Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells

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Abstract CHOP (GADD153) is a member of the C/EBP family and a stress-induced protein. To investigate the role of CHOP in cellular growth, we expressed CHOP conditionally in M1 myeloblastic leukemia cells that do not express p53 protein. More than 60% of M1 cells died through apoptosis 72 h after CHOP induction. Site-directed mutagenesis revealed that this process requires leucine zipper domain but neither intact basic region nor trans-activation domain. CHOP-mediated apoptosis accompanied downregulation of bcl-2 mRNA and overexpression of Bcl-2 delayed the process. Our results indicate that CHOP can induce apoptosis in a p53-independent manner.

Key words: CHOP; GADD153; Apoptosis; p53; Leucine zipper; Bcl-2

1. Introduction

CHOP (C/EBP-homologous protein), also known as GADD153, is a member of the C/EBP family, a group of leucine zipper proteins that share extensive amino acid homology with the DNA binding domain of C/EBP. The family currently consists of six members including C/EBP (C/EBPα), NF-IL6 (C/EBPβ), Ig/EBP (C/EBPγ), NF-IL6β (C/EBPδ), CRP1, and CHOP [1–8]. These members can form homo- and heterodimers, all of which recognize the same nucleotide sequences except for CHOP. CHOP can form dimers with other family members, but the heterodimers cannot bind to the C/EBP binding consensus because of several amino acid substitutions in the DNA binding domain. This finding suggests that CHOP may act as a negative modulator of the activity of other C/EBP family members [8]. CHOP is expressed at undetectable levels in growing mammalian cells but is markedly increased by treatment with genotoxic agents, calcium ionophore, lipopolysaccharide, and nutrient deprivation [9–13]. Microinjection of CHOP protein into NIH3T3 cells induces G_1/S arrest of the cell cycle [14,15]. This requires both a leucine zipper domain and a basic region. In myxoid liposarcoma, a tumor of human adipose tissue, a t(12;16) chromosomal translocation results in the expression of an oncogenic fusion protein between CHOP and the amino terminus of a RNA binding protein, TLS or FUS [16,17]. These studies suggest that CHOP may play a role in an inducible growth arrest pathway that is triggered by DNA damage or metabolic stress.

Murine M1 myeloblastic leukemia cells undergo apoptosis following exposure of genotoxic stresses such as methyl methanesulfonate and ultraviolet irradiation [18]. IL-6 or LIF also induces terminal differentiation and growth arrest of M1 cells, ultimately culminating in apoptosis [18]. In both cases the dramatic induction of CHOP mRNA coincided with the induction of apoptosis. It is well known that p53 plays a pivotal role in DNA damage-inducible growth arrest and apoptosis [19]. However, M1 cells do not express p53 protein [20], indicating that apoptosis of M1 cells cannot be mediated by the p53-dependent pathway. This finding suggests that CHOP may mediate growth arrest and apoptosis in M1 cells by a p53-independent pathway. To investigate the role of CHOP in M1 cells, we took advantage of the inducible expression system by which CHOP protein can be expressed conditionally by adding isopropyl β-D-thiogalactoside (IPTG). The present study demonstrates that conditional expression of CHOP protein is able to induce apoptosis in M1 cells.

2. Materials and methods

2.1. Cells and cell culture

Murine M1 myeloblastic leukemia cells were cultured in Eagle's minimal essential medium and 10% fetal calf serum. The presented data were obtained using the M1Mock clone 3, M1wtCHOP clone 19, M1L133A/L140A clone 2, M1Δ105-115 clone 18, M1Δ1-88 clone 10, M1CHOP/puro clone 11, M1CHOP/Bcl-2 clone 18; however, for each cell variant two other independent cell lines were examined (M1Mock/7, 20; M1wtCHOP/3, 14; M1L133A/L140A/9, 10; M1Δ105-115/3, 10; M1Δ1-88/3, 21; M1CHOP/puro/16, 24; M1CHOP/Bcl-2/4, 7), where the results were similar to the presented data. Cells were seeded at 1.0×10^5/ml except that cells were seeded at 5.0×10^5/ml for RNA extraction within 24 h after addition of IPTG. M1 transfectants were treated with 5 mM IPTG in all experiments. Viability was determined by trypan blue exclusion and counted in a hemacytometer.

2.2. Plasmid construction and site-directed mutagenesis

The hamster CHOP (GADD153) cDNA plasmid was used as a template for subsequent PCR amplification or site-directed mutagenesis. The LacSwitch-inducible mammalian expression system (Stratagene) was used for conditional expression of CHOP. To construct pOPRSVI-wild-type (wt) CHOPFlag and pOPRSVI-Δ1-88 CHOP-Flag, 5′ PCR primers and 3′ PCR primer were devised to introduce an optimal Kozak consensus sequence and FLAG (DYKDDDDK,

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Abbreviations: CHOP, C/EBP-homologous protein; GADD153, growth arrest- and DNA damage-inducible gene 153; C/EBP, CCAAT/enhancer-binding protein; IPTG, isopropyl β-D-thiogalactoside

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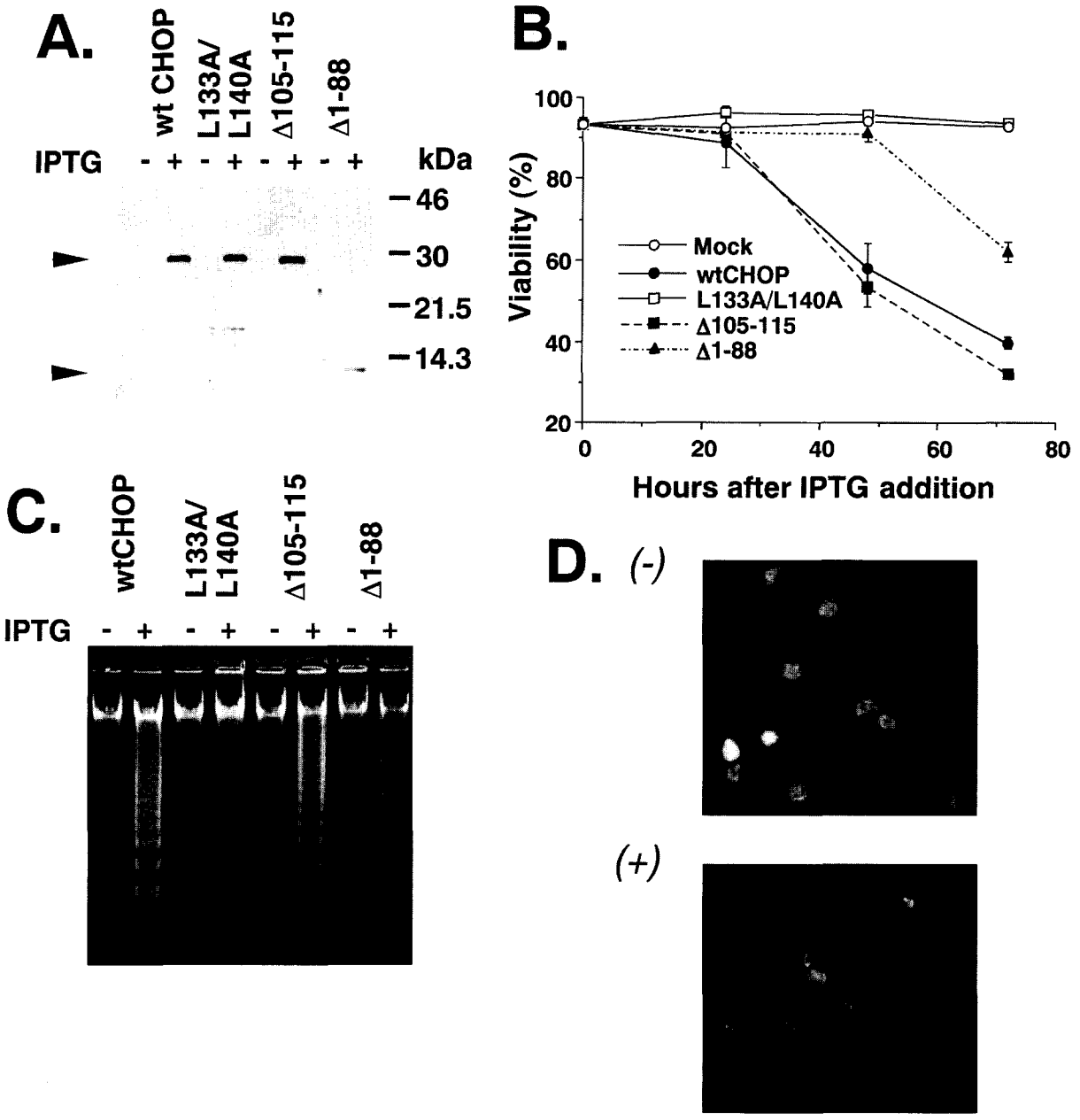

Fig. 1. IPTG-induced expression of CHOP triggers apoptosis in M1 cells. A: Establishment of M1CHOP derivative clones. The indicated clones were either treated (+) or untreated (−) with 5 mM IPTG for 8 h and subjected to Western blot analysis. B: Viability of M1CHOP derivative cells treated with IPTG. Viability was determined at 0, 24, 48 and 72 h time points by trypan blue exclusion. Each value represents the mean of three independent experiments. The error bars represent standard deviation. C: Induction of DNA fragmentation in M1CHOP derivative cells. The indicated clones were either treated (+) or untreated (−) with IPTG for 72 h and harvested for DNA extraction. Genomic DNA (5 μg/lane) was electrophoresed on a 2% agarose gel and visualized under UV light. D: Visualization of apoptotic cells by Hoechst staining. M1wtCHOP cells were cultured with (+) or without (−) IPTG for 72 h, fixed in glutaraldehyde, stained with Hoechst 33258 and observed under fluorescent microscopy. Magnification: ×200.

Kodak) epitope, respectively. The upstream primer sequences were 5′-GGTCGACCACCATGGCAGCTGAGTCCCTG-3′ (for pOPRSVI-wtCHOPFlag) and 5′-GGTCGACCACCATGGCTCAGGAGGAAGAG-3′ (for pOPRSVI-Δ1-88CHOPFlag). The common downstream primer sequence was 5′-CGTCGACTCACTTGTCATCGTCGTCCTTGTAGTCTACTTGTTGCAGATTTAC-3′. After cloned into pT7Blue-T vector (Novagen), these PCR-amplified products were verified by automated DNA sequencing utilizing dye-labeled dideoxynucleotides. Following digestion, each fragment was cloned into pOPRSVI operator vector. Wild-type CHOPFlag fragment was also inserted into PUC19 vector and then this vector was used to generate L133A/L140ACHOPFlag and Δ105-115 CHOPFlag. The sequences of the oligonucleotides used to generate the mutation and deletion are as follows: L133A/L140A, 5′-AAAGTGGCTCAGGCAGCTGAGGAGAACGAGCGGGCCAAGCAGGAAATCGAG-3′; Δ105-115, 5′-CGAACCAGGAAACGGAAAAAGCAGCGCATGAAGGAG-3′.

2.3. Stable transfections

To prepare doubly transfected clones, 10^7 exponentially growing M1 cells were cotransfected via electroporation with 20 μg of P3′SS lac repressor-expressing vector and 20 μg of pOPRSVI-CHOPFlag derivative. Cells were placed in growth medium for 24 h, then transferred to 24-well plates, and placed under selection with 75 μg of hygromycin B (Boehringer) and 400 μg of geneticin (BRL) per ml for 14 days. Single colonies were expanded and tested for expression of CHOPFlag derivative by Western blot analysis after 8 h treatment

with IPTG. To generate CHOP/Bcl-2 or CHOP/puro cell lines, M1wtCHOP-19 cells were cotransfected with 20 μg of PSVT-B [21] and 1 μg MSCV pac, selected under 5 μg/ml puromycin (Sigma), and expanded as described above. MSCV pac was employed for expression of the puromycin resistance gene.

2.4. DNA fragmentation assay

Genomic DNA was extracted by using SepaGene (Sanko Junyaku). Following treatment with RNase A (20 μg/ml) for 1 h, DNA was precipitated with polyethylene glycol and the purified DNA (5 μg/lane) was fractionated on a 2% agarose gel with TAE (40 mM Tris-acetate, 1 mM EDTA) as the running buffer. The electrophoresed gel was stained with ethidium bromide (0.5 μg/ml) and visualized under UV light.

2.5. Hoechst staining

M1wtCHOP cells were cultured with or without 5 mM IPTG for 72 h, fixed in glutaraldehyde (1% in PBS), stained with Hoechst 33258 (1 mM in PBS, Sigma), and observed under fluorescent microscopy.

2.6. Western blot analysis

To detect CHOPFlag derivative protein, cells were lysed directly in 1×Laemmli buffer. After boiling for 5 min, cell lysates (2×10^5 cells per lane) were separated by SDS-PAGE (15–25% gradient polyacrylamide), electrophoretically transferred to nitrocellulose membranes, and then analyzed for immunoreactivity with a monoclonal antibody specific for the FLAG-epitope tag (M2, Kodak) by the enhanced chemiluminescence detection system (Amersham).

2.7. Northern blot analysis

Cytoplasmic RNA was obtained by a guanidine isothiocyanate-cesium chloride gradient centrifugation method [22]. Total RNA (20 μg/lane) was separated on a 1.0% agarose gel containing 6.0% formaldehyde. After transfer to a Hybond N^+ nylon membrane (Amersham) in 10×SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate), hybridization and wash were performed according to the method of Church and Gilbert [23], using cDNA probes labeled by the random primer method. The probes for bcl-2 and bax were RT-PCR products prepared from M1 cells as follows: bcl-2, nucleotides 1892–2433; bax, nucleotides 35–579. The probes for c-myc, c-myb and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were the same as used previously [24]. CHOP probe was the complete coding cDNA fragment of hamster CHOP. Human bcl-2 probe was the 0.95Kb *Xho*I fragment from PSVT-B.

3. Results and discussion

3.1. IPTG-induced expression of CHOP triggers apoptosis in M1 cells

Attempts to establish M1 cell lines that constitutively express high levels of CHOP protein have been unsuccessful probably due to the antimitotic activity of this protein. To circumvent this problem, we developed clonal derivatives of M1 cells which contain the hamster CHOP gene under control of the lac repressor. Mock clones expressing lac repressor and pOPRSVI vector without an insert were selected as negative controls. Moreover, three CHOP derivatives were constructed to define the functional domain of CHOP protein. The first derivative, L133A/L140A, lacks the functional leucine zipper domain in which two of three leucines composing leucine zipper are substituted by alanines. The second derivative, Δ105–115, lacks the central portion of the basic region corresponding to DNA binding domain in other C/EBPs. The third derivative, Δ1–88, lacks a large portion of the trans-activation domain. At the same time, FLAG-epitope was tagged to the C-terminus of each CHOP derivative protein in order to discriminate the endogenous one. Stable expression of CHOP derivative proteins were driven by addition of IPTG in M1 cells (Fig. 1A). While 133A/140A and Δ105–115 proteins were expressed at comparable levels to wild-type (wt) CHOP protein, Δ1–88 protein level was extremely lower than other CHOP derivatives. Expression of CHOP derivative proteins reached at a maximum level within 4 h after IPTG addition and lasted for more than 72 h (data not shown). Following treatment with IPTG, viability of M1CHOP derivative cells was determined. As shown in Fig. 1B, the viability of wtCHOP and Δ105–115 cells began to decrease 48 h after addition of IPTG. By 72 h, more than 60% of wtCHOP and Δ105–115 cells died, whereas more than 90% of Mock and L133A/L140A cells survived (Fig. 1B). Although Δ1–88 cells did not lose viability for up to 48 h, its viability dropped to about 60% during next 24 h. The late decrease of viability by Δ1–88 cells was considered to be due to low expression level of its proteins. Then we examined, by gel electrophoresis, genomic DNA from M1CHOP derivative cells after treatment with IPTG. The characteristic 'ladder' pattern of DNA (Fig. 1C) was observed 72 h after IPTG addition to M1CHOP derivative cells except L133A/L140A cells. Hoechst staining of wtCHOP cells revealed nuclear morphological features associated with apoptosis including condensed nuclei and fragmented chromatin (Fig. 1D). These studies indicated that IPTG-treated M1CHOP derivative cells except L133A/L140A cells died through apoptosis and CHOP-mediated apoptosis requires leucine zipper domain but neither intact basic region nor trans-activation domain of CHOP protein. On the other hand, CHOP-mediated growth arrest and oncogenic transformation by TLS-CHOP require an intact basic region, suggesting that DNA binding by CHOP may be implicated in these activities [14,15]. Recently, it was demonstrated that CHOP-C/EBP and CHOP-NF-IL6 dimers bind to unique DNA control elements different from C/EBP consensus and activate unknown genes [25]. This transcriptional activity requires the intact basic region of CHOP protein. However, CHOP-mediated apoptosis does not require an in-

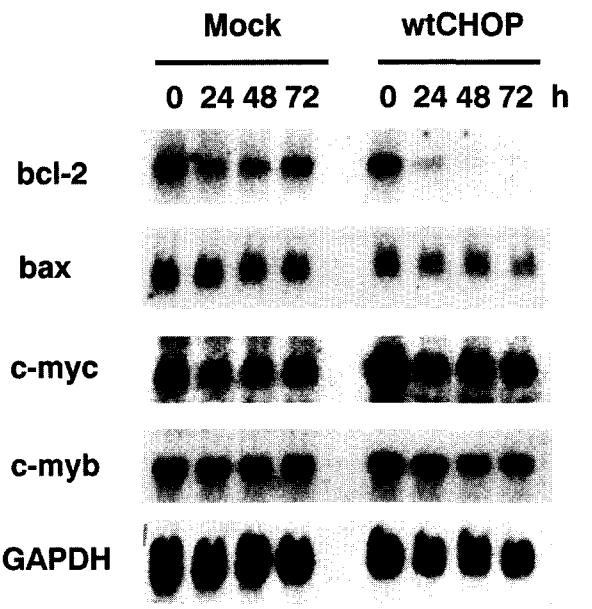

Fig. 2. Effect of conditional expression of CHOP on apoptosis- and growth-related genes. M1Mock and M1wtCHOP cells were stimulated with IPTG (5 mM) and total RNA (20 μg/lane) prepared at the indicated times was analyzed by Northern blot hybridization with the indicated cDNA probes.

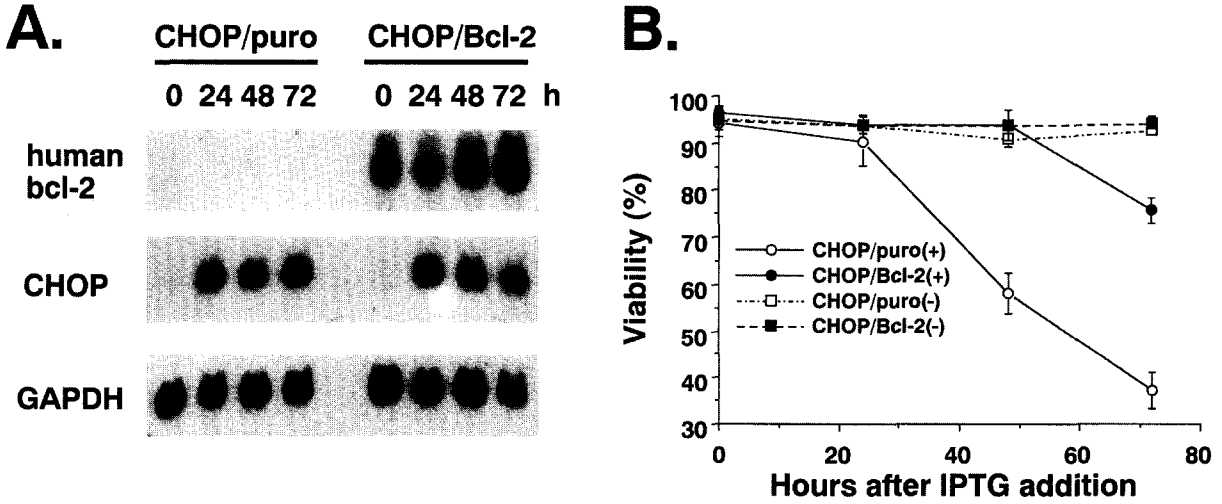

Fig. 3. Effect of ectopic expression of Bcl-2 on CHOP-mediated apoptosis in M1 cells. A: Establishment of M1CHOP/puro and M1CHOP/Bcl-2 clones. Expression of the human bcl-2 and CHOP transgenes in M1CHOP/puro and M1CHOP/Bcl-2 cells were analyzed by Northern blot hybridization. B: Viability of M1CHOP/puro and M1CHOP/Bcl-2 cells treated with IPTG. M1CHOP/puro and M1CHOP/Bcl-2 cells were treated with (+) or without (−) IPTG (5 mM). Viability was determined at 0, 24, 48 and 72 h time points after addition of IPTG by trypan blue exclusion. Each value represents the mean of three independent experiments. The error bars represent standard deviation.

tact basic region. This finding suggests that the mechanisms underlying CHOP-mediated growth arrest shown in previous reports [14,15] and apoptosis in this study are quite different. In the case of CHOP-mediated growth arrest, CHOP may activate the transcription of several genes and cause cell cycle arrest, while in the case of CHOP-mediated apoptosis, CHOP may act as a dominant negative inhibitor of other transcription factors and repress the expression of those target genes which is essential for cell survival.

3.2. Bcl-2 mRNA reduction precedes CHOP-mediated apoptosis

We examined the effect of CHOP proteins on the expression of apoptosis- and growth-related genes (Fig. 2). Bcl-2, which promotes cell survival, and Bax, which promotes cells death, have been implicated as major players in the control of apoptotic pathways [26]. As shown in Fig. 2, bcl-2 mRNA reduction preceded wtCHOP-mediated cell death, but bax mRNA levels remained unaltered during progression of apoptosis. Other clones expressing M1CHOP derivatives except L133A/L140A also showed downregulation of bcl-2 mRNA prior to cell death (data not shown). Decrease in the ratio of bcl-2 to bax may be in part responsible for CHOP-mediated apoptosis as described in p53- or TGF-β1-induced apoptosis [27,28]. While both c-myc and c-myb expression are markedly reduced following p53- or TGF-β1-mediated apoptosis in M1 cells [20,28], both mRNA levels were unaffected in CHOP-mediated apoptosis.

3.3. Bcl-2 delayed the CHOP-mediated apoptosis in M1 cells

We next investigated the effect of Bcl-2 on CHOP-mediated apoptosis. Overexpression of Bcl-2 can prevent or markedly delay cell death induced by a variety of apoptotic stimuli, such as growth factor deprivation, irradiation, and treatment with glucocorticoids, calcium ionophores and chemotherapeutic drugs [29]. A human Bcl-2 expression vector was transfected into M1wtCHOP cells, together with a puromycin resistance gene. Several M1CHOP/Bcl-2 clones expressing human Bcl-2 were selected and viability was determined following treatment with IPTG. M1CHOP/puro clones expressing only puromycin resistance gene were also isolated as controls. M1CHOP/puro and M1CHOP/Bcl-2 clones expressed similar levels of CHOP following stimulation with IPTG (Fig. 3A). While CHOP/puro cells lost viability in a similar kinetics to wtCHOP cells, CHOP/Bcl-2 cells did not die until 48 h following addition of IPTG (Fig. 3B). However, the viability of CHOP/Bcl-2 cells was decreased to about 75% at 72 h after addition of IPTG. These results establish that high levels of Bcl-2 effectively delay but do not completely block CHOP-mediated apoptosis in M1 cells. This finding may suggest the presence of some other genes for cell survival which are negatively regulated by CHOP protein, besides bcl-2.

In conclusion, we have demonstrated that CHOP induces apoptosis in M1 myeloblastic leukemia cells in p53-independent manner and Bcl-2 delayed this process. p53 is the most commonly mutated gene in human cancer [30], and cells lacking intact p53 protein are resistant to killing by radiation and chemotherapy [31]. To understand the mechanism underlying CHOP-mediated apoptosis in M1 cells may provide a novel therapeutic strategy against p53-inactivated malignancies.

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