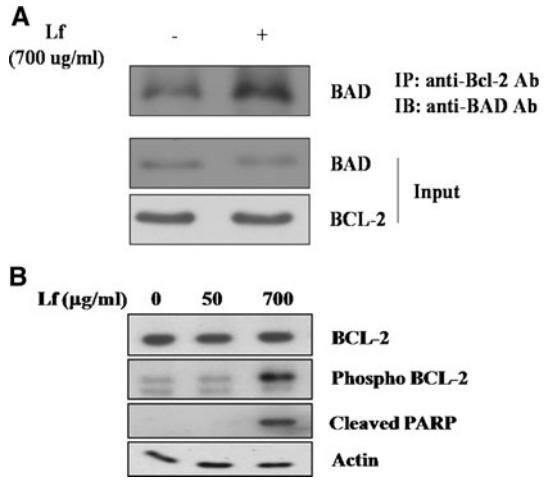

Fig. 4 Lf-induced apoptosis was correlated with the expression and activity of Bcl-2 family proteins. **a** Jurkat ells were treated with 700 μg/ml of Lf. The cells were then lysed, and immunoprecipitated with anti-Bcl-2 antibody followed by immunoblotting with anti-Bad antibody. Input lysates were also analyzed by immunoblotting with the same antibodies. **b** Jurkat cells were treated with the indicated concentrations of Lf for 24 h. After incubation, the cells were harvested, and levels of Bcl-2, phospho-Bcl-2, cleaved PARP, and actin were determined by immunoblot analysis

checkpoint in the phenomenon of Lf-induced apoptotic cell death. We assessed the effects of Lf on the association of Bcl-2 with one of its binding partners, such as Bad, using co-immunoprecipitation experiments. According to our results, Lf increased the level of interaction between Bcl-2 and Bad (Fig. 4a). This indicates that Bad may function as a sensitizer via direct interaction with the anti-apoptotic molecule Bcl-2, thus allowing Bax and/or Bak to induce a permeabilization of the mitochondrial outer membrane, and ultimately promoting apoptosis. However, total protein levels of Bcl-2 and Bad did not change significantly. The mechanism by which Lf-induced E2F1 directs Bcl-2 activity towards apoptosis was further studied. As shown in Fig. 4b, PARP was clearly cleaved in response to the high Lf level. The level of Bcl-2 phosphorylation in cells treated with a high Lf level was clearly higher than in the control cells, in which apoptosis could not be induced. Therefore, Lf-induced increase in Bcl-2 phosphorylation might explain how Lf enhanced the interaction between Bcl-2 and Bad. Thus, it appears likely that Lf may induce a shift in the cell growth balance towards apoptosis.

Discussion

Apoptosis is a crucial function in a variety of biological processes in both normal and dysfunctional cells. Recent studies have demonstrated the involvement of Lf in cell proliferation via cell cycle arrest or apoptosis in a variety of mammalian cells (Xiao et al. 2004). Lf concentration has been shown to be critically important to the ultimate fate of the cell. It has also been reported that the binding of iron to Lf appears to induce conformational changes (Baker and Baker 2009). It is, therefore, likely that the local concentration and structural features of Lf would constitute the key effectors affecting its biological functions. Indeed, it has been previously noted that relatively low levels of iron-saturated human Lf stimulated cell cycle progression by facilitating entry into the S phase of the cell cycle in MCF-7 cells (Lee et al. 2009b). This phenomenon may be associated with cell survival. By way of contrast, we have demonstrated herein that the Jurkat cell death induced by high levels of Lf occurs via a p53-independent signaling pathway, and is accompanied by E2F-dependent gene activation and the activation of Bcl-2 signaling. The Bcl-2 family-mediated signaling pathway is subsequently linked to the intrinsic apoptotic pathway, facilitating cytochrome c release.

Since the Bcl-2 family proteins are the principal important factors in the direct cytochrome c release pathway, we have attempted to explore the functions of Bcl-2 family proteins in Lf-induced apoptosis. The BH3-only proteins have been generally linked to in pro-apoptotic activity, and are also known to stimulate Bax and Bak to release cytochrome c. Two major competing models of action of the BH3-only proteins in the activation of Bax and/or Bak have been proposed (Adams and Cory 2007; Danial 2007). In the direct activation model, the BH3-only proteins are split into two categories: activators including Bim and Bid that bind directly to Bax and Bak to trigger their activation, and sensitizers including Bad and Noxa that bind to Bcl-2 or Bcl-xl and release Bim or Bid to activate Bax and Bak. Alternatively, the indirect activation model holds that all BH3-only proteins bind to the pro-survival Bcl-2 or Bcl-xl, thereby essentially inhibiting Bax and Bak. Because both activation models propose that the BH3-only protein Bad binds to Bcl-2 or Bcl-xl and inactivates their functions, we attempted to determine whether

the interaction between Bad and Bcl-2 was augmented by Lf treatment. As anticipated, Lf treatment did increase the interaction between Bcl-2 and Bad protein, thereby indicating the release of Bax or Bak from Bcl-2 (Fig. 4). Our results show that Bad may neutralize pro-survival Bcl-2 via direct interaction, thereby inducing cell death as a response to Lf.

It has been reported that JNK-mediated Bcl-2 phosphorylation can inhibit its binding to Bax (Shitashige et al. 2001). Therefore, Bcl-2 phosphorylation and its inactivation by Bad may exert a synergistic apoptotic-stimulatory effect by virtue of Bax and Bak activation, although the effects of Bcl-2 phosphorylation on the interaction between Bcl-2 and Bad remain to be thoroughly elucidated. Bax and Bak would then be released from the anti-apoptotic molecules to undergo the mitochondrial intra-membranous homo-oligomerization required for their activation and subsequent cytochrome c release.

Lf is proposed to function as a mediator of the immune response as well as a signal transducer that regulates cell growth (Brock 2002; Oh et al. 2001). Lf is an essential element in the human body, and is usually utilized as both a particular transporter of iron from ingested food and a scavenger of bacterial iron; thus, Lf can be present in the body in either a natural or an iron-bound form. Many previous studies have shown that different forms of Lf may have differing effects on cell proliferation and growth (Damiens et al. 1999; Huang et al. 2008; Naot et al. 2005; Xiao et al. 2004). Collectively, our results show that the Jurkat cell death induced by human Lf with a relatively low iron involves a mitochondrially regulated apoptotic pathway. Therefore, Lf plays a crucial role in the apoptotic process, and this information may prove invaluable in the development of novel strategies for the treatment of leukemia-like pathological processes.

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