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Activation of mitogen-activated protein kinase family proteins accompanied by down-regulation of BCL-2 in anti-IgM-induced apoptosis in WEHI-231 B lymphoma cells

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ABSTRACT

Cross-linking of membrane immunogloblin (mIg) in WEHI-231 B lymphoma cells induced growth arrest in the G1 phase of the cell cycle, followed by induction of apoptosis. The kinetics of anti-IgM-induced activation of three species of the mitogen-activated protein kinase (MAPK) family differed among them: enhancement of extracellular signal-regulated kinase (ERK)1/ERK2 was rapidly induced by treatment of WEHI-231 cells with anti-IgM, while that of c-Jun N-terminal kinase (JNK)1 was late and sustained. The extent of p38 MAPK activation was rapid, but small, and transient. Bcl-2 was completely down-regulated by anti-IgM treatment, whereas the level of Bcl-xL and Bax-α was almost unaltered. The induction of apoptosis appeared to correlate with the down-regulation of Bcl-2. The anti-IgM-induced apoptosis as well as the Bcl-2 down-regulation was reversed by the addition of CD40-ligand (CD40-L). These observations suggest that Bcl-2 down-regulation is involved in anti-IgM-induced apoptosis, probably through the activation of MAPK family proteins.

INTRODUCTION

Cross-linking of membrane immunogloblin (mIg) in immature B cells rapidly results in cell death or anergy leading to B cell unresponsiveness unless appropriate signals such as CD40-ligand (CD-40L) are provided by T cells[1, 2]. WEHI-231 B lymphoma cells, representing an immature B cells, have been widely used as a model for analysis of B cell unresponsiveness and clonal deletion[3, 4], since stimulation of WEHI-231 cells with anti-IgM antibody (Ab) causes growth arrest in the G1 phase of the cell cycle, followed by induction of apoptosis. Anti-IgM-induced apoptosis in WEHI231 B lymphoma cells was rescued by anti-CD40 stimulation or CD40-L[5].

The inductin of apoptosis has been cosidered to be regulated by the balance of bcl-2 family gene products; apoptosis-promoting products (Bax-α and Bad) and apoptosis-inhibiting products (Bcl-2 and Bcl-xL)[6]. Anti-IgM-induced apoptosis is accompanied by down-regulation of Bcl-2 level[7]. Enforced over-exprssion of both Bcl-2 and Bcl-xL protects anti-IgM-mediated apoptosis of immature B lymphoma cells, WEHI-231[8, 9] and CH31[10]. CD-40L-mediated rescue of anti-IgM-mediated

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apoptosis has been shown through upregulation of Bcl-xL[11]. Bax-α, acting as an inhibitor of apoptosis induction, is homo- or heterodimerized with itself or Bcl-2/Bcl-xL on the mitochondrial membrane[12].

A family of mitogen-activated protein kinases (MAPK) has been demonstrated to regulate the proliferation or apoptosis in a positive or negative way depending on cell types or activational stage of the cells[13~15]. The family comprises three distinct kinases; extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK. There are some controversies concerning the role of each kinase in apoptosis induction in B cells. The activity of ERK2 was increased immediately by anti-IgM stimulation in WEHI-231 cells, whereas that of both JNK1 and p38 MAPK was augmented only slightly during 60 min following the stimulation[16]. Lee and Koretzky[17] have demonstrated that anti-CD40 stimulation interferes with the increase in anti-IgM-mediated ERK2 activation, whereas the anti-IgM-induced ERK2 activation has been shown to be unaltered by anti-CD40 stimulation, as reported by Kashiwada et al[18]. In contrast, Graves et al.[19] have shown that anti-IgM-mediated late and sustained increase in JNK activity correlates with the induction of apoptosis in human B104 lymphoma cells.

In the present study, we carefully checked the kinetics of both the activity of the MAPK family and levels of bcl-2 gene family members over a long period in WEHI-231 cells. Our results show that ERK1/ERK2 activation is rapid, but transient, whereas a late andsustained increase in JNK activity appears to parallel the apoptosis induction, accompanied by down-regulation of Bcl-2. Our finding provide a new insight into the signal transduction pathway leading to apoptosis induction.

MATERIALS AND METHODS

Cell Cultures:

WEHI-231 B lymphoma cells ($\mu^+ \varkappa^+$) were maintained in RPMI-1640 medium containing 10% v/v fetal bovine serum (JRH Biosciences, Lenexa, Australia), L-glutamine (2 mM), kanamycin (100 μ/ml), and 2-mercaptoethanol (50 μM) at 37°C in humidified air with 5% CO2.

Reagents:

[γ^{32}-P]-ATP (3000 Ci/mmol) was from NEN Life Science Products (Boston, MA, USA). Myelin basic protein (MBP) was purchased from Upstste Biotechnology Associate (Lake Placid, NY, USA) and ATF2 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). A glutathione S-transferase (GST)-c-Jun(1-79) cDNA[20] and CD40-L-CD8 fusion protein[21] were kind gifts from Dr. Roger J. Davis (University of Massachusetts Medical Center, Worcester, MA, USA) and from Dr. Peter Lane (Basel Institute for Immunology, Bazel, Switzerland), respectively. The following Abs were used: rat anti-mouse IgM heavy chain-specific mAb (Bet 1)(William. E. Paul, Laboratory of Immunology, NIH, USA), mouse anti-rat Bcl-2 mAb (Medical and Biological Laboratories, Nagoya, Japan), rabbit anti-human Bcl-xL Abs, mouse anti-human ERK1 mAb, and mouse anti-rat ERK2 mAb (Transduction Laboratories, Lexington, KY, USA), rabbit anti-human Bax-α (N-terminal) (Upstate Biotechnology Assosiates, Lake Placid, NY, USA), rabbit anti-human JNK1 Abs and rabbit antimouse p38 Abs (Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), rabbit anti-actin Abs (Sigma BioSciences, St. Louis, MO, USA), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG Fc and HRP-goat anti-mouse IgG (Cappel Research Products, Durham, NC, USA). The other reagents were from commercially available sources.

Assessment of apoptosis and cell cycle by flow cytometric analysis:

Percentages of apoptotic cells of total were determined as previously described[22]. Briefly, WEHI-231 cells cultured with anti-IgM for various times were washed with phosphate buffered saline (pH 7.4), fixed in ethanol, followed by incubation with RNase. The cells were stained with propidium iodide and analyzed on a flow cytometer (FACSCalibur, Nippon Becton Dickinson Company Ltd, Tokyo). CELLQuest software (Becton Dickinson Immunocytometry System, San Jose, CA, USA) for cell cycle analysis was used for histogram analysis. Cell nuclei containing less than G1 DNA content were considered to be apoptotic.

In vitro immune complex kinase assay:

WEHI-231 cells treated with anti-IgM for various time periods were solubilized in a lysis buffer (10 mM Tris-HCl, pH 7.4/1% Triton-X100/10 μg/ml aprotinin/10 μg/ml leu-

peptin/1 mM phenyl sulfonylfluoride/1 mM Na3 VO4). The lysates were incubated with anti-ERK1/ERK2, anti-JNK1, anti-p38, MAPK antibodies, and then immunoprecipitated as previously described[23]. The immunoprecipitate was suspended in kinase buffer (20 mM HEPES, pH 7.4/100 mM NaCl/5 mM MnCl2/5 mM MgCl2) with 10 μCi of [γ-32P] ATP in the presence of substrates (MBP, GST-cJun, GST-ATF2, respectivily), followed by incubation for 15 min at 30°C. Sample were separated on 10% SDS/PAGE and then autoradiographed. In some experiments, the level of phosphates incorporated into proteins was measured by BAS 2000 (Fuji Photo Film Co., Ltd, Tokyo, Japan).

Western blotting analysis:

Western blotting was done as previously described[22]. Briefly, samples were separated by SDS/PAGE and proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA), which was blocked with 5% nonfat dry milk in PBS. Membranes were blotted with primary antibody specific Bcl-2, Bcl-xL, Bax-α, and actin. After washes, the bound primary antibody was detected by horseradish-peroxidase-conjugated secondary antibody and enhanced chemiluminescence according to the manufacturer's recommendations (Amersham Life Science, Buckinghamshire, UK).

RESULTS

1. Anti-IgM treatment induces G1 arrest followed by apoptosis in WEHI-231 B lymphoma cells

WEHI-231 B lymphoma cells were cultured with 1 μg/ml anti-IgM for various times and cell cycle analysis was done by the propidium iodide staining method. Cells containing DNA less than 2N were considered to be apoptotic. As shown in Figure 1, apoptotic cells were observed at 36 h (50% vs 5% in medium) following anti-IgM stimulation with increase at 48 h (60%). Anti-IgM-induced apoptosis was preceded by the accumulation of G1 phase at 14 h (55% vs 35% in medium) and 21 h (80%) with sustained accumulation up to 48 h. The cells in both S phase and G2/M phase were inversely declined during 14 h to 48h. These results indicate that mIg cross-linking induces growth arrest of phase of the cell cycle, followed by apoptosis induction.

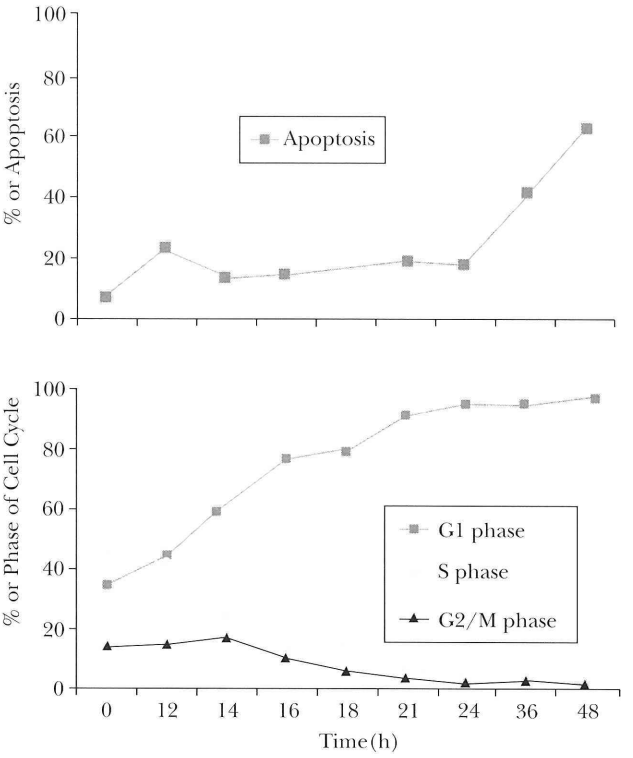

Fig. 1 Induction of both G1 accumulation and apoptosis in WEHI-231 B lymphoma cells induced by anti-IgM stimulation. WEHI-231 B lymphoma cells were cultured with or without 1 μg/ml anti-IgM for various times and cell cycle analysis was done by the PI staining method.

2. ERK1/ERK2, JNK1, and p38, MAP Kinase Activation by anti-IgM stimulation

We checked whether anti-IgM stimulation causes the activation of these kinases by in vitro kinase assay usingspecific substrates for each kinase. The protein level of each kinase, as determined by Western blotting, was unchanged by anti-IgM treatment for up to 24 h (data not shown). ERK1 activation was augmented 7 fold at 5 min with its peak value at 15 min (9-fold) and declined rapidly at 30 min (2-fold), returning to the baseline level at 60 min (Figure 2A). The profile of ERK2 activation was similar to that of ERK1, although theintensity of activity was less than ERK1. No enhancement of ERK1/ERK2 activities was observed from 1 h to 24h following anti-IgM stimulation (data not shown). Although JNK1 activity was not increased up to 6h, considerable increase was obtained during 8h to 20h with a peak value (3 fold) at 12h (Figure 2B). The enhancement of p38MAPK activity displayed two peaks: rapid and transient increase

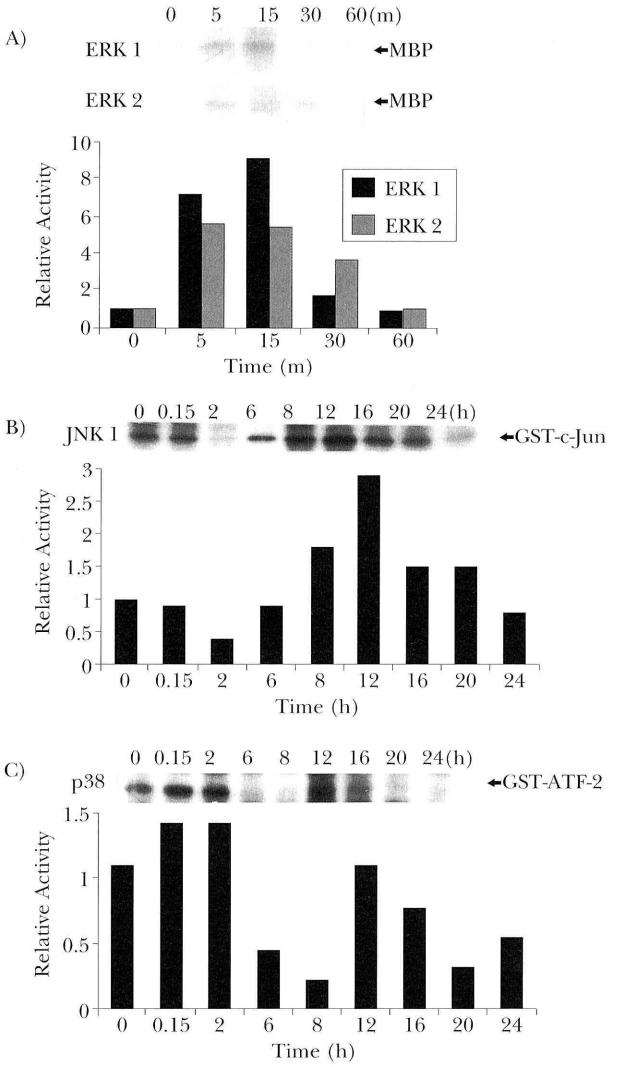

Fig. 2 Potentiation of ERK1/ERK2, JNK1, and p38 MAPK activity by anti-IgM stimulation. WEHI-231 cells cultured with or without 1 μg/ml anti-IgM for the indicated times were lysed with a Triton-X lysis buffer. The lysates (200 μg/sample) were immunoprecipiated with anti-ERK1/ERK2 Ab (A), anti-JNK1 Ab (B), or anti-p38 MAPK Ab (C), respectively. Kinase activity from each sample was determined by an in vitro kinase assay using specific substrates for each kinase. The activity of kinases was expressed as follows: level in stimulated group/level in unstimulated group. The data represent one of several essentially similar results.

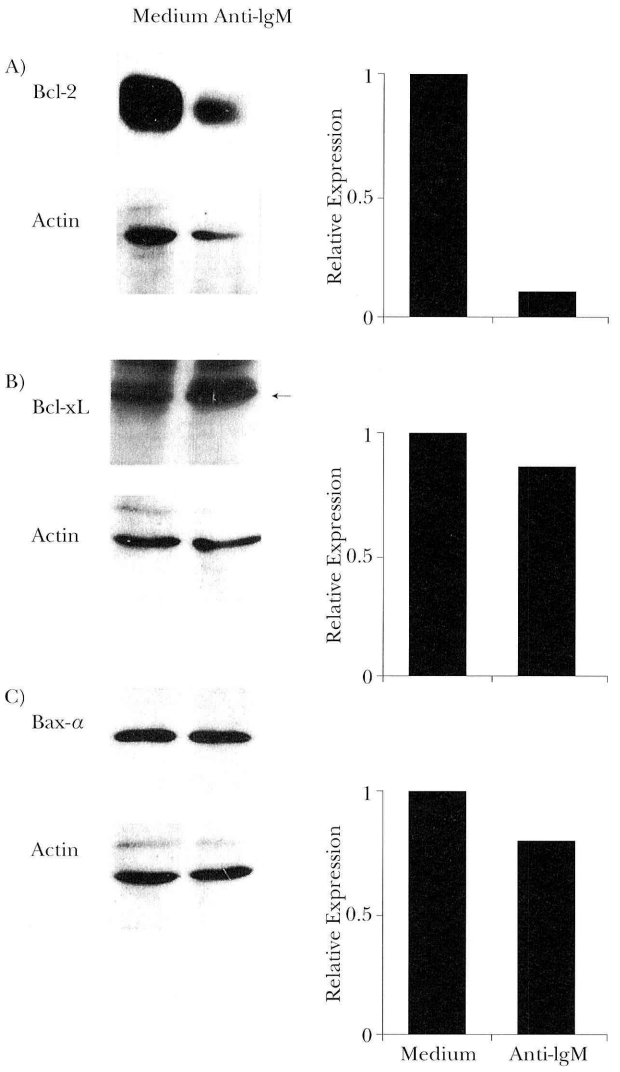

Fig. 3 Down-regulation of Bcl-2 by anti-IgM stimulation. WEHI-231 cells cultured with or without anti-IgM for 48h were lysed with a lysis buffer. Western blotting was used to determine the level Bcl-2 (A), Bcl-xL (B), and Bax-α(C), which was normalized by actin level as follows: (Bcl-2 level in stimulated group/actin level in stimulated group)/(Bcl-2 level in nstimulated group/actin level in unstimulated group).

(1.6-fold) at 10 min and late one (2 fold) at 12hr (Figure 2C). These results indicate that the kinetics of activation of anti-IgM mediated MAPK family proteins differs among them: ERK1/ERK2 activation is rapid and transient, whereas JNK1 activation is late but relatively sustained. However, p38 MAPK activation profile is bimodal.

3. Down-regulation of Bcl-2 following anti-IgM stimulation

bcl-2 family gene products have been shown to be critical for the outcome of the cells, survival or apoptosis: products of bax, bad, and bcl-xs promote apoptosis, whereas those of bcl-2 and bcl-xL inhibit apoptosis[6]. The level of bcl-2 family gene products, Bcl-2, Bcl-xL, and

Bax-α, were therefore examined in anti-IgM treated cells by Western blotting. The Bcl-2 level was down-regulated by more than 95% at 48 h compared with control (Figure 3). However, the level of both Bcl-xL and Bax-α was only slightly (10% and 20%, respectively) down-modulated, suggesting that Bcl-2 is selectively down-regulated in anti-IgM treated WEHI-231 cells. These observations support the notion that the radio of Bcl-2 to Bax-α is critical for the fate of the B lymphoma cells[12].

4. Restoration of anti-IgM-induced apoptosis and Bcl-2 down-regulation by CD40-L

CD40-L has been shown to protect anti-IgM-mediated apoptosis in B cells[5]. WEHI-231 cells were cultured with or without anti-IgM in the presence of CD40-L for 24 h and assayed by the PI staining method and Western blotting. As shown in Figure 4A, the induction of apoptotic cells was confirmed to be reversed by CD40-L, as indicated by the percentage of apoptotic cells. Concomitant addition of CD40-L restores the down-regulation of Bcl-2 induced by anti-IgM (Figure 4B), suggesting that CD40-L functions upstream of Bcl-2 in the anti-IgM-mediated apoptosis induction pathway.

DISCUSSION

Immature B cells are susceptible to apoptosis induction. Lymphoma cell lines representing immature B cells, WEHI-231 or CH-31, have been used as a model for the analysis of the interplay of the signaling cascade via the receptor[3, 4]; cross-linking of antigen receptor induces apoptosis, whereas concurrent stimulation with other receptors blocks the apoptosis induction. However, these complex signaling pathways leading to apoptosis or cell survival remain largely unknown.

Recent studies have shown that both the Bcl-2 family members and MAPK family protein activation participate in the survival or apoptosis induction in various cell types including B cells[6]. For example, overexpression of Bcl-2[10] or Bcl-xL[8, 9, 11] protects apoptosis induction,

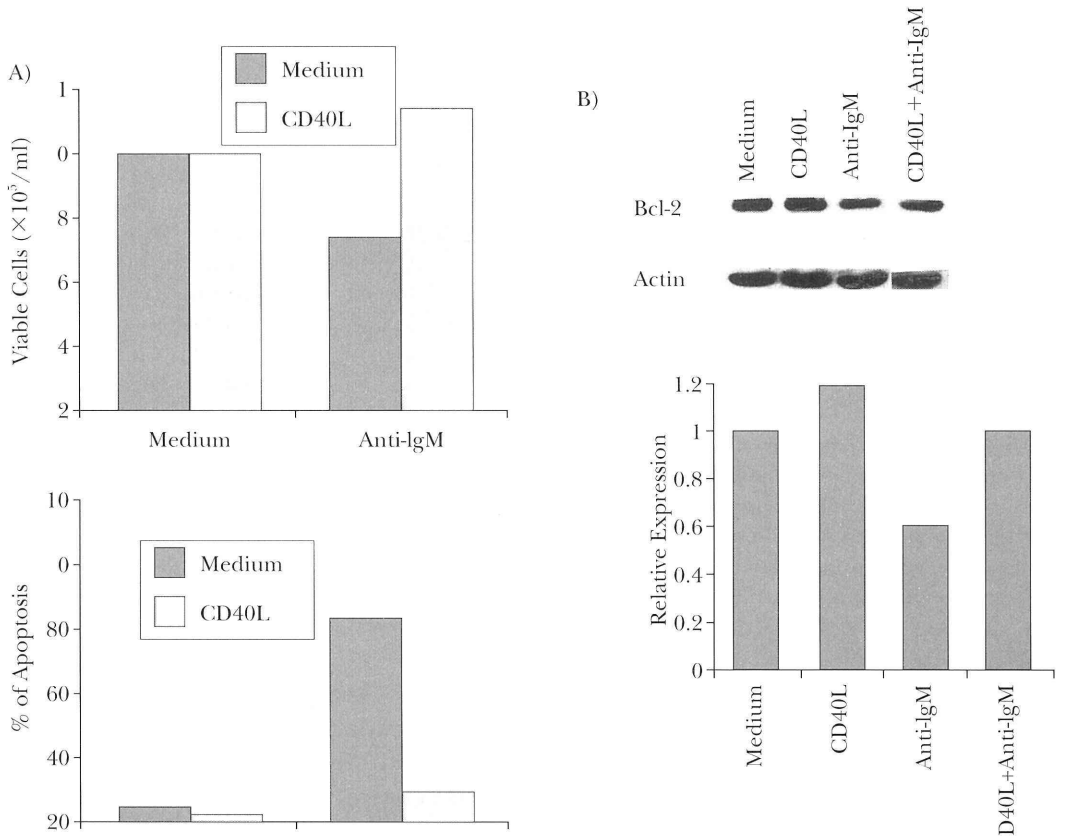

Fig. 4 CD40-L reverses both anti-IgM induced apoptosis and Bcl-2 down-regulation. WEHI-231 cells cultured with or without anti-IgM in the presence or absence of CD40-L for 48 h were assayed for apoptosis (A) and Bcl-2 level (B), as described in Figure1 & 3.

whereas Bax-α overexpression accelerates the cell death[12, 24]. Many recent studies have demonstrated that activation of ERKs, JNKs, and p38, MAP Kinase is involved in receptor-mediated apoptosis or proliferation, depending upon the cell types or activational stage of the cells[13~15]. The activation of MAPK family proteins either protects or promotes apoptosis induction, depending upon the differentiation stage or cell types used. Thus, activation leads to apoptosis in PC12 cells[15],whereas it results in protection of apoptosis in thymocytes[25]. In regard to these observations, some controversyexists concerning B cells: ERK activation is implicated in anti-IgM-mediated apoptosis[16, 17], whereas other investigators claim late sustained p38 and JNK activation play a role in apoptosis induction[19, 26].

In the present study, we carefully checked the time-course of both anti-IgM-mediated apoptosis induction and MAPK protein activation in WEHI-231 B lymphoma cells. Both ERK1 and ERK2 activation was immediately induced by anti-IgM stimulation as reported by others. Although only slight enhancement of JNK activation has been reported at early time points in WEHI-231 B lymphoma cells, we clearly demonstrated a late and sustained potentiation of JNK1 activation. Our observation agrees with the findings of Graves et al.[19] that late and sustained increase in JNK activation correlates with anti-IgM-induced apoptosis in B104 human lymphoma cells. In agreement with the findings that p38 MAPK activation parallels JNK activation in several cell lines[15, 26], our findings indicate that anti-IgM-induced p38 activation pattern is similer JNK activation kinetics with additional early enhancement.

Although activation of the ERK pathway appears to be selectively stimulated by anti-IgM in WEHI-231 B lymphoma cells in the short term assay shown in Figure 2A, as well as other reports[16~18], it remains unknown how early activationis linked to late events such as apoptosis. We found that a late and sustained increase in JNK1 activation was induced in anti-IgM-stimulated WEHI-231 B lymphoma cells, which appeared to parallel the induction of apoptosis. These findings agree with the observation that both JNK and p38MAPK activation correlate with anti-IgM-induced apoptosis in human B lymphoma cells[19]. Alternatively, the possibility equally exists that anti-IgM-mediated late induction of JNK activation is a result, rather than a cause, of a complex apoptotic signaling cascade. Interestingly, however, anti-IgM-mediated apoptosis induction has recently been shown to be inhibited by p38 inhibitor[26]. Experiments are under way to determine whether dominant-negative mutant JNK affects the anti-IgM-induced apoptosis in B lymphoma cells.

Anti-IgM-induced apoptosis appears to parallel the Bcl-2 down-regulation, since a relatively specific down-regulation of Bcl-2, but not Bcl-xL and Bax-α, was obtained in anti-IgM-stimulated B lymphoma cells. In agreement with our observation, enforced expression of mouse bcl-2 blocked anti-IgM-mediated apoptosis[8]. Moreover, Hartley et al.[27] demonstrated that bcl-2 transgene blocked clonal deletion of self-reactive immature B cells using radiation chimera made by reconstituting lethally irradiated hen egg lysozyme (HEL)-transgenic mice with bone marrow cells from anti- HEL antibody transgenic mice with or without bcl-2 transgene. On the contrary, Ishida et al.[11] have reported that bcl-xL mRNA is competely down-modulated with considerable decline in bcl-2 mRNA, whereas Western blotting revels complete susppression of Bcl-2 with slight decline of bcl-xL in anti-IgM-treated WEHI-231 cells (Figure 3), suggesting that these products are regulated by both transcriptional and post-transcriptional control. The expression of Bcl-2 and Bcl-xL is reciprocally regulated during B cell development. Although both products have been shown to protect anti-Ig-mediated immature B cell apoptosis, the mechanisms by which Bcl-2 and Bcl-xL inhibit or delay the clonal deletion of self-reactive B cells have been suggested to differ[6, 8, 27].

CD40, a glycoprotein, is exprssed in B lymphocytes, and follicular dendritic cells, and some epithelial cells. Stimulation through CD40 by anti-CD40 mAb or CD40-L leads to B cell proliferation or protects apoptosis from germinal center B cells or immature B cells[1, 28]. CD40-L-mediated reversal of anti-IgM-induced apoptosis accompanies the restration of Bcl-2 down-modulation, further suggesting that Bcl-2 plays a pivotal role in anti-Ig-induced apoptosis.

Our findings clearly demonstrate that anti-IgM-induced apoptosis involves activation of

MAPK family proteins, which appear to be linked to Bcl-2 down-regulation. The signal transduction pathway though CD40 interferes with the mIg-mediated apoptotic signals, at least the regulation of Bcl-2 levels. These studies may be valuable for analysis of an tigen-mediated clonal deletion and further contribute to the formation of a strategy for intervention in autoimmune diseases.

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Abbreviations used in this paper:

Membrane immunoglobulin, mIg; Monoclonal antibody, mAb; Mitogen-activated protein kinase, MAPK; Extracellular signal-regulated kinase, ERK; c-Jun N-terminal kinases, JNK

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抗 IgM 抗体誘導性アポトーシスにおける BCL-2 低下に伴う MAP キナーゼファミリータンパク質の活性化

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要旨：WEHI-231 リンフォーマ細胞膜上の抗原受容体を坑 IgM 抗体で架橋すると，G1 期の停止，続いてアポトーシスが誘導された．抗 IgM 抗体刺激後の mitogen-activated proteinkinase（MAPK）ファミリータンパク質「extracellular signal-regulated kinase（ERK), c-Jun N terminal kinase（JNK), p38 MAPK」の活性化の時間経過を調べてみると，各々のタンパク質で活性化の経過が異なることが示された．すなわち，ERK1 および ERK2 の活性化は刺激後，速やかに（5 分後）に認められ，15 分後にピーク（6〜9 倍）を示した．JNK1活性化は 12 時間後にピーク（3 倍）を示し，20 時間後まで持続した．p38 MAPK については，10 分後（1.6倍），12 時間後（2 倍）と二峰性の活性化が観察された．アポトーシス誘導に伴って，Bcl-2 レベルは低下したが，Bcl-xL および Bax レベルには変化は認められなかった．さらに，Tumor necrousis factor（TNF）受容体ファミリーに属している CD40 リガンドを加えることにより抗 IgM 抗体誘導性アポトーシスを解消させるとBcl-2 レベルの低下は認められなくなった．これらの事実は Bcl-2 レベルの低下が抗 IgM 抗体誘導性アポトーシスに深く関与しているということを示唆している．また Bcl-2 レベルの低下には MAPK ファミリータンパク質の活性化が寄与していると推定された．

〈**キーワード**〉B 細胞リンパ腫，アポトーシス，MAPK