Combined effects of aberrant MEK1 activity and BCL2 overexpression on relieving the cytokine dependency of human and murine hematopoietic cells

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The MEK1 oncoprotein plays a critical role in Ras/Raf/ MEK/MAPK-mediated transmission of mitogenic signals from cell surface receptors to the nucleus. In order to examine this pathway's role in leukemic transformation, a conditionally active (-estradiol-inducible) form of the MEK1 protein was created by ligating a cDNA encoding an N-terminal truncated form of MEK1 to the hormone-binding domain of the estrogen receptor (ER). We introduced this chimeric MEK1:ER oncoprotein into cytokine-dependent human TF-1 and murine FDC-P1 hematopoietic cell lines. Two different types of cells were recovered after drug selection in medium containing either cytokine or - estradiol: (1) cells that expressed the MEK1:ER oncoprotein but remained cytokine-dependent and (2) MEK1-responsive cells that grew in response to MEK1:ER activation. Cytokine-dependent cells were recovered 10^2 to 10^4 times more frequently than MEK1-responsive cells depending upon the particular cell line. To determine whether BCL2 overexpression could synergize with the MEK1:ER oncoprotein in relieving cytokine dependence, the cytokine-dependent MEK1:ER-expressing cells were infected with a BCL2-containing retrovirus, and the frequency of MEK1-responsive cells determined. BCL2 overexpression, by itself, did not relieve cytokine dependency of the parental cells, however, it did increase the frequency at which MEK1-responsive cells were recovered approximately 10-fold. MEK1:ER+BCL2 cells remained viable for at least 3 days after estradiol deprivation, whereas viability was readily lost upon withdrawal of -estradiol in the MEK1-responsive cells which lacked BCL2 overexpression. The MAP kinases, ERK1 and ERK2 were activated in response to MEK1:ER stimulation in both MEK1:ER and MEK1:ER+BCL2 cells. As compared to the cytokine-dependent MEK1:ER and BCL2 infected cells, MEK1-responsive BCL2 infected cells expressed higher levels of BCL2. While both MEK1-responsive MEK1:ER and MEK1:ER+BCL2 infected cells expressed cDNAs encoding the autocrine cytokine GM-CSF, more GM-CSF cDNAs and bioactivity were detected in the MEK1-responsive MEK1:ER+BCL2 cells than in the MEK1-responsive cells lacking BCL2 or cytokine-dependent cells. These conditionally transformed cells will be useful in furthering our understanding of the roles MEK1 and BCL2 play in the prevention of apoptosis in hematopoietic cells. *Leukemia* (2000) **14,** 1080–1096.
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Introduction

The proliferation of many hematopoietic precursor cells is promoted by interleukin-3 (IL-3), granulocyte/macrophage-colony stimulating factor (GM-CSF), as well as certain other growth factors.[1–3] The loss of cytokine dependency by hematopoietic cells may be an important factor in the development of leukemias.[1,4–11] Cytokine-dependent human and murine hematopoietic cell lines have been previously isolated.[12–14]

The human IL-3/GM-CSF-dependent TF-1 cell line was isolated from a patient with an erythroleukemia. The murine IL-3/GM-CSF-dependent FDC-P1 cell line was isolated from the bone marrow of a normal DBA/2 mouse and resembles cells with a CFU granulocyte/macrophage morphology.[12–14] Since spontaneous factor-independent cells are rarely recovered from TF-1 and FDC-P1 cell lines, these cell lines represent attractive model systems to analyze the effects various oncogenes have on signal transduction, loss of cytokine dependence and leukemogenesis.[1,4–11]

IL-3 and GM-CSF exert their biological activity by binding to the IL-3 and GM-CSF receptors, respectively (IL-3R and GM-CSFR).[15–21] These receptors activate a Janus (Jak2) protein tyrosine kinase which leads to the phosphorylation and dimerization of signal transducers and activators of transcription (STATs).[22,23] These STATs translocate to the nucleus where they regulate the expression of various immediate–early genes. In addition to Jak-STAT activation, receptor ligation promotes phosphorylation of the Shc protein.[24] Phosphorylated Shc results in the recruitment of a complex consisting of Cbl/Grb2/Sos and the guanidine nucleotide exchange factor Vav.[25] This complex leads to the stimulation of Ras as well as additional pathways.[1] Activated Ras can promote the sequential activation of Raf, MEK and the MAP (ERK1 and ERK2) kinases.[26–29]

Certain cytokines and interferons stimulate Jak activity which can activate the Raf kinase pathway. Moreover, for optimal activation of STAT binding activity, STAT dimers must be phosphorylated on key serine residues by the MAP kinases, ERK1 and ERK2, indicating that there is cross-talk between the Jak-STAT and Raf/MEK/MAPK signaling pathways.[27,30]

Raf proteins transmit their regulatory signals to MEK1, a dual specific serine/threonine and tyrosine kinase which phosphorylates the downstream MAP kinases (ERK1 and ERK2). Activation of ERK1 and ERK2 ultimately results in the activation of transcription factors, such as Elk-1 and CREB, that regulate gene expression.[31–34] N-terminal deletion of the MEK1 negative regulatory domain results in a constitutively activated oncoprotein that can transform NIH-3T3 and Chinese hamster ovary cells and abrogate the cytokine dependency of certain hematopoietic cells.[35–39] In addition, evidence suggests that the Raf pathway is intimately associated with the control of the apoptotic machinery in myelo–monocytic cells.[26,40]

The efficiencies of abrogation of cytokine dependency of hematopoietic cell lines by activated MEK1 are much lower than that observed with v-Ha-Ras, v-Raf and other activated oncoproteins.[36,39,41–43] The nature of the inability of activated MEK1 to relieve the cytokine dependency of most hematopoietic cells has not been elucidated. We are interested in the transforming capacity of MEK1 because it is located at pivotal positions in both signal transduction as well as apoptotic pathways.

A member of the apoptotic regulatory proteins, BCL2, has

been described as a repressor of cell death.[44,45] The *bcl-2* gene was isolated from the chromosomal breakpoint of t(14;18)-bearing follicular B cell lymphomas.[44,45] B cells bearing this translocation exhibit increased production of BCL2 and extended cell survival. BCL2 and related BCL-X_L have been shown to protect hematopoietic cell lines from apoptosis following growth factor withdrawal.[46]

Cytokine stimulation has been documented to suppress apoptosis via different mechanisms. The prevention of apoptosis has been associated with the carboxyl region of the IL-3R $_c$ chain which is also responsible for the activation of the Raf/MEK/MAPK cascade.[26,40,47] Stimulation of appropriate target cells by IL-3 leads to phosphorylation of Bad.[48–50] Unphosphorylated Bad normally forms heterodimers with anti-apoptotic factors, such as BCL2 or BCL-X_L, to induce cell death. When Bad is phosphorylated, it is bound and sequestered by the 14–3-3 family of proteins preventing its interaction with BCL2 or BCL-X_L.[48–50] Thus, Bad is no longer associated with BCL2 or BCL-X_L which allows them to bind Bax, resulting in the prevention of apoptosis. Raf, Akt (also known as protein kinase B (PKB)), ERK2, p90^{Rsk}, and protein kinase A (PKA) have all been shown to phosphorylate Bad.[49,51] Recent studies have also suggested roles for MEK1 in some of these apoptotic pathways.[52] Thus, signaling pathways have been implicated in the regulation of anti-apoptotic pathways. Elucidation of the sites of interaction and overlap of these pathways may aid in our understanding, prevention and treatment of leukemia.

Materials and methods

Cell lines and growth factors

Cells were maintained in a humidified 5% CO_2 incubator with Iscove's modified Eagle's medium (IMEM, Life Technologies; Gaithersburg, MD, USA) complemented with 5% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA, USA). The IL-3/GM-CSF-dependent murine myeloid cell line FDC-P1[13,14] was cultured in medium supplemented with 10% WEHI-3B(D$^-$) conditioned medium (WCM) as a source of IL-3. The human TF-1 cell line[11,12] was purchased from the ATCC (Rockville, MD, USA) and grown in RPMI 1640 (RPMI; Life Technologies) containing 5% fetal bovine serum supplemented with 1 ng/ml rGM-CSF (R&D systems, Minneapolis, MN, USA). MEK1-dependent FD/ MEK1:ER and TF/ MEK1:ER cells were grown in RPMI + 5% BCS + 1 M -estradiol or 500 nM 4-Hydroxy-Tamoxifen (4-HT; Sigma, St Louis, MO, USA).

Retroviral infection of cells

Plasmid DNAs containing recombinant retroviruses were transfected into the retroviral packaging cell lines 2 or PA317 with lipofectin (Life Technologies). The retroviruses were passed sequentially from one cell line to the other to amplify their titers as described.[6–8,39] FDC-P1 and TF-1 cells were infected with viral stocks as described.[6–8,39] The nomenclature of the FDC-P1 or TF-1 cells is FD/ MEK1:ER or TF/ MEK1:ER, for cells infected with the MEK1:ER virus.[53–55] After the ER is the designation of whether the cells are IL-3-dependent (IL3), GM-CSF-dependent (GM) or MEK1-responsive (Est). C refers to a clone isolated by limiting dilution and pool refers to a pool of cells. The following G418-resistant (*neo^r*) retro-

viruses were used in this study: (1) LNL6, an empty retroviral vector[53,55] and (2) BCL2, the LNL6 retroviral vector encoding sequences for the human BCL2 protein.[46] *Neo^r* FDC-P1 or TF-1 cells were isolated by selection in medium containing G418 (2 mg/ml; Life Technologies) and either cytokine (IL-3 or GM-CSF) or -estradiol.

Cell proliferation and ^{3}H-thymidine incorporation

Cells were washed three times with phosphate-buffered saline (PBS) before being set up for growth curves or proliferation assays.[39] Growth curves were performed in 5 ml of medium with the indicated supplements in T25 tissue culture flasks (Corning, Elmira, NY, USA). Cell proliferation assays were performed in 96-well flat-bottom plates (Corning) with initially 10^4 cells/well that were incubated for either 1 (FDC-P1) or 3 (TF-1) days in the presence of the indicated supplements. During the last 6 h of incubation, DNA proliferation was measured by adding ^{3}H-thymidine (6.7 Ci/mmol; NEN, Boston, MA, USA) as described.[7,8,39]

In some cases MEK1:ER cells were also cultured with 4-OH Tamoxifen, also dissolved in ethanol which is an estrogen-receptor antagonist but activates the MEK1:ER fusion protein.[39,54,56,57] MEK1:ER-infected cells were also treated with the MEK1 inhibitor, PD98059 (New England Biolabs (NEB), Beverly, MA, USA)[58,59] which was dissolved in dimethyl sulfoxide (DMSO; Sigma). Control cultures were set up with DMSO and either cytokine or -estradiol to measure the toxicity of the solvent. In some cases, cells were treated with the phorbol ester, phorbol 12-myristate-13-acetate (PMA; Sigma) which activates protein kinase C (PKC) as a control.[60–63]

Analysis of cell cycle distribution

Approximately 1×10^7 exponentially growing cells were washed three times with PBS and then incubated at approximately 2×10^5 cells/ml in 25 ml of phenol red-free medium containing BCS but lacking cytokine or -estradiol. This initial time point was designated (–24 h). Twenty-four hours later at the T_0 point, IL-3 (10%) or 4-HT (250 nM) was added to the cultures. Aliquots of approximately 1×10^6 cells were subsequently removed at 12 h intervals starting at the –24 h point. Cell pellets prepared from the different incubation times and culture conditions were resuspended in PBS and then fixed and permeabilized in 70% ethanol overnight. The next day the cells were collected by centrifugation and washed with PBS containing 1% BSA. The cell pellets were resuspended in 0.5 ml of solution containing propidium iodide (50 g/ml), RNase (240 g/ml) and Triton X-100 (0.01% v/v) and stained for 30 min in the dark. The cell suspensions were passed through a 25-gauge needle once before cell cycle analysis. The percentage of cells in the various phases of the cell cycle was analyzed on a Becton Dickinson FACS analyzer. The percentage of cells in the different phases of the cell cycle was statistically analyzed using the ModFit 5.02 program.[64]

Polymerase chain reaction amplification of cytokine mRNA transcripts

Total cytoplasmic RNA was prepared as described[3,39,42,43] and 1 g was included in a 20 l cDNA synthesis reaction con-

taining reverse transcriptase buffer, 1 mM of each dNTP, 20 mg/ml oligo-dT and 20 units Mo-MuLV reverse transcriptase. After incubation at 42°C for 40 min, the reaction was terminated by addition of H_2O. For PCR amplification, 5 l cDNA was included in a 50 l reaction mixture containing PCR buffer, dNTPs, 1–2 units Taq polymerase and 1 mM of each oligonucleotide primer. The primers for murine IL-3 were: AATCAGTGGCCGGGATACCC and CGAAATCATCCAGATCTCG defining a 200 bp cDNA fragment which could readily be distinguished from a genomic IL-3 DNA fragment by size (1 kb). The primers for murine GM-CSF were: CCTGAGGAGGATGTGGCTGC and CTGTCCAAGCTGAGTCAGCG defining a 601 bp fragment. The primers for murine 2-microglobulin were TTCTCTCACTGACCGGCCTG and CAGTAGACGGTCTTGGGCTC defining a 308 bp fragment. The primers for human GM-CSF were: CCTGGACTGGCTCCCAGCAG and GATGTGGCTGCAGAGCCTGC defining a 425 bp fragment. The primers for human -actin were: GAGAAGAGCTATGAGCTGCCT and TTCTGCATCCTGTCAGCAATG defining a 236 bp fragment. The primers for human IL-3 were CTCCTGCCGATCCAAACATGAG and AGAGGTTTCAGAAGTTCTGCTG defining a 575 bp fragment. Thirty-five cycles of PCR were performed to detect the cDNAs. The PCR products were electrophoresed on 1% agarose gels and visualized after ethidium bromide staining of the gel. Bands were verified by Southern hybridization using a full-length probe.

Preparation of cell extracts and analysis by Western blotting

Cells were washed twice with cold PBS and lysed on ice in Gold lysis buffer (GLB) containing 20 mM Tris (pH 7.5), 137 mM NaCl, 5 mM Na_2EDTA, 1% (vol/vol) Triton X-100, 15% (vol/vol) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 g/ml aprotinin, 10 g/ml leupeptin, 1 mM sodium orthovanadate, 1 mM ethylene glycol-bis (-aminoethyl ether)-N,N,N ,N tetra-acetic acid (EGTA), 10 mM sodium fluoride, 1

mM tetrasodium PP_i, and 100 M -glycerophosphate. Insoluble material was removed by centrifugation at 15 000 *g* as described.[39,54,56,57] All chemicals were purchased from Sigma unless otherwise indicated.

Cellular proteins were analyzed by electrophoresis through polyacrylamide SDS gels followed by Western immunoblotting on to polyvinylidene difluoride membranes (PVDF, Immobilon P; Millipore, Medford, MA, USA). Western blots were incubated with the appropriate primary Ab at a dilution of 1:500 (ERK2, BCL-2; Santa Cruz Biotechnology (SCB), Santa Cruz, CA, USA) to 1:1000 (ER; SCB) and then washed in Tris-buffered saline containing 0.05% (vol/vol) Tween-20. Antigen–antibody complexes were visualized by using 1:10 000-diluted protein A coupled to horseradish peroxidase as indicated in the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA).[39,54,56,57] The blots were exposed to Kodak XAR5 X-ray film (Kodak, Rochester, NY, USA) for 30 to 60 s.

Determination of MEK activity

MEK activity was measured by immunoprecipitating the MEK1:ER protein from 100 g total lysate with an ER Ab (SCB) and protein A-sepharose beads as described.[39,54] The immunoprecipitates were then washed three times and used in a kinase reaction for 30 min at 30°C in a reaction mix containing: 100 mM HEPES, pH 7.4, 25 mM $MgCl_2$, 1 mM DTT, 50 M ATP, 10 M ^{32}P-ATP and 2 g of bacterially expressed rp42, an enzymatically-inactive form of MAP kinase (ERK2; Upstate Biotechnology, Lake Placid, NY, USA). The reaction mixtures were electrophoresed through a 10% polyacrylamide gel, transferred to PVDF and exposed to X-ray film. The levels of MEK1:ER proteins used in the kinase assays were often confirmed by probing the kinase blot with the ER Ab.

Table 1 Abrogation of cytokine dependency of MEK1:ER clones after BCL2 infection

Initial cytokine-dependent cell line	Retrovirus[a]	Selection conditions[b]		
		Cytokine + G418[c] (%)	-Estradiol + G418 (%)	DMEM + G418 (%)
FDC-P1	BCL2	384/384 (100)	0/384 (0)	0/384 (0)
	LNL6[d]	384/384 (100)	0/384 (0)	0/384 (0)
	Mock	0/384 (0)	0/384 (0)	0/384 (0)
FD/ MEK1:ERc2[e]	BCL2	384/384 (100)	126/384 (33)	0/192 (0)
	LNL6	384/384 (100)	6/384 (1.5)	0/192 (0)
	Mock	0/384 (0)	0/192 (0)	0/192 (0)
TF-1	BCL2	192/192 (100)	0/192 (0)	0/192 (0)
	LNL6	192/192 (100)	0/192 (0)	0/192 (0)
	Mock	0/192 (0)	0/192 (0)	0/192 (0)
TF/ MEK1:ERc3[e]	BCL2	192/192 (100)	52/192 (27)	0/192 (0)
	LNL6	192/192 (100)	9/192 (5)	0/192 (0)
	Mock	0/192 (0)	0/192 (0)	0/192 (0)

[a]The retroviral stocks contained between 10^5 and 10^6 G418^r retroviruses/ml.
[b]1×10^4 to 1×10^5 cells were plated in each well of a 96-well plate 2 days post infection in the presence of the indicated selective growth conditions. Cytokine + G418 indicates: 10% IL-3 + 2 mg/ml G418 for FDC-P1 cells and 2 mg/ml GM-CSF + G418 for TF-1 cells. -Estradiol + G418 indicates 1 M -estradiol + G418. G418 indicates 2 ng/ml of G418 was added to the basal media + 5% FBS.
[c]Number of wells positive for growth/total number of wells examined.
[d]LNL6 is a retroviral vector which lacks an oncogene.
[e]FD/ MEK1:ERc2 and TF/ MEK1:ERc3 were determined to express the MEK1:ER kinases, respectively, however, they were cytokine-dependent clones.[39] The number of times the particular infection was done is indicated by the total number of wells 96. Similar results were obtained after infection of other cytokine-dependent FD/MEK1:ER and TF/ MEK1:ER clones.

Determination of p42/44^{MAPK} activity

Cells were deprived of either cytokine or -estradiol for 24 h in phenol red-free medium that contained 5% charcoal-stripped BCS. The cells were then pulsed with cytokine or -estradiol for varying time points. Extracts were prepared as previously described.[39] ERK2 activity was determined, after immunoprecipitation of 100 g of total cell lysate with an ERK2 antibody (SCB), using an *in vitro* radioactive kinase assay with 43 mM HEPES, pH 7.4, 43 mM MgCl$_2$, 200 M ATP, 2.3 g myelin basic protein (MBP; Promega, Madison, WI, USA), 10 M -^{32}P-ATP and 0.5 mM DTT. The reactions were electrophoresed through a 14% polyacrylamide gel, transferred to PVDF and exposed to X-ray film. The levels of ERK2 proteins used in the kinase assays were confirmed by probing the blot with the ERK2 Ab.

*Isolation of low molecular weight DNA for apoptosis assays*

1×10^7 cells (4×10^5 cells/ml) were grown in the indicated conditions for 1 day.[39] The cells were pelleted by centrifugation, resuspended in 500 l of lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% Triton X-100) and placed on ice for 10 min. The lysate was then centrifuged for 10 min at 14 000 r.p.m., and the pellet was discarded. Twenty-five l of 4 mg/ml protease K (Sigma) were added to the supernatant, and the mixture was incubated overnight at room temperature. The solution was phenol extracted twice followed by chloroform extraction once. The aqueous phase was saved each time. The nucleic acids were ethanol precipitated and subsequently resuspended in H$_2$O. The concentration of the nucleic acids was determined and 40 g was treated with 10 l of 0.1 mg/ml RNase A (Sigma) for 5–10 min. The remaining DNA was electrophoresed in an agarose gel with 1× TBE (1× = 0.09 M Tris borate, 0.002 M EDTA) running buffer and visualized with ethidium bromide.

Results

Synergy between BCL2 and MEK1 in the abrogation of cytokine dependency of human and murine hematopoietic cells

It has been reported that the *bcl-2* and *raf* oncogenes can interact and may be involved in the prevention of apoptosis in hematopoietic cells.[65–67] Moreover, there is increasing evidence indicating that ERK2, a downstream target of MEK1, phosphorylates BCL2 which, in turn, stimulates the anti-apoptotic activity of BCL2.[68–70] The aim of the following experiments was to determine whether BCL2 overexpression could increase the frequency of hematopoietic cells which could proliferate in response to activated MEK1:ER. Uninfected human TF-1 and murine FDC-P1 cells are strictly cytokine-dependent, as they do not proliferate in the absence of IL-3 or GM-CSF. In the absence of appropriate cytokines these cell lines undergo apoptosis within 1 to 2 days, respectively. The overall design for the following experiments was to infect cytokine-dependent FD/ MEK1:ER and TF/ MEK1:ER clones, which express the MEK1:ER oncoprotein, with either a BCL2-containing retrovirus or the empty retroviral vector (LNL6) and determine whether BCL2 overexpression could increase the frequency of isolation of estradiol-responsive cells.
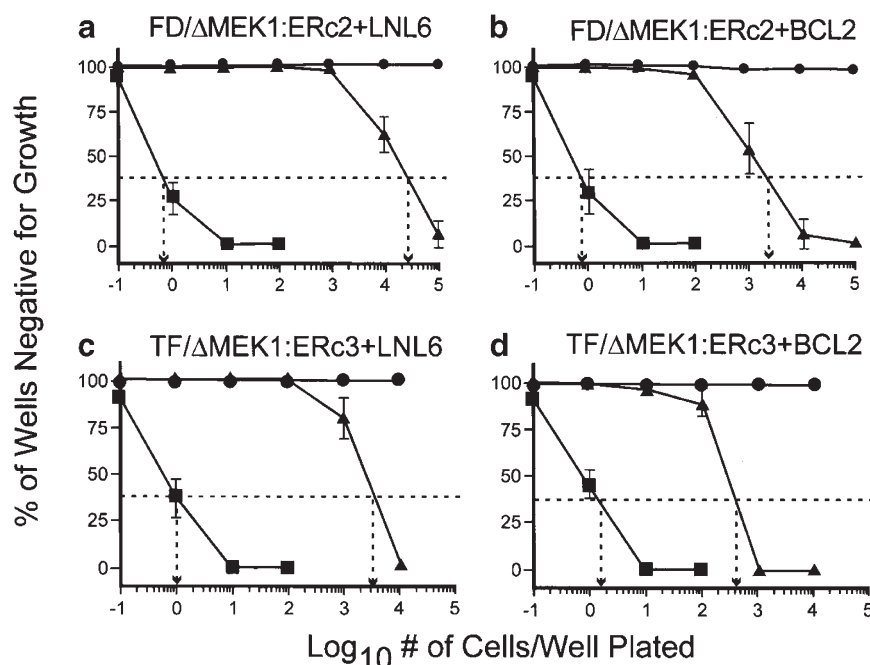
Figure 1 Isolation of MEK1-responsive cells after infection of cytokine-dependent MEK1:ER FDC-P1 and TF-1 cells with a BCL2 containing retrovirus. Limiting dilution analysis of: panel (a) FD/ MEK1:ER(IL3)c2+LNL6, (b) FD/ MEK1:ER(IL3)c2+BCL2, (c) TF/ MEK1:ER(GM)c3+LNL6 and (d) TF/ MEK1:ER(GM)c3+BCL2. Growth in medium containing: ( ), (IL-3, panels a and b), (GM-CSF, panels c and d), -estradiol ( ) or no supplement ( ). Dotted line equals 37% of wells negative for growth from which the differential plating efficiency was estimated by Poisson distribution. Arrow on the X-axis indicates where the experimental data line intersects with the 37% negative for growth line and is an estimate of the number of cells/well required to form a colony. These experiments were performed on four pools isolated from each type of MEK1:ER infected cells. Similar results were observed with two other clones of BCL2 and LNL6 infected FD/ MEK1:ER and TF/ MEK1:ER cells.

Table 2 Frequency of isolation of MEK1-responsive cells after infection with an empty retroviral vector or a BCL2 containing retrovirus[a]

| Initial cytokine-dependent cell line	MEK1-responsive cells after LNL6 infection	MEK1-responsive cells after BCL2 infection	Fold enhancement with BCL2
FDC-P1	1 in 10^7	1 in 10^7	–
FD/ MEK1:ERc2	1 in 2.5 × 10^4	1 in 2.5 × 10^3	10 ×
TF-1	1 in 10^4	1 in 10^4	–
TF/ MEK1:ERc3	1 in 3.5 × 10^3	1 in 4 × 10^2	9

[a]Determined by Poisson analysis as shown in Figure 1.

Three control infections were performed, infection of the parental FDC-P1 and TF-1 cells with: (1) the BCL2 retrovirus, (2) the empty retroviral vector and (3) a mock infection. Infection of parental FDC-P1 and TF-1 cells with BCL2 and LNL6 retroviruses resulted in the isolation of cells which grew in the presence of cytokine and G418 but not in the absence of the required cytokine (eg -estradiol + G418) (Table 1). Thus, parental FDC-P1 and TF-1 cells did not become factor-independent after infection with BCL2 or the empty retroviral vector. In addition, no mock-infected cells grew in

G418-containing medium indicating that the selection conditions were sufficient to isolate retrovirally-infected cells.

Next, cytokine-dependent FD/ MEK1:ER and TF/ MEK1:ER cells were infected with the BCL2 virus, the empty retroviral vector as well as mock infected (Table 1). BCL2 infection increased the recovery of MEK1-responsive FD/ MEK1:ER and TF/ MEK1:ER cells as compared with infection with the empty retroviral vector. -Estradiol was necessary for the recovery of the cytokine-independent clones as no clones were recovered in the absence of -estradiol and cytokine under G418 selection conditions. Thus, even in the presence of constitutive BCL2 overexpression, the cells remained conditionally factor-independent as they required -estradiol to proliferate.

Six different types of hematopoietic cells were isolated after these infections: (1) cells which were infected with the empty retroviral vector (LNL6) that remained cytokine-dependent; (2) cells which were infected with BCL2 that remained cytokine-dependent, (3) cells which were infected with MEK1:ER and LNL6 that remained cytokine-dependent; (4) cells which were infected with MEK1:ER and BCL2 which remained cytokine-dependent; (5) cells which were infected with MEK1:ER and LNL6 that grew in response to -estradiol in the absence of exogenous cytokines; and (6) cells which were infected with MEK1:ER and BCL2 that proliferated in response to -estradiol in the absence of exogenous cytokines.
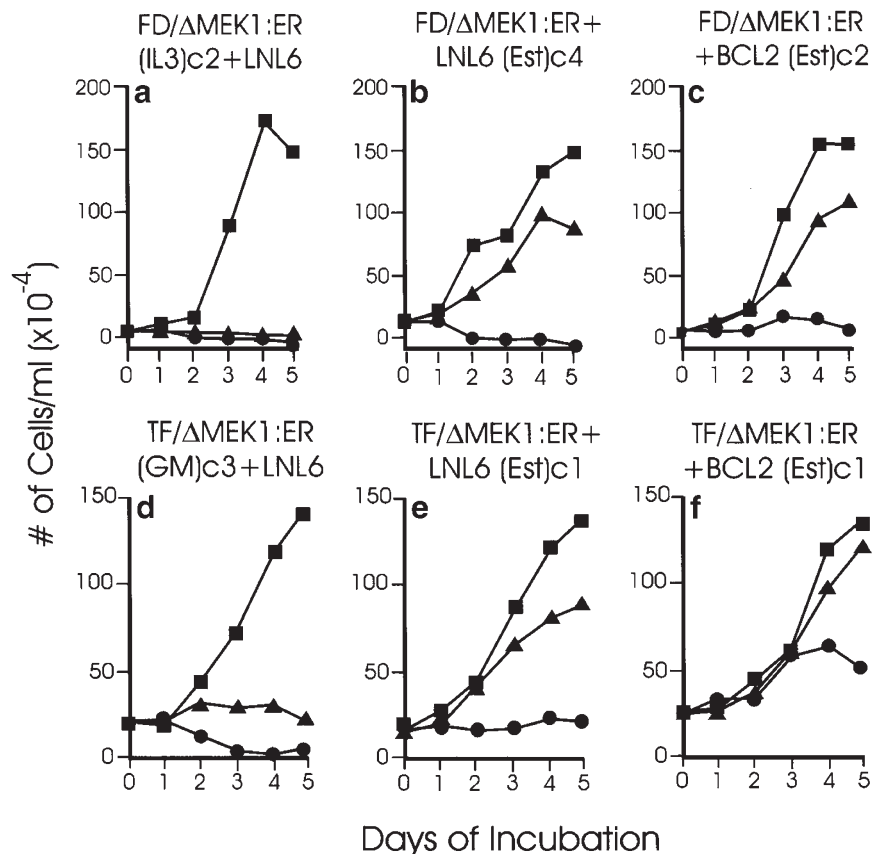
Figure 2 Growth of MEK1:ER and BCL2 infected cells. The growth properties of three different types of MEK1:ER infected cells are compared. Panel (a) IL-3-dependent FD/ MEK1:ER(IL3)c2+LNL6 cells, panel (b), MEK1-responsive FD/ MEK1:ER+LNL6(Est)c4 cells, panel (c) MEK1-responsive FD/ MEK1:ER+BCL2(Est)c2 cells, panel (d), GM-CSF-dependent TF/ MEK1:ER(GM)c3+LNL6 cells, panel (e), MEK1-responsive TF/ MEK1:ER+LNL6(Est)c1 cells, panel (f), MEK1-responsive TF/ MEK1:ER+BCL2(Est)c1 cells. Growth in medium containing IL-3: (, panels a, b and c), GM-CSF (, panels d, e and f), -estradiol ( ), or no supplement ( ). The data presented in Figure 2 are from triplicate sets of experiments using two different clones.
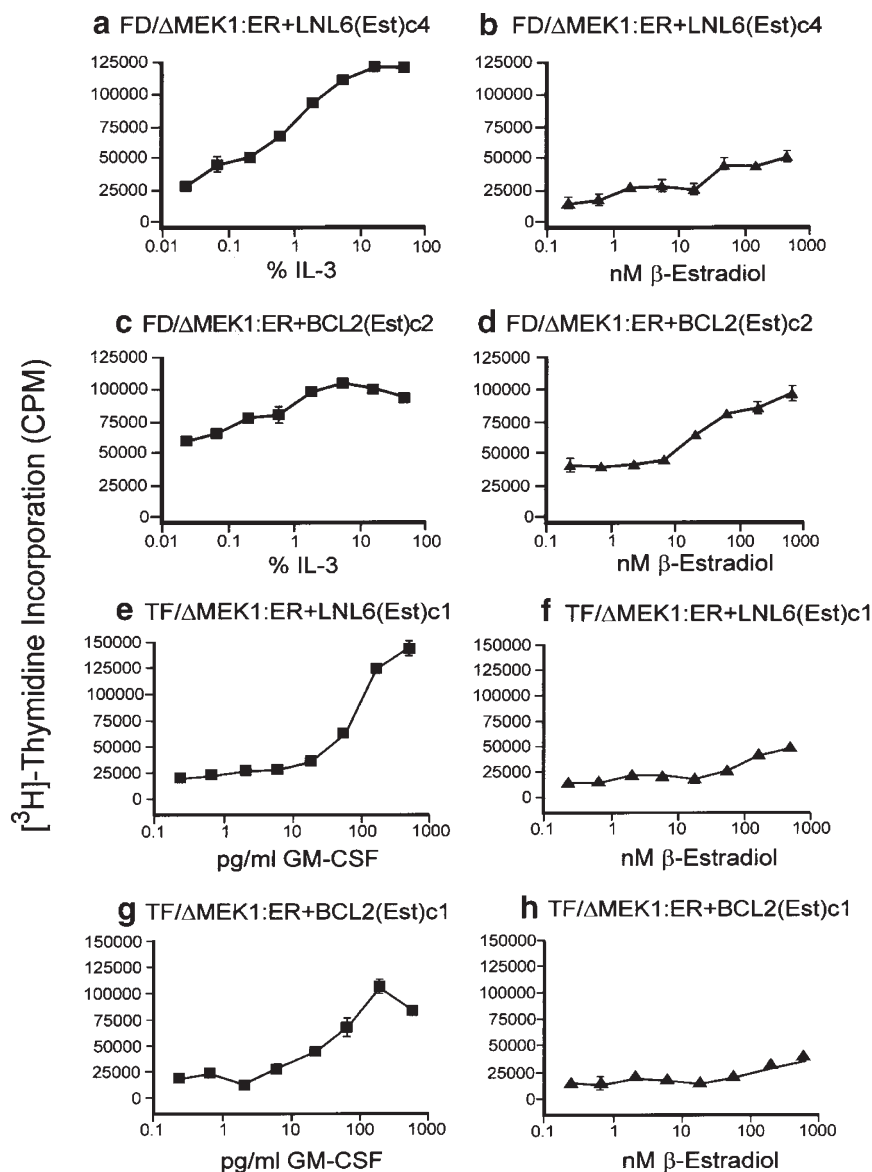
Figure 3 Stimulation of ³H-thymidine incorporation by -estradiol and cytokines in the MEK1-responsive MEK1:ER and BCL2 infected cells. ³H-thymidine incorporation was measured in the different MEK1-responsive cells. Symbols: ( ) % IL-3 (% WEHI-3B conditioned supernatant, panels a and c), ( ) pg/ml GM-CSF (panels e and f). ( ) nM -estradiol. Panels (a and b) FD/ MEK1:ER+LNL6(Est)c4, panels (c and d) FD/ MEK1:ER+BCL2(Est)c2, panels e and f) TF/ MEK1:ER+LNL6(Est)c1, and panels (g and h) TF/ MEK1:ER+BCL2(Est)c1. Similar results were observed with four other FD/ MEK1:ER+LNL6(Est) clones, six other FD/ MEK1:ER+BCL2(Est) clones, three other TF/ MEK1:ER+LNL6(Est) clones and pools and four other TF/ MEK1:ER+BCL2(Est) clones and pools.

Isolation of MEK1-responsive cells from pools of MEK1:ER and BCL2 infected cells

To determine the frequency of isolation of MEK1-responsive cells from MEK1:ER and BCL2 infected FDC-P1 cells, limiting dilution analysis was performed on the pools of infected cells isolated in the presence of IL-3 and G418 (Table 2 and Figure 1, panels a and b). The differences from the experiments shown in Table 1 vs Figure 1 and Table 2 are that the MEK1-responsive cells recovered in Table 1 were from direct selection in -estradiol and G418, whereas the MEK1-responsive cells recovered in Figure 1 and Table 2 were from pools of infected cells. Approximately 1 in 2×10^4 FD/ MEK1:ER(IL3)c2+LNL6 cells could give rise to a colony which grew in -estradiol (Figure 1, panel a). BCL2 overexpression increased the frequency of isolation of FD/

MEK1:ER(IL3)c2 cells (FD/ MEK1:ER(IL3)c2+BCL2; Figure 1, panel b) that would grow in direct response to MEK1:ER activation 10-fold in comparison to cells that were infected with the empty retroviral vector. In contrast, cytokine-independent cells were not isolated from pools of parental FDC-P1 cells infected with either BCL2 or the empty retroviral vector (Table 2 and data not presented). These results have been summarized in Table 2.

In order to determine whether a similar scenario was true with the human TF/ MEK1:ER cells, limiting dilution analysis was performed with these cells. Limiting dilution analysis indicated that 1 in 3.5×10^3 TF/ MEK1:ER(GM)c3+LNL6 cells would grow in the absence of exogenous GM-CSF in the presence of -estradiol (Figure 1, panel c). Approximately ninefold more MEK1-responsive cells were recovered from BCL2 infected TF/ MEK1:ER(GM) cells (TF/ MEK1:ER(GM)c3+

BCL2; Figure 1, panel d) than from empty retroviral vector infected TF/ MEK1:ER cells indicating that BCL2 over-expression increased the recovery of cells which grew in response to MEK1:ER. As controls, the subcloning efficiency of empty vector and BCL2 infected parental cells were examined. The pools of LNL6 and BCL2 infected parental TF-1 cells did not give rise to cytokine-independent clones (Table 2 and data not presented).

Growth properties of BCL2 and MEK1:ER infected FDC-P1 and TF-1 cells

To determine the growth properties of the MEK1:ER cells which overexpressed BCL2, growth curve experiments were performed. Cytokine-dependent and FD/ MEK1:ER(IL3)c2+ LNL6 cells proliferated in the presence but not the absence of IL-3 (Figure 2, panel a). While -estradiol did not promote growth in cytokine-dependent FD/ MEK1:ER(IL3)c2+LNL6 cells (Figure 2, panel a), it did stimulate the growth of the MEK1-responsive FD/ MEK1:ER+LNL6(Est)c4 cells (Figure 2, panels b), however, these MEK1-responsive cells did not grow in the absence of -estradiol or IL-3 indicating that they were conditionally transformed to MEK1-responsive growth.

IL-3 and -estradiol stimulated the growth of the MEK1-responsive FD/ MEK1:ER+BCL2(Est)c2 cells (Figure 2). However, these cells displayed a definitive difference from their MEK1-responsive FD/ MEK:ER+LNL6 siblings as they did not die as rapidly in the absence of IL-3 and -estradiol (Figure 2, panel c).

Similarly, the cytokine-dependent TF/ MEK1:ER(GM)c3+ LNL6 cells did not proliferate in the absence of GM-CSF and -estradiol (Figure 2, panel d). Only a slight increase in cell numbers was observed when the cytokine-dependent cells were cultured in -estradiol. In contrast, the MEK1-responsive TF/ MEK1:ER+LNL6(Est)c1 cells proliferated in the presence of either GM-CSF or -estradiol (Figure 2, panel e). Finally, the growth characteristics of the TF/ MEK1:ER+BCL2(Est)c1 cells were examined. While elevated numbers of these cells were observed when they were cultured in either GM-CSF or -estradiol, relatively high numbers of these viable cells were also observed when they were cultured in the absence of GM-CSF and -estradiol (Figure 2, panel f). However, these cells did not continue to proliferate past 4 days in the absence of GM-CSF and -estradiol.

The ability of -estradiol and IL-3/GM-CSF to stimulate ³H-thymidine incorporation in BCL2 and MEK1:ER infected hematopoietic cells

The sensitivities of the MEK1-responsive FD/ MEK1:ER and TF/ MEK1:ER cells to cytokine and -estradiol were examined using ³H-thymidine incorporation assays, a measurement of the ability of the activated MEK1 oncoprotein to promote DNA synthesis. The FD/ MEK1:ER+LNL6(Est)c4 cells incorporated ³H-thymidine in a dose-dependent fashion in response to IL-3 (Figure 3, panel a). A lower level of ³H-thymidine incorporation was observed in these cells in response to -estradiol (Figure 3, panel b). In contrast, the FD/ MEK1:ER+BCL2(Est)c2 cells had a higher basal level of ³H-thymidine incorporation than the FD/ MEK1:ER+LNL6(Est)c4 MEK1-responsive cells and only showed an approximate two-fold increase in response to either IL-3 or -estradiol (Figure 3, panels c and d).

Similarly, ³H-thymidine incorporation was observed in a dose-dependent fashion in response to either -estradiol or GM-CSF in the MEK1-responsive TF/ MEK1:ER+LNL6(Est)c1 and TF/ MEK1:ER+BCL2(Est)c1 cells; however, the responses were higher with GM-CSF than with -estradiol (Figure 3, panels e–h).

Stimulation of ³H-thymidine incorporation by 4-Hydroxy-Tamoxifen

In order to determine whether endogenous estrogen receptors could be synergizing with MEK1 in the MEK1-responsive cells to lead to cytokine independence, the ability of the estrogen-receptor antagonist 4-OH Tamofixen (4-HT) to promote growth of the MEK1-responsive cells was examined (Figure 4). 4-HT inhibits endogenous estrogen receptors but activates the MEK1:ER fusion protein. MEK1-responsive FD/ MEK1:ER +LNL6(Est)c4 (Figure 4, panel a) and FD/ MEK1:ER+BCL2(Est) c2 (Figure 4, panel b) cells incorporated ³H-thymidine in response to 4-HT. Similar results were observed with the TF/ MEK1:ER+LNL6 and TF/ MEK1:ER+BCL2 clones (Figure 4, panels c and d). These experiments eliminated the possibility that endogenous estrogen-receptors functioned either alone or in synergy with the activated MEK1:ER protein to stimulate proliferation of the MEK1-responsive cells.
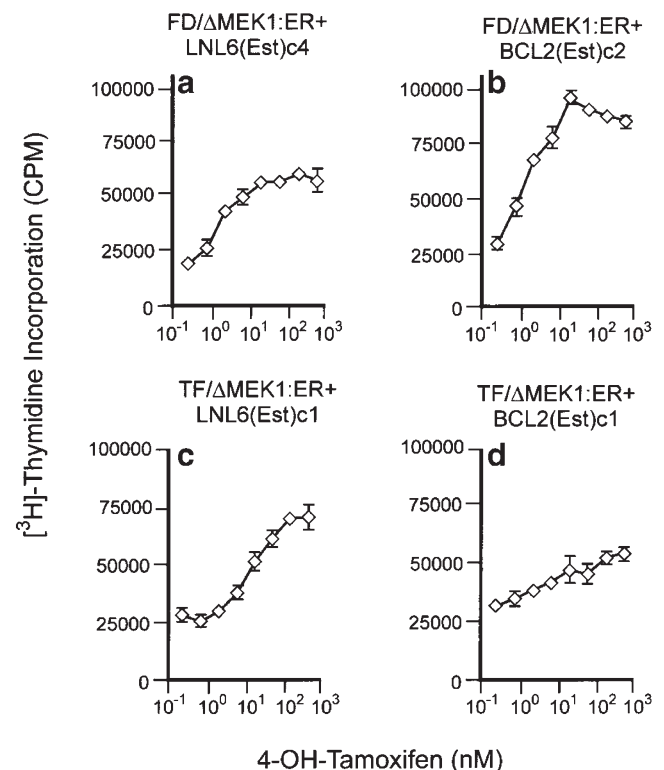
Figure 4 Stimulation of ³H-thymidine incorporation by 4-OH Tamoxifen in the estradiol-responsive MEK1:ER and BCL2 cells. The ability of 4-HT to promote ³H-thymidine incorporation was measured in the MEK1-responsive cells. Symbol: ( ) 4-HT. Panels (a) FD/ MEK1:ER+LNL6(Est)c4, (b) FD/ MEK1:ER+BCL2(Est)c2, (c) TF/ MEK1:ER+LNL6(Est)c1, and (d) TF/ MEK1:ER+BCL2(Est)c1. Similar results were observed with four other FD/ MEK1:ER+LNL6, six other FD/ MEK1:ER+BCL2(Est) clones, three other TF/ MEK1:ER+LNL6(Est) clones and pools and four other TF/ MEK1:ER+BCL2(Est) clones and pools.

A FD/BCL2
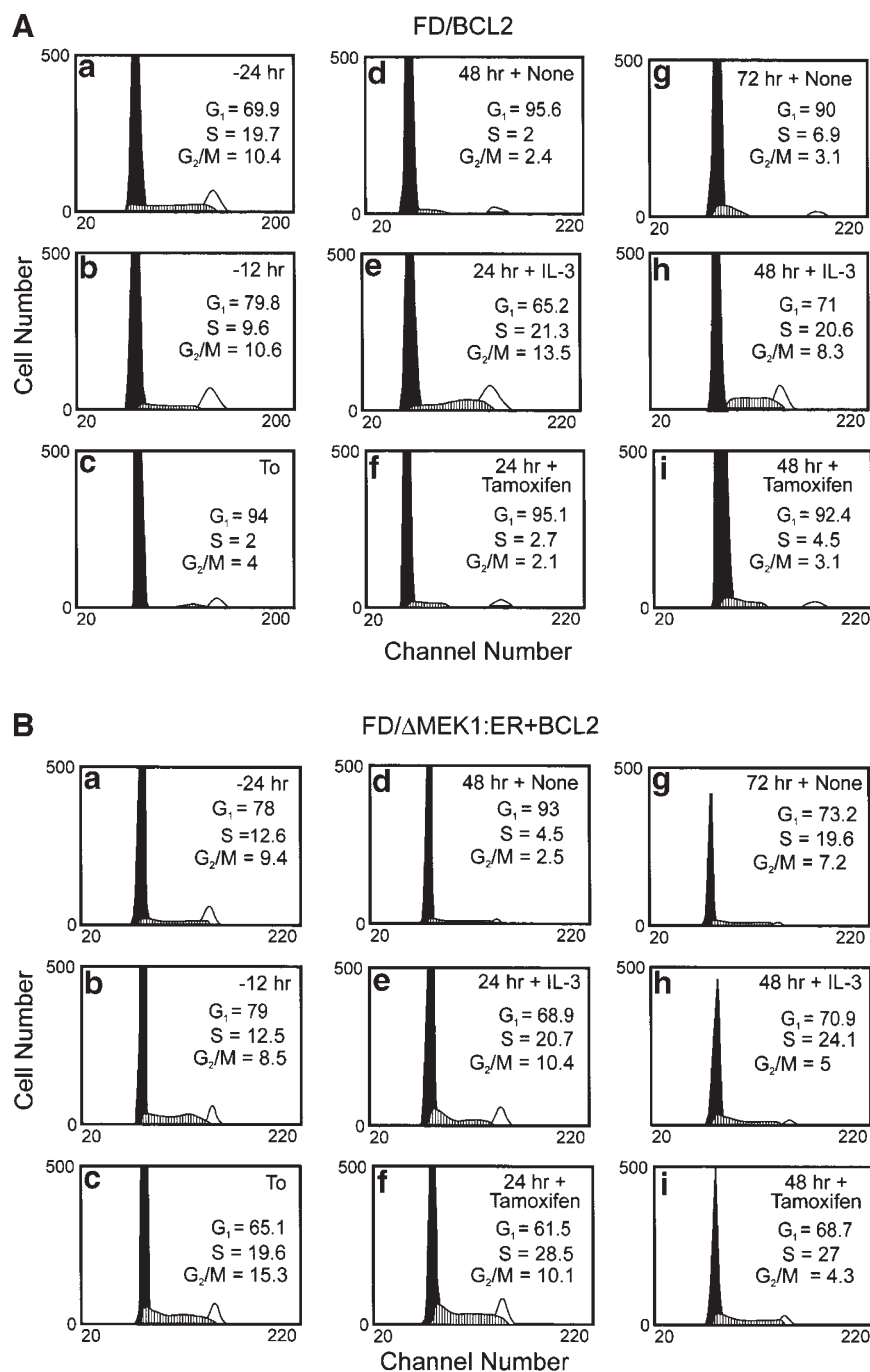
B FD/ΔMEK1:ER+BCL2

Figure 5 Modfit histogram analysis of cell cycle distribution. Top panels: Cell cycle progression in cytokine-dependent FD/BCL2(IL3) cells. Bottom panels: FD/ MEK1:ER+BCL2(Est)c2 cells. In these histograms, the cell number was cut off at 500 in order to show the proportion of cells in S and G_2/M better. The actual percentages of the cells in the different phases are presented in Figure 6. Panel (a) represents the start of the experiments (−24 h). Panel (b) represents −12 h before the start of addition of cytokines or 4-HT. Panel (c) represents the time of addition (T_0) of cytokine or 4-HT. Panel (d) represents cells cultured without IL-3 and 4-HT for an additional 24 h. Panel (e) represents cells cultured with IL-3 for 24 h. Panel (f) represents cells cultured with 4-HT for 24 h. Panel (g) represents cells cultured without IL-3 and 4-HT for an additional 48 h. Panel (h) represents cells cultured with IL-3 for 24 h. Panel (i) represents cells cultured with 4-HT for 48 h. Shaded peak: G_1; stripped peak: S; and open peak: G_2/M. Cells in panels (d, e and f) were collected at the same time and cells in panels (g, h and i) were collected at the same time. The percentages of cells in the G_1, S and G_2/M phases of the cell cycle are presented in each panel for each condition and time point.

Effects of MEK1:ER and BCL2 expression on cell cycle progression

Raf and downstream MEK and ERK have been shown to influence the cell cycle by regulating the expression of the p21[Cip1] cell cycle inhibitory protein.[71] In addition, cell cycle and apoptotic regulatory proteins are typically the targets of anti-neoplastic drugs. Therefore, in order to examine the effects of aberrant MEK1 activation and BCL2 expression on cell cycle progression, we performed cell cycle analyses on

both the cytokine-dependent and MEK1-responsive FDC-P1 cells. This analysis allowed the examination of the percentage of cells in each phase of the cell cycle in response to cytokine stimulation or MEK1 activation (Figures 5 and 6). Initially, cells were starved of IL-3 or -estradiol for 24 h and then stimulated with either IL-3 or 4-HT, which was used in place of -estradiol. After staining with propidium iodide, the percentage of cells in the individual phases of the cells cycle was examined by FACS and the Modfit 5.02 computer program. A diagrammatic example of the effects of MEK1 on cell cycle distribution is presented in Figure 5. Two examples, FD/BCL2(IL3) (top panel) and FD/ MEK1:ER+BCL2(Est)c2 (bottom panel), are presented to show how these cell lines differ in their distribution of cells in the various cell cycle phases after stimulation with IL-3 or 4-HT. Note that after 24 h of starvation, a significant percentage of the FD/ MEK1:ER+ BCL2(Est)c2 cells still remained in S and G_2/M phases (bottom, panel c). In contrast, the proportion of FD/BCL(IL3) cells in the S and G_2/M phases was greatly diminished (top, panel c). Moreover, the effects of 4-HT on cell cycle progression differed greatly between the cell lines. A significant percentage of the cytokine-dependent FD/BCL2 (IL3) cells did not enter S phase in response to 4-HT while a significant percentage of the MEK1-responsive FD/ MEK1:ER+BCL2(Est)c2 cells entered S and G_2/M phases in response to 4-HT.

The extent of cell cycle progression was examined in the other cytokine-dependent and MEK1-responsive MEK1:ER and BCL2 infected FDC-P1 cells. These data represent the average of three to six time course experiments and are presented graphically in Figure 6. Initially (designated –24 h), approximately 25% of the IL-3-dependent and MEK1-responsive cells were in the S phase of the cell cycle which is charac-

teristic of this cell line when it is proliferating exponentially (Figure 6, panels b, e, h, k and n).

The level of cytokine-dependent cells in S phase dropped to approximately 5% after 24 h of cytokine deprivation (Figure 6, panels b, e and h). Likewise, the percentage of cytokine-dependent cells in G_2/M decreased from approximately 10% to 5% 24 h after withdrawal of cytokine (Figure 6, panels c, f and i) while the percentage of cells in G_1 increased from approximately 65% to 90% (Figure 6, panels a, d and g). Thus, removal of cytokine from the cytokine-dependent cells resulted in the cells exiting the S and G_2/M phases and entering the G_1 phase of the cell cycle.

Upon stimulation with IL-3 at T_0, each of the cytokine-dependent cell lines entered the cell cycle (Figure 6, panels a–i). In contrast, while only very few parental FDC-P1 and FD/BCL2(IL3) cells entered the cell cycle in response to 4-HT, a small but significant percentage (12%) of the FD/ MEK1:ER(IL3)c2 cells responded to 4-HT and entered S phase (Figure 6, panels b, e and h).

In sharp contrast to the cytokine-dependent cells, cytokine/ -estradiol deprivation of the MEK1-responsive FD/ MEK1:ER+LNL6(Est)c4 (Figure 6, panel k) and FD/ MEK1:ER+BCL2(Est)c2 (Figure 6, panel n) cells did not result in as many cells exiting S and G_2/M phases of the cell cycle. After 24 h of deprivation, higher percentages of the MEK1-responsive cells, approximately 12.5% of the FD/ MEK1:ER+LNL6(Est)c4 cells (Figure 6, panel k) and 20% of the FD/ MEK1:ER+BCL2(Est)c2 cells (Figure 6, panel n) remained in S phase. Treatment of the MEK1-responsive cells with IL-3 resulted in approximately 40% of the cells entering S phase by 24 h. In addition, unlike in the cytokine-dependent cells where treatment with 4-HT only had a modest effect,
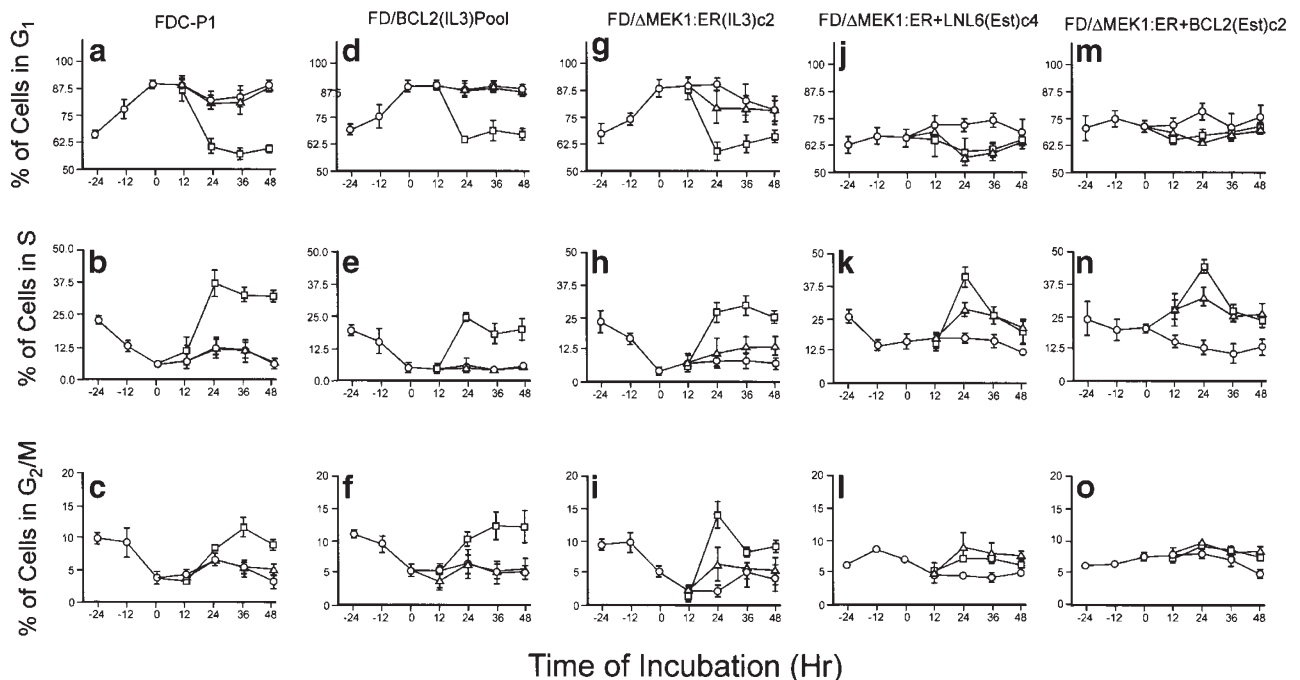
Figure 6 Effects of MEK1 and BCL2 on cell cycle progression in FDC-P1 cells. The percentage of cells in the G_1, S and G_2/M phases of the cell cycle of the indicated cell lines were statistically determined by the Modfit 5.02 computer program after deprivation of cytokine or -estradiol and then treatment with either IL-3 ( ), 4-HT ( ) or no addition ( ). –24 indicates the start of the experiments, 0 indicates when either IL-3 or -estradiol was added. Panels (a, b and c) FDC-P1, panels (d, e and f) FD/BCL2(IL3)Pool, panels (g, h and i) FD/ MEK1:ER(IL3)c2, panels (j, k and l) FD/ MEK1:ER+LNL6(Est)c4 and panels (m, n and o) FD/ MEK1:ER+BCL2(Est)c2 cells. These experiments were repeated three to six times and then averaged together.

treatment of the MEK1-responsive cells resulted in 25% to 30% of the FD/ MEK1:ER+LNL6(Est)c4 (Figure 6, panel k) and FD/ MEK1:ER+BCL2(Est)c2 (Figure 6, panel n) cells entering S phase, respectively. In summary, the cytokine-dependent cells seemed to be blocked from entering S phase in response to 4-HT. BCL2 overexpression alone in the parental FDC-P1 cells was not able to relieve this block in the cell cycle, but in synergy with MEK1:ER, BCL2 overexpression was able to lead to cell cycle progression. This same ability was seen in the MEK1-responsive cells infected with only the empty retroviral vector, FD/ MEK1:ER+LNL6(Est)c4. As will be discussed, these cells were found to overexpress endogenous BCL2.
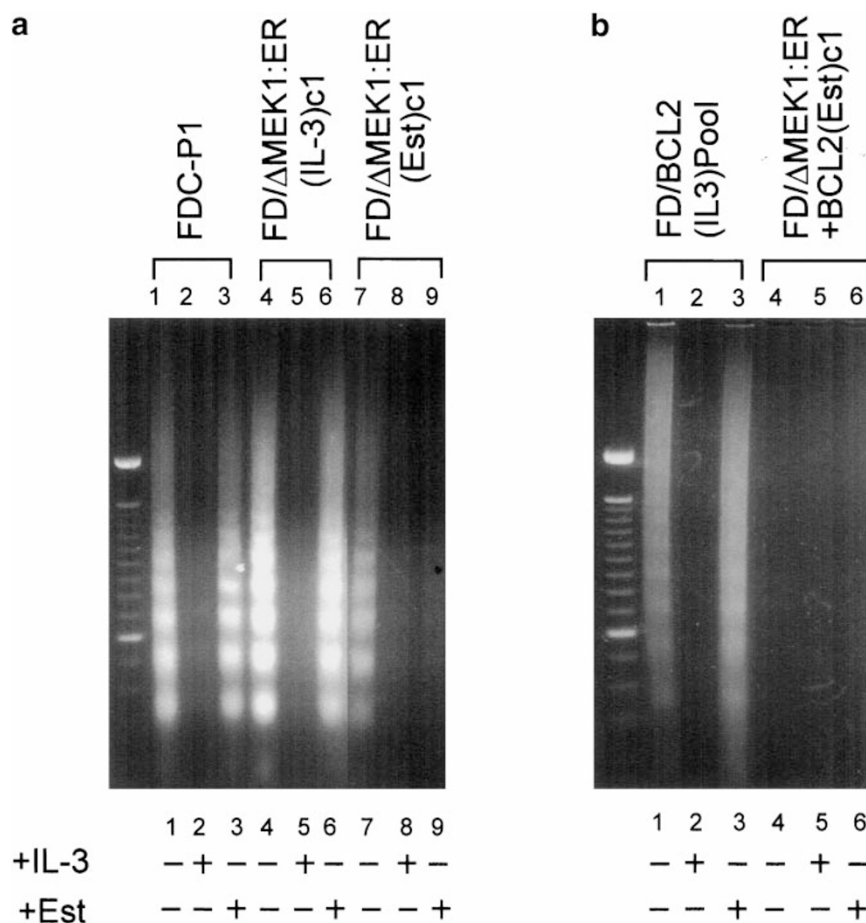
BCL2 overexpression prevents apoptosis in MEK1:ER infected cells

BCL2 expression has been linked to the prevention of apoptosis in hematopoietic cells.[1] In order to determine whether BCL2 overexpression would synergize with MEK1:ER in the prevention of apoptosis, low molecular weight DNA was prepared from cells grown for one day under the indicated conditions (Figure 7). In these experiments, the presence of an apoptotic DNA ladder indicates that the cells have undergone apoptosis, whereas the absence of an apoptotic ladder suggests that the cells have not undergone apoptosis. When the IL-3-dependent cell lines FDC-P1 (Figure

7, panel a, lanes 1 and 3), FD/ MEK1:ER(IL3)c1 (Figure 7, panel a, lanes 4 and 6), or FD/BCL2 (Figure 7, panel b, lanes 1 and 3) were cultured in the absence of IL-3, the cells underwent apoptosis. In contrast, when the same cells were cultured in IL-3 (Figure 7, panel a, lanes 2, 5, and panel b, lane 2) the cells did not undergo apoptosis. Interestingly, the apoptotic DNA ladder obtained from the FD/BCL2 cells was not as intense as that observed from the parental FDC-P1 and FD/ MEK1:ER(IL3)c1 cells. This reproducible result suggests BCL2 was hindering apoptosis in the FD/BCL2 cells but was unable to completely block apoptosis.

When the MEK1-responsive FD/ MEK1:ER(Est)c1 cells were cultured in the absence of IL-3 or -estradiol the cells underwent apoptosis (Figure 7, panel a, lane 7). In contrast, when these cells were cultured with either IL-3 (Figure 7, panel a, lane 8) or -estradiol (Figure 7, panel a, lane 9), the cells did not undergo apoptosis. Similar results were obtained when 4-HT was used in place of -estradiol (data not presented). Thus, MEK1:ER activation prevented the appearance of an apoptotic DNA ladder in the MEK1-responsive cells in the absence of IL-3.

Apoptosis was not observed when the estradiol-responsive FD/ MEK1:ER+BCL2(Est)c2 cells were cultured in the absence of IL-3 or -estradiol (Figure 7, panel b, lane 4). Nor was apoptosis observed when these cells were cultured in the presence of IL-3 (Figure 7, panel b, lane 5) or -estradiol (Figure 7, panel b, lane 6). The FD/ MEK1:ER+BCL2(Est)c2 cells typi-

Figure 7 BCL2 overexpression prevents apoptosis in MEK1:ER infected cells. Low molecular weight DNA was prepared from the indicated cell lines that had been grown in the indicated conditions for 1 day and electrophoresed on a 1.5% agarose gels. These experiments were repeated two to six times and similar results were observed with the different cell lines.

cally had to be deprived of cytokine and β-estradiol for 48 h before a detectable apoptotic ladder was visible. Thus, BCL2 overexpression in conjunction with ΔMEK1:ER expression suppressed apoptosis in these cells to a greater degree than BCL2 alone (Figure 7, panel b, lanes 4–6). As seen in Figure 2, the FD/ΔMEK1:ER+BCL2(Est)c2 cells typically took several days to die after cytokine withdrawal.
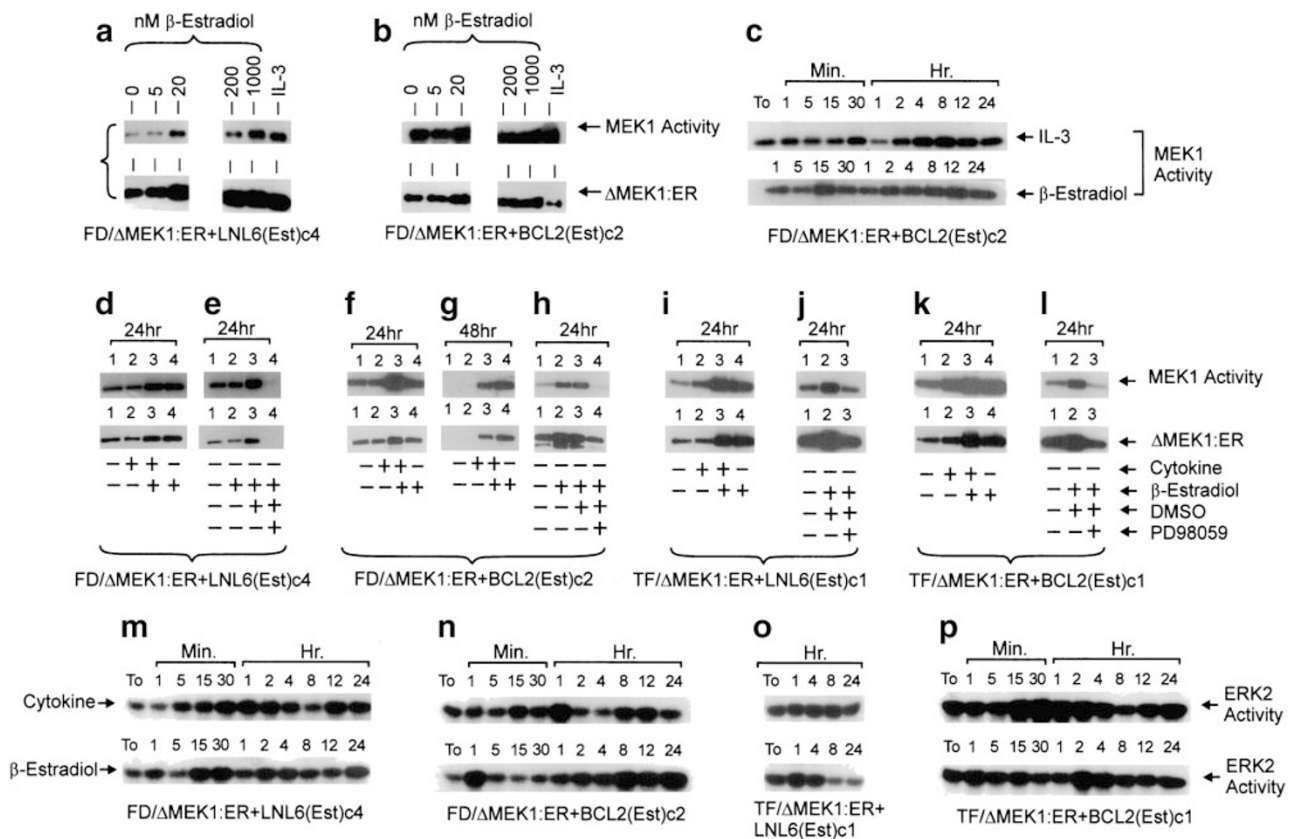
Effects of ΔMEK1:ER and BCL2 on MEK1 and downstream kinase activities

The levels of MEK1 kinase activity were examined in the MEK1-responsive FDC-P1 and TF-1 cells. A dose–response curve of β-estradiol on MEK1 activity was performed with the FD/ΔMEK1:ER+LNL6(Est)c4 and FD/ΔMEK1:ER+BCL2(Est)c2 cells by depriving them of β-estradiol for 24 h and then treating them with varying concentrations of β-estradiol for 4 h. The ΔMEK1:ER encoded kinase was regulated by β-estradiol in the FD/ΔMEK1:ER+LNL6(Est)c4 cells (Figure 8) as higher levels of kinase activity were detected when the cells were stimulated with increasing concentrations of β-estradiol (Figure 8, panel a). In contrast, after 24 h of cytokine-deprivation and then treatment with β-estradiol there did not appear to be a significant difference in the level of MEK1 kin-

ase detected in the FD/ΔMEK1:ER+BCL2(Est)c2 cells (Figure 8, panel b). This result is consistent with the cell cycle data presented in Figure 5 which indicated that a higher proportion of these cells remained in the S phase of the cell cycle after cytokine withdrawal. Often, culture of the FD/ΔMEK1:ER+BCL2(Est)c2 cells in the absence of β-estradiol for 2 days was necessary to reduce the level of ΔMEK1:ER kinase activity to background levels (see Figure 8, panel g below). The ΔMEK1:ER kinase blots were subsequently probed with an ER Ab to measure the amount of ΔMEK1:ER protein used in the kinase assay. The level of the ΔMEK1:ER protein was seen to increase with higher concentrations of β-estradiol. This is believed to be a result of the ability of β-estradiol to stabilize the ΔMEK1:ER protein.[54,56,57]

The kinetics of MEK1 activation were examined in FD/ΔMEK1:ER+BCL2(Est)c2 cells which had been deprived of cytokine and β-estradiol for 24 h. As observed in panel c, the basal level of MEK1 activity in the FD/ΔMEK1:ER+BCL2(Est)c2 cells was reasonably high, but upon treatment with IL-3 or β-estradiol, MEK1 activity increased initially at 15 min and again from 4 to 12 h after stimulation.

MEK1 activity was also examined in MEK1-responsive FD/ΔMEK1:ER+LNL6(Est)c4 and FD/ΔMEK1:ER+BCL2(Est)c2 cells which had been cultured for 24 or 48 h in the absence of IL-3 and β-estradiol or in the presence of IL-3, IL-3 and β-

Figure 8 β-estradiol induces MEK1 and downstream map kinase activities in MEK1-responsive ΔMEK1:ER and BCL2 infected cells. Panel (a) FD/ΔMEK1:ER+LNL6(Est)c4 and panel (b) FD/ΔMEK1:ER+BCL2(Est)c2 cells were deprived of β-estradiol for 24 h and then cultured with the indicated concentrations of β-estradiol or IL-3 (10% WCM) for 4 h. Lysates were prepared and MEK1 activity determined. Panel (c) MEK1 activity was determined in FD/ΔMEK1:ER+BCL2(Est)c2 cells that were deprived of β-estradiol for 24 h and then stimulated with either IL-3 (10% WCM), or 1 μM β-estradiol. MEK1 activity was determined in cells cultured for either 24 or 48 h in the indicated conditions in panels (d, e, f, g, h, i, j, k and l), cells were cultured with β-estradiol (1 μM), cytokines (10% IL-3, panels d, e, f, g and h or 500 pg/ml rGM-CSF, panels i and k). Some cells (panels e, h, j and l) were also treated with β-estradiol (1 μM) and PD98059 (50 μM) or 0.05% DMSO (used to dissolve PD98059). Panels (m, n, o and p) ERK2 activity in cells which were deprived of β-estradiol for 24 h and then stimulated with cytokines IL-3 (10%, panels m and n), GM-CSF (500 pg/ml panels o and p) or β-estradiol (1 μM) for the indicated time periods.

estradiol or -estradiol alone (Figure 8, panels d–h). For these experiments the cells were not previously deprived of cytokine or -estradiol. When the MEK1-responsive FD/ MEK1ER+LNL6(Est)c4 and FD/ MEK1:ER+BCL2(Est)c2 cells were cultured in the absence of either IL-3 or -estradiol for 24 hrs, a background basal level of MEK1 activity was observed (Figure 8, panels d and f, lane 1). Likewise, a basal level of MEK1 activity was observed when they were cultured with IL-3 for 24 h (lane 2). In contrast, when these cells were cultured with -estradiol and IL-3 (lane 3) or -estradiol (lane 4), higher levels of MEK1 activity were observed. When the FD/ MEK1:ER+BCL2(Est)c2 cells were deprived of -estradiol for 48 h (Figure 8, panel g, lane 1) or cultured in the presence of IL-3 for 48 h (lane 2), no MEK1 activity was detected. This is most likely due to the fact that when these cells are cultured in the absence of -estradiol for a prolonged period of time, the level of the MEK1:ER protein decreased significantly (see MEK1:ER protein levels, bottom part of Figure 8, panel g), as -estradiol is necessary for the presence and stability of the protein. In contrast, when the FD/ MEK1:ER+BCL2(Est)c2 cells were grown in the presence of either -estradiol and IL-3 (Figure 8, panel g, lane 3) or -estradiol (lane 4) for 48 h, MEK1 activity was detected.

The effects of the MEK1 inhibitor, PD98059, on MEK1 activity in FD/ MEK1:ER+LNL6(Est)c4 (Figure 8, panel e) and FD/ MEK1:ER+BCL2(Est)c2 (Figure 8, panel h) cells were examined. MEK1 activity was detected in higher levels in the cells that had been treated for 24 h with -estradiol (Figure 8, panels e and h, lane 2) or -estradiol and DMSO (Figure 8, panels e and h, lane 3) than with -estradiol and the MEK1 inhibitor (Figure 8, panels e and h, lane 4). Thus the MEK1 inhibitor suppressed the levels of MEK1 activity in response to -estradiol.

Similarly, the level of MEK1 activity in the MEK1-responsive TF/ MEK1:ER+LNL6(Est)c1 and TF/ MEK1:ER+BCL2(Est)c1 cells (Figure 8, panels i–l) was usually detected at higher levels when the cells were treated with GM-CSF and -estradiol or

-estradiol (Figure 8, panels i and k, lanes 3 and 4) than when they were treated with GM-CSF alone (Figure 8, panels i and k, lane 2). Likewise, the MEK1 inhibitor also suppressed MEK1 activity in these cells (Figure 8, panels j and l, lane 3).

The ability of cytokine and -estradiol to induce ERK2 activity was examined in the FD/ MEK1:ER+LNL6(Est)c4, FD/ MEK1:ER+BCL2(Est)c2, TF/ MEK1:ER+LNL6(Est)c1, and TF/ MEK1:ER+BCL2(Est)c1 estradiol-responsive cells (Figure 8, panels m–p). Both -estradiol and cytokine induced ERK2 activity as early as 15–30 min after addition to the cytokine-deprived cells. The level of ERK2 activity typically peaked initially at 1–2 h after stimulation but then peaked again around 8–12 h after stimulation and remained elevated for up to 24 h (Figure 8, panels m–p).

Expression of the BCL2 oncoprotein in the MEK1:ER and BCL2 infected cells

The expression of the BCL2 oncoprotein was examined in the parental FDC-P1 and TF-1 cells infected with BCL2 as well as the MEK1:ER+LNL6 and MEK1:ER+BCL2 cells. Overexpression of BCL2 was detected in the parental cells infected with BCL2 alone (Figure 9, panels a and c). However, no cytokine-dependent clones were ever recovered from these pools.

In the FD/ MEK1:ER(IL3)c2 cells, endogenous expression of BCL2 was barely detectable (Figure 9, panel b). The level of BCL2 in these cells appeared to increase at 15–30 min after IL-3 or 4-HT stimulation but then rapidly fell to background levels by 2 h. In contrast, the FD/ MEK1:ER cells infected with BCL2 expressed constitutively elevated levels of BCL2 as compared to uninfected FD/ MEK1:ER cells. The levels of the overexpressed BCL2 in these cells did not vary significantly with the different treatments. Interestingly, the MEK1-responsive FD/ MEK1:ER+BCL2 cells, were found to express higher levels of BCL2 as compared to the cytokine-dependent FD/ MEK1:ER+BCL2 cells (Figure 9, panel f and data not shown).
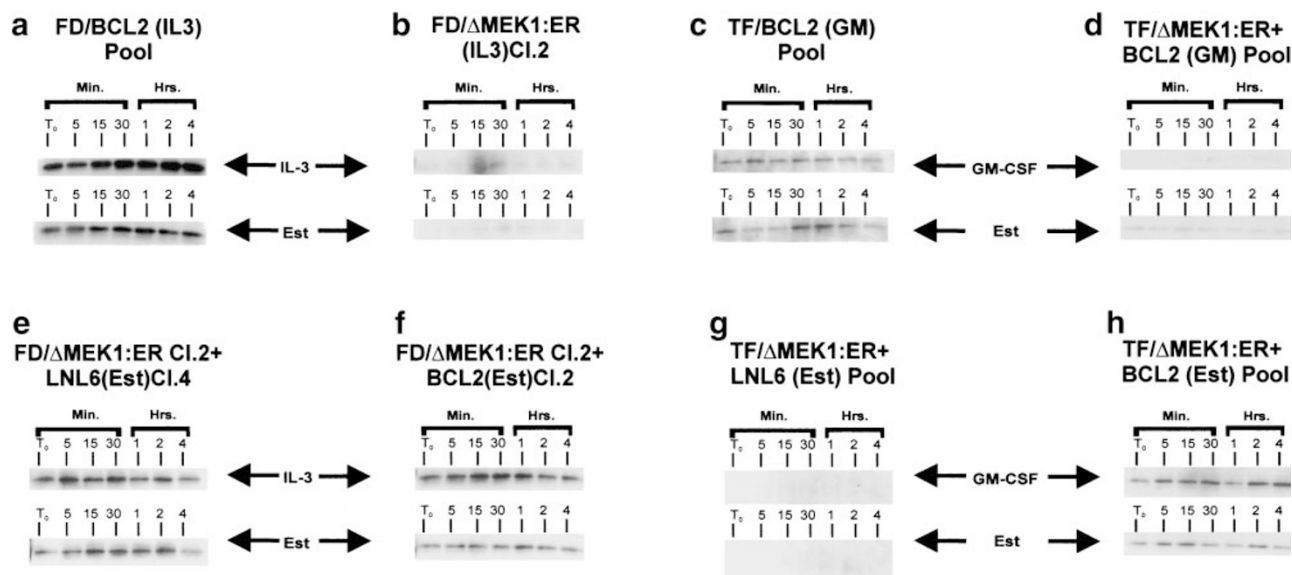
Figure 9 Expression of the BCL2 oncoprotein in MEK1-responsive cells. The expression of the BCL2 oncoprotein was examined in parental+BCL2, MEK1:ER+LNL6 and MEK1:ER+BCL2 cells by Western blot analysis. To examine the level of BCL2 protein expression, cells were deprived of -estradiol and serum for 4 h and then treated with the indicated supplements. BCL2 was immunoprecipitated, run on 14% polyacrylamide gels and blotted with the BCL2 Ab. Panel (a) FD/BCL2(IL3), panel (b) FD/ MEK1:ER(IL3)c2, panel (c) TF/BCL2(GM), panel (d) TF/ MEK1:ER+BCL2(GM)Pool, panel (e) FD/ MEK1:ER+LNL6(Est)c4, panel (f) FD/ MEK1:ER+BCL2(Est)c2, panel (g) TF/ MEK1:ER+LNL6(Est)Pool and panel (h) TF/ MEK1:ER+BCL2(Est) Pool.

As expected, the level of BCL2 detected in the cytokine-dependent FD/ MEK1:ER+LNL6 cells was similar to that observed in the uninfected FD/ MEK1:ER cells (data not shown). Surprisingly though, it was found that the MEK1-responsive FD/ MEK1:ER+LNL6(Est)c4 cells expressed levels of endogenous BCL2 comparative to the levels observed in the MEK1-responsive FD/ MEK1:ER+BCL2 cells (Figure 9, panel e).

Much like that observed in the FD/ MEK1:ER+BCL2 cells, BCL2 levels were found to be constitutively elevated in the TF/ MEK1:ER+BCL2 cells, with the MEK1-responsive cells expressing the higher levels of BCL2 as compared to the cytokine-dependent TF/ MEK1:ER+BCL2 cells (Figure 9, panels d and h). In contrast to the MEK1-responsive FD/ MEK1:ER+LNL6(Est)c4 cells, the MEK1-responsive TF/ MEK1:ER+LNL6(Est) cells did not express elevated levels of BCL2 and therefore were transformed to cytokine independence by some additional mechanism (Figure 9, panel g).

Autocrine growth factor synthesis by MEK1:ER and BCL2 infected cells

To determine whether there was a possible autocrine component to the growth induced by MEK1:ER and BCL2, the presence of autocrine growth factors was determined by examining the ability of the supernatants to stimulate the growth of the parental cell lines (Figure 10). The supernatant prepared from FD/ MEK1:ER+BCL2(Est)c2 and TF/ MEK1:ER+BCL2(Est)c1 cells readily supported ^{3}H-thymidine incorporation in the parental FDC-P1 (Figure 10, panel a) and TF-1 cells (Figure 10, panel b). The cytokine secreted by MEK1-responsive FD/ MEK1+BCL2(Est)c2 and TF/ MEK1:ER+BCL2(Est)c1 cells was determined to be GM-CSF by neutralization experiments with GM-CSF and IL-3 antibodies (data not presented). Moreover, the level of GM-CSF production was sufficient to support the growth of parental FDC-P1 (Figure 10, panel c) and TF-1 (Figure 10, panel d) cells. The significance of this autocrine growth factor production was further examined by treatment of the cells with an GM-CSF antibody. Treatment of MEK1-responsive FD/ MEK1:ER+BCL2(Est)c2 and TF/ MEK1:ER+BCL2(Est)c1 cells with the anti-GM-CSF antibody reduced ^{3}H-thymidine incorporation by approximately 50%. In contrast, treatment of the cells with an IL-3 antibody or control antibody, of the same immunoglobulin subclass, did not inhibit ^{3}H-thymidine incorporation (Figure 10, panel e). Autocrine GM-CSF secretion was also detected in the MEK1-responsive FD/ MEK1:ER+LNL6(Est)c4 and TF/ MEK1: ER+LNL6(Est)c1 cells. However, the level of GM-CSF secretion was 2.5- to 5-fold lower than that detected in the MEK1-responsive cells which express the BCL2 oncogene (data not presented, see below).

Presence of GM-CSF cDNAs in the MEK1:ER and BCL2 infected cells

To provide further evidence that the bioactivity that was detected was indeed GM-CSF, the presence of GM-CSF and IL-3 cDNAs was examined by RT-PCR (Figure 11). As a control, the presence of IL-3 and GM-CSF cDNAs were examined in the T cell thyoma cell line, EL4, prior to and after treatment with the phorbol ester, PMA, for 24 h. While GM-CSF cDNAs were detected in unstimulated EL4 cells, their levels increased upon stimulation with PMA. In addition, IL-3 cDNAs were
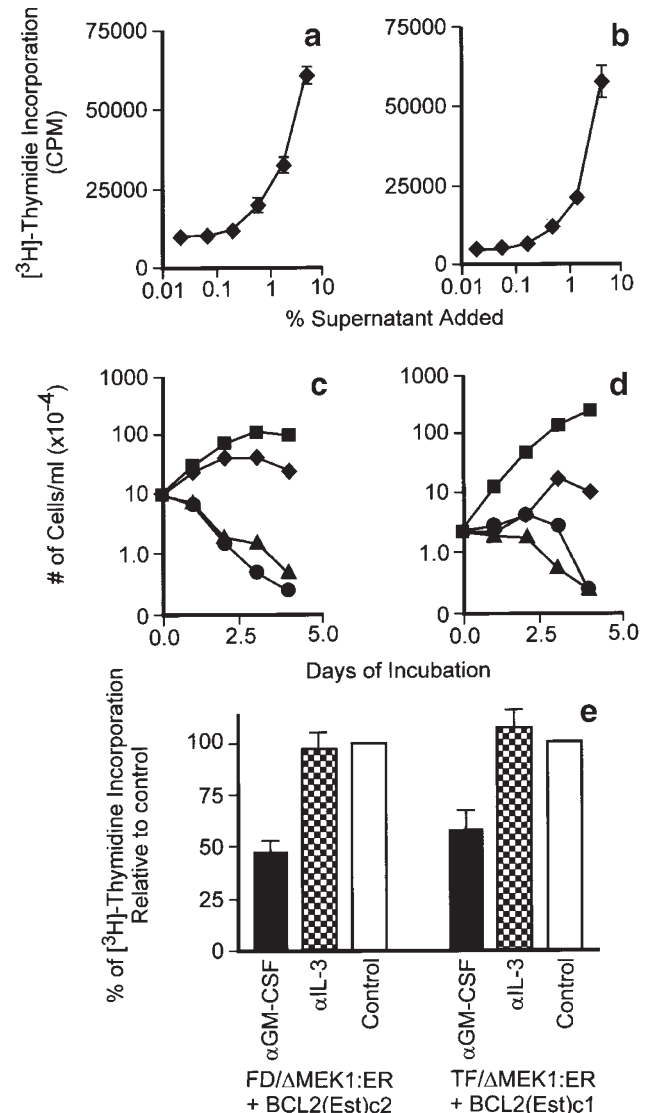
Figure 10 Detection of autocrine growth factor activity in MEK1:ER and BCL2 infected cells. The ability of the supernatants to support ^{3}H-thymidine incorporation in the parental cells was determined (panels a and b). Panel (a) ( ) supernatant from FD/ MEK1:ER+BCL2(Est)c2 cells was titered on the FDC-P1 cell line. Panel (b) ( ) supernatant from the TF/ MEK1:ER+BCL2(Est)c1 cells was titered on the TF-1 cell line. The ability of the supernatant prepared from FD/ MEK1:ER+BCL2(Est)c2 and TF/ MEK1:ER+BCL2 (Est)c1 cells to support the growth of the respective parental cells was determined (Panels c and d). Panel (c) FDC-P1 cells cultured with: ( ) 10 % WCM), (10 % supernatant from the FD/ MEK1:ER+BCL2(Est)c2 cells), (1 M -estradiol) and (no addition). Panel (d) TF-1 cells cultured with: (500 pg/ml GM-CSF), (10 % supernatant from the TF/ MEK1:ER+BCL2(Est)c2 cells), (1 M -estradiol) and (no addition). Panel (e) Inhibition of MEK1:ER+BCL2-mediated growth by GM-CSF antibodies. The effects of monoclonal antibodies to GM-CSF and IL-3 on ^{3}H-thymidine incorporation was determined (Panel e). Controls were an -murine IL-6 Ab for the FD/ MEK1:ER+BCL2(Est)c2 cells and -human IGF1R for the human TF/ MEK1:ER+BCL2(Est)c1 cells.

detected upon PMA stimulation. cDNAs encoding either IL-3 or GM-CSF were not detected in FDC-P1, FD/BCL2(IL3)Pool, FD/ MEK1:ER(IL3)c1, TF-1, TF/BCL2(GM)Pool, TF/ MEK1: ER(GM)Pool or TF/ MEK1:ER+BCL2(GM)Pool cells which remained cytokine-dependent. cDNAs encoding GM-CSF but
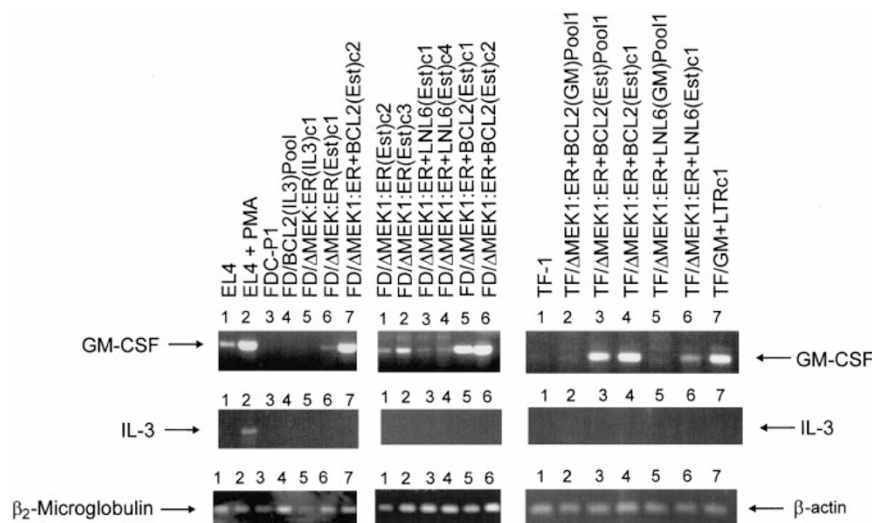
Figure 11 Presence of autocrine GM-CSF transcripts in MEK1:ER and BCL2 infected cells. The identity of the cytokines secreted by MEK1:ER and BCL2 infected cells was further confirmed by RT-PCR analysis performed on mRNA samples from the cells. The EL-4 thymoma cell line was used as a control. Higher levels of GM-CSF cDNA transcripts were detected when EL-4 cells were stimulated with PMA than in unstimulated EL4 cells. Similar amounts of mRNAs were used in the RT-PCR analysis as determined by the relatively equal levels of β_2-microglobulin and β-actin cDNAs detected.

not IL-3 were detected in the MEK1-responsive cells implicating a possible autocrine mechanism of transformation. Usually higher levels of GM-CSF cDNAs were detected in the MEK1-responsive FD/ MEK1:ER+BCL2(Est)c2 and TF/ MEK1:ER+BCL2(Est)c1 cells than in the FD/ MEK1:ER+LNL6(Est)c4 and TF/ MEK1:ER+LNL6(Est)c1 cells. Thus, conversion of hematopoietic cells to MEK1-responsive growth in the presence of constitutive BCL2 expression was associated with the production of autocrine GM-CSF.

Discussion

We recently reported that infection of two different cytokine-dependent hematopoietic cell lines, murine FDC-P1 and human TF-1, with a MEK1:ER containing retrovirus resulted in the isolation of cells with two growth factor phenotypes, cytokine-dependent and MEK1-responsive (cytokine-independent).[39,74] However, both types of cells expressed the MEK1:ER oncoprotein. Due to the relatively low frequency of isolation of MEK1-responsive cells, we sought to examine if there were other genes which could interact with the activated MEK1 oncoprotein to relieve the cytokine-dependence of FDC-P1 and TF-1 cells. *Bcl-2* was chosen as a gene to examine as other investigators have previously shown that members of the Raf/MEK/MAPK pathway may interact with BCL2 in order to regulate apoptosis.[65,66] Raf has been shown to associate with BCL2, and it has been suggested that the association of Raf with BCL2 allows for the phosphorylation of BCL2 by Raf, as well as the translocation of Raf to the mitochondria where it may phosphorylate additional targets.[65,66] Moreover, the downstream ERK2 and p90[Rsk] kinases may phosphorylate BCL2 at a key serine residue near the amino terminal end. Phosphorylation of BCL2 at this site is believed to convey BCL2 with its anti-apoptotic ability.[68–70,72,73] Earlier analysis of the cytokine-dependent and MEK1-responsive FDC-P1 and TF-1 cells indicated that MEK1 activation in the MEK1-responsive cells resulted in the suppression of apoptosis, whereas MEK1 activation in the cytokine-dependent cells was unable to significantly suppress apoptosis (data not published).

In this study, the cytokine-dependent MEK1:ER cells were infected with a retrovirus encoding BCL2 or the empty retroviral vector, LNL6, as a control. Infection with the BCL2 retrovirus increased the recovery of MEK1-responsive MEK1:ER cells approximately 10-fold as compared to infection with the empty retroviral vector. Thus, BCL2 overexpression could compliment aberrant MEK1 activation and increase the recovery of cytokine-independent cells. Although BCL2 overexpression was able to prolong survival of these cells, the growth of these MEK1-responsive cells was conditional as removal of -estradiol eliminated growth and eventually induced apoptosis.

The possibility of endogenous estrogen receptors functioning in synergy with MEK1 and BCL2 was excluded by the use of the estrogen receptor antagonist 4-OH Tamoxifen. The MEK1-responsive cells maintained the ability to incorporate ^{3}H-thymidine even when endogenous estrogen receptor activation was antagonized.

Cytokine-dependent FDC-P1 and FD/BCL2 cells were found to exit the cell cycle after cytokine withdrawal and only re-enter the cell cycle when IL-3 was added. In contrast, a small percentage of cytokine-dependent FD/ MEK1:ER+LNL6 cells were found to remain in S phase after IL-3 withdrawal as measured by both cell cycle FACS analysis and ^{3}H-thymidine incorporation. These same cytokine-dependent FD/ MEK1:ER+LNL6 cells entered the cell cycle when IL-3 was added as was observed in the cytokine-dependent cells which lacked the MEK1:ER protein, but these cells also enter S phase in response to 4-HT. Although some of these cells were able to enter S phase, these cytokine-dependent FD/ MEK1:ER+LNL6 cells were unable to enter G_2/M and complete the cell cycle. In contrast, the MEK1-responsive FD/ MEK1:ER+LNL6 and FD/ MEK1:ER+BCL2 cells showed a less drastic cell cycle progression profile as a significant percentage of the cells remained in S and G_2/M phases after cytokine withdrawal and addition of no growth factor. This is likely due to the high level of autocrine growth factor synthesis which promoted growth for a limited time period in the absence of exogenous cytokines and 4-OH-Tamoxifen.

The effects of cytokine withdrawal on the inhibition of cell

growth in the two different types of conditionally transformed factor-independent cells was varied, as the MEK1-responsive FD/ MEK1:ER+LNL6 cells would undergo apoptosis after 1 day of cytokine withdrawal whereas the BCL2-infected MEK1-responsive cells took longer to undergo apoptosis. In both cases apoptosis was prevented in the MEK1-responsive cells when they were treated with either IL-3 or -estradiol. This indicated that while activated MEK1:ER oncoproteins were necessary for estradiol-responsive growth, BCL2 over-expression could result in an increase in cell viability when the conditionally transformed cells were deprived of both cytokine and -estradiol. This suppression of apoptosis could be due not only to the presence of BCL2 alone, but also to the high level of GM-CSF expression. In addition, while both MEK1:ER+LNL6 and MEK1:ER+BCL2 transformed cells expressed GM-CSF, higher levels were detected in the MEK1:ER cells which overexpressed BCL2.

Activated MEK1 resulted in the activation of the downstream MAP kinases (ERK1 and ERK2). In the MEK1:ER infected cells, activation of ERK2 was observed to increase approximately 15–30 min after MEK1:ER activation. Interestingly, in MEK1-responsive FD/ MEK1:ER cells there was a second peak of ERK2 activity around 8–12 h after addition of -estradiol which remained elevated for up to 24 h. This is likely due to the induction of an autocrine loop as treatment of the cells with -estradiol induced the expression of GM-CSF. The level of GM-CSF secreted from the MEK1-responsive FD/ MEK1:ER+LNL6 and FD/ MEK1:ER+BCL2 cells was found to be sufficient for the maintenance and proliferation of the parental FDC-P1 and TF-1 cells. In addition, the autocrine GM-CSF was required for the proliferation of the MEK1-responsive cells. These cells suggest a synergistic role for underlying anti-apoptotic signals in conjunction with proliferative signal transduction alterations during the development of myelodysplastic disease and provide an interesting model to elucidate the effects of MEK1 and BCL2 oncoproteins on hematopoietic cell growth, cell cycle progression, prevention of apoptosis and malignant transformation. Identification of pathways which cross-talk with the Ras/Raf/MEK/MAPK pathway to promote cellular transformation and prevent apoptosis will aid in our understanding of the mechanisms responsible for tumor progression and will provide targets for chemotherapy.

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