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Advances in Brief

Acceleration of Apoptosis in Transforming Growth Factor β1-treated M1 Cells Ectopically Expressing B-*myb*

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Abstract

Inappropriate expression of genes involved in cell proliferation can result in altered regulation of apoptosis, a process of programmed cell death. Since B-*myb* has recently been implicated in the cell cycle progression we wanted to examine its role in the apoptotic process. For this purpose we used transforming growth factor β1 (TGF-β1)-treated M1 myeloid leukemia cell lines that continuously express murine B-*myb*. It was found that in cells overexpressing B-*myb*, TGF-β1-induced apoptosis was accelerated as assessed by cell viability and DNA fragmentation into nucleosomal fragments. A DNA ladder was detected after 24 h of TGF-β1 treatment in these cells, whereas it was not detected until after 36 h in the parental M1 cells. It was further determined by Northern blot analysis that this higher sensitivity of B-*myb* overexpressing clones was not due to a change in the expression of TGF-β receptor type I or in the kinetics of the regulation of c-*myc*, c-*myb*, *bcl-2*, and/or *bax*.

Introduction

B-*myb* is a member of the *myb* family of transcription factors and has a DNA-binding domain highly homologous to c-*myb* (1, 2). It is cell cycle regulated with the peak expression at the transition from G_1-S of the cell cycle (3, 4). Interestingly, the overexpression of human B-*myb* was shown to reduce growth factor requirements and induce a transformed phenotype in BALB/c 3T3 fibroblasts (5). Treatment with an antisense oligomer complementary to the B-*myb* gene significantly inhibits the proliferation of myeloid or lymphoid cell lines (6). All these results indicate that, like c-*myb*, B-*myb* is a growth-promoting gene. Since there have been reports that other growth-promoting genes, when expressed concurrently with growth arrest signals, can cause cells to undergo apoptosis, we wanted to determine if B-*myb* could also affect this process. It is important to identify and characterize genes that regulate this active program that controls cell numbers since therapeutic modulation of apoptosis may ultimately become a major approach in controlling malignancy. We chose the TGF-β1[3]-treated murine myeloid leukemia cell line M1 to evaluate B-*myb* effects on programmed cell death because it had been shown that treatment of M1 cells with TGF-β1 induces rapid growth arrest and apoptosis (7, 8) and that c-*myb* and c-*myc* oncogenes can accelerate this process (7). Our results show that overexpression of B-*myb* accelerates programmed cell death in TGF-β1-treated M1 cells without affecting the regulation of expression of c-*myb* and c-*myc*.

Materials and Methods

Cells and Viruses. The murine myeloid leukemia cell line M1 has been described previously (7). The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated horse serum. Murine amphotropic GP+*env*Am12 (9) and ecotropic GP+E-86 (10) packaging cell lines were maintained in DMEM containing 10% fetal bovine serum. The M1 cells were seeded at a concentration 1.5×10^5/ml with or without human recombinant TGF-β1 (10 ng/ml; R&D Systems, Inc., Minneapolis, MN). Viable cell numbers were determined using trypan blue dye exclusion and counting in a hemocytometer.

Recombinant retroviruses were prepared by transfecting plasmids pLXSN (11) or pLB-*myb*SN into amphotropic packaging cell line GP+*env*Am12 by the calcium phosphate method. After 48 h, conditioned medium-containing virus was used to infect the ecotropic packaging cell line GP+E-86 in the presence of 4 μg/ml of Polybrene (Sigma Chemical Co., St. Louis, MO). Infected cells were selected by culturing in medium containing 400 μg/ml of G418 (GIBCO, Gaithersburg, MD). Selected cells producing recombinant retroviral particles were treated with mitomycin C (10 ng/ml; Sigma) and used to infect M1 cells by cocultivation. The infected M1 cells were subsequently selected for resistance to G418 (400 μg/ml).

Construction of a Retroviral Vector Expressing B-*myb*. A retroviral vector for expression of murine B-*myb* was prepared by digestion of pMB21 (1) with *Hind*III, followed by conversion of *Hind*III ends to the *Bam*HI sites and digestion with *Bam*HI to obtain a 2.6-kilobase fragment of B-*myb* cDNA. This was subsequently subcloned into the *Bam*HI cloning site of retroviral vector LXSN (11).

Analysis for DNA Fragmentation. Briefly, 2×10^6 cells, treated with TGF-β1 for indicated times, were lysed [0.5% SDS-0.1 M NaCl-1 mM EDTA-50 mM-Tris-HCl (pH 8.0)] and incubated for 4 h at 50°C in the presence of 0.1 mg/ml proteinase K (GIBCO). The samples were then extracted with chloroform-isoamyl alcohol, precipitated with ethanol, treated with RNase for 1 h at 37°C, reextracted, reprecipitated, and finally dissolved in 10 mM Tris-1 mM EDTA (pH 7.4). Equal amounts of DNA were electrophoresed on a 2% agarose gel containing 1 mg/ml ethidium bromide and visualized by UV fluorescence.

Northern (RNA) Analysis. Total RNA was prepared from $3–5 \times 10^7$ cells using TRIzol reagent (GIBCO) as described in the instructions from the manufacturer. Ten-μg samples of total RNA were electrophoresed on a 1.2% agarose gel containing formaldehyde (0.7%), blotted onto nylon membrane, UV cross-linked using Stratalinker 1800 (Stratagene, La Jolla, CA), and hybridized. The following hybridization probes were labeled using a random priming kit (GIBCO): 2.0-kilobase *Nco*I fragment of c-*myb* (12); 2.6-kilobase *Bam*HI-*Hind*III fragment of B-*myb* cDNA (1); 1.4-kilobase *Sst*I-*Hind*III fragment of c-*myc* (13); cDNA for TGR-I (14); *Eco*RI fragment of murine *bcl-2* (15); murine *bax* cDNA(16) and rat glyceraldehyde 3-phosphate dehydrogenase cDNA (17).

***In Vitro* Translation.** A 2.6-kilobase *Bam*HI-*Hind*III fragment of B-*myb* cDNA was subcloned into pBlueII SK(+) (Stratagene) yielding pBS-B-*myb*, which was translated *in vitro* using TNT T3 Coupled Reticulocyte Lysate System (Promega, Madison, WI) in the presence of [^{35}S]cysteine according to the manufacturer's protocol. Five μl of reaction mixture were mixed with an equal volume of 2 × SDS gel loading buffer and subjected to SDS-PAGE (7%) electrophoresis. The gel was dried and exposed to X-ray film.

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[3] The abbreviations used are: TGF, transforming growth factor; TGR-I, TGF-β receptor type I.

Results and Discussion

To analyze the effect of B-*myb* gene expression on the TGF-β1-induced programmed cell death in M1 cells we constructed a retroviral vector LB-*myb*SN, where a full length B-*myb* cDNA was placed under the control of Moloney murine leukemia virus LTR. The protein coding capacity of the B-*myb* cDNA was checked by carrying out *in vitro* transcription and translation on the plasmid, pBluescriptII SK(+), which contained B-*myb* cDNA sequence downstream of the T3 promoter (BS-B-*myb*). As shown in Fig. 1*A*, we detected the protein of predicted size. Viruses, transiently expressed and packaged in amphotropic cell line GP+*env*Am12, were used to infect GP+E86 cells, an ecotropic cell line. These cells were subsequently subjected to G418 selection. Infection of the M1 cell line was carried out by cocultivation with B-*myb*-virus-producing GP+E86 cells, followed by selection of infected clones in medium containing G418 (400 μg/ml). Southern blot analysis of selected M1/B-*myb* clones confirmed that each clone contained 1–2 integrated retroviral constructs carrying B-*myb* cDNA of expected size (data not shown). Although the data that follow were largely obtained using three representative clones having a single virus integration, similar results were obtained with all selected M1/B-*myb* clones. As a negative control, M1 cells were also infected with retroviruses containing the vector LXSN lacking the B-*myb* insert (M1/LXSN). Northern blot analysis using the murine B-*myb* probe revealed that all infected cell lines expressed high levels of B-*myb* mRNA transcribed from the retroviral vector and that endogenous levels of B-*myb* were comparable in all cell lines including parental M1 cells (Fig. 1*B*). The ectopic expression of B-*myb* did not affect the doubling time and the morphology of the untreated parental M1 cell line (data not shown), but TGF-β1 treatment resulted in a much more rapid loss of viability in all B-*myb*-expressing clones comparing to the parental M1 cell line and the cell line infected with the vector LXSN (Fig. 2*A*). The acceleration of cell death in B-*myb* clones was confirmed by the appearance of DNA fragmentation into specific nucleosomal fragments characteristic of apoptosis. Apoptotic DNA ladder was detected in B-*myb*-expressing clones after 24 h, whereas in M1 and M1/LXSN cells it was not detected until after 36 h (Fig. 2*B*).

Considering the previous results of Selvakumaran *et al.* (7) that showed that both c-*myb* and c-*myc* could positively modulate apoptosis, we wanted to see if the acceleration of programmed cell death by B-*myb* was a consequence of direct or indirect effects on regulation c-*myb* or c-*myc* expression. As shown in Fig. 3, the kinetics of down-regulation of both c-*myb* and c-*myc* mRNAs in B-*myb* expressing clones was similar to that observed in the parental M1 cell line. It was interesting that although c-*myb* and B-*myb* accelerate TGF-β1-induced apoptosis similarly, the kinetics of mRNA expression of the two related endogenous genes differs in the cytokine-treated M1 cells; although the level of c-*myb* mRNA is suppressed dramatically after 1 h in TGF-β1, the level of endogenous B-*myb* mRNA is down-regulated gradually and later (Fig. 3).

Since TGF-β1 exerts its biological effects on cell growth through its cell surface receptors, type I (TGR-I) and type II (TGR-II) (18, 19), we wished to determine if the increased sensitivity of M1/B-*myb* clones to the TGF-β1 treatment was due to increased expression of mRNA encoding one of the receptors, type I (TGR-I). As shown in the Northern blot in Fig. 4*A*, however, we did not observe any differences in the expression of message for TGR-I in the cells ectopically expressing B-*myb* compared to the parental cell line M1 or M1/LXSN.

Since recent studies demonstrated that *bcl-2* and *bax* genes are the

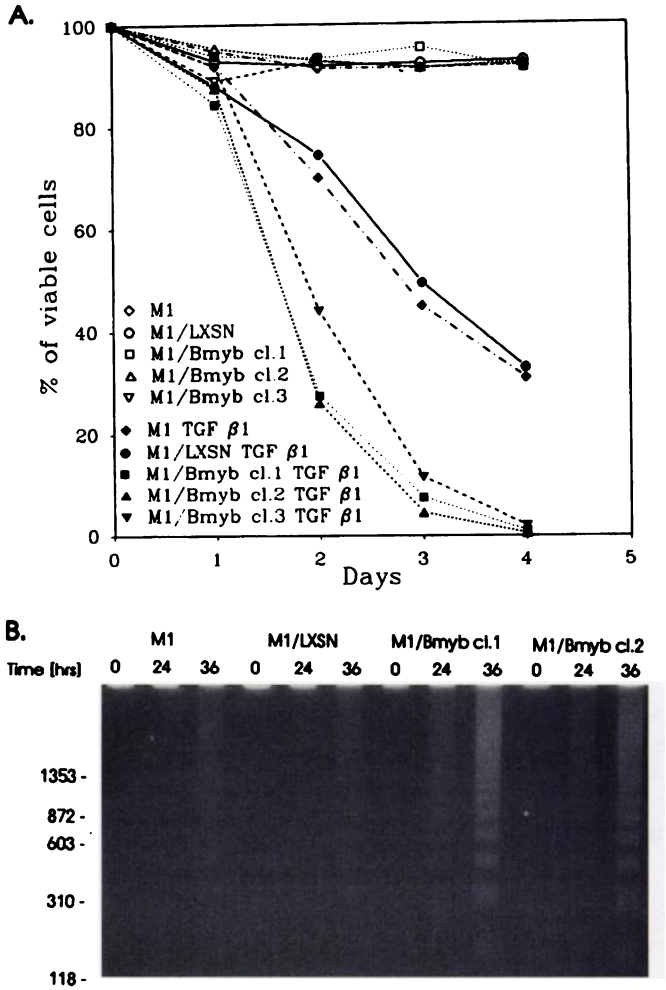

Fig. 2. Effect of deregulated expression of B-*myb* on TGF-β1-induced loss of viability and apoptosis. *A*, percentage of viable cells at the indicated times following TGF-β1 treatment of M1, M1/LXSN, and M1/B-*myb* clones. The cells seeded at 1.5×10^5/ml with TGF-β1 (10 ng/ml) were harvested and viability was assessed at the indicated time points by trypan blue dye exclusion. *Points*, mean from three independent experiments with SDs up to 7% of each value. In *B*, the cells treated with TGF-β1 for indicated time were harvested for DNA extraction and analyzed as described in "Materials and Methods." *Ordinate*, migration of the size markers in base pairs.

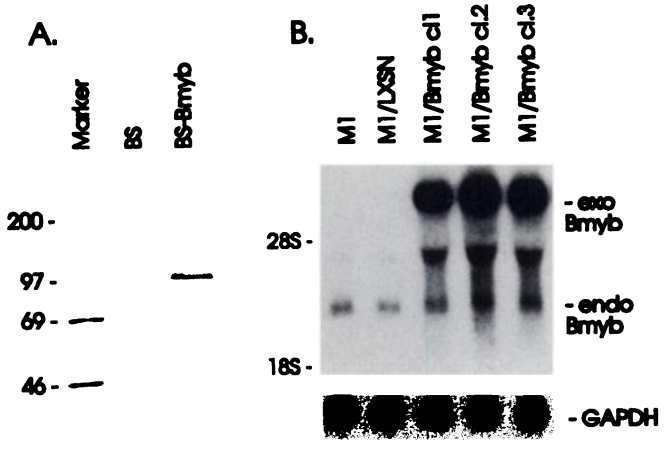

Fig. 1. *A*, *In vitro* translation product of B-*myb* cDNA which was subcloned into pBluescriptII SK(+) under the control of T3 promoter (*BS-Bmyb*), pBluescriptII SK(+) without insert (*BS*) was used as a negative control. *Ordinate*, size of protein markers in kilodaltons. *B*, Northern blot analysis of total RNAs from parental M1 cells and cell lines infected with retroviruses carrying LXSN or LXSN expressing B-*myb* gene. Ten μg of total RNA from each cell line were analyzed by Northern blot hybridization using the probes for murine B-*myb* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a control for integrity and loading consistency.

major players in the control of programmed cell death (16), we wanted to determine how the regulation of these genes is affected by deregulated expression of B-*myb* in M1 cells. For this purpose we analyzed (by Northern blot hybridization) total RNAs isolated from control M1/LXSN cells as well as cell lines overexpressing B-*myb* treated with TGF-β1 for indicated times. As shown in Fig. 4*B*, the regulation of transcription of both genes *bcl-2* and *bax* was similar in both cell lines.

p53 is also an important central molecule in the regulation of programmed cell death as well as the cell cycle but is not expressed in parental M1 cells (20). To resolve the question of whether deregulated expression of B-*myb* in M1 cells may relieve the transcriptional block imposed on p53 transcription, we analyzed total RNAs isolated from parental M1 cells and from M1/B-*myb* cells treated for different times with TGF-β1. However, we did not detect p53 mRNA by Northern blot hybridization in any of the cultures suggesting that the pathway leading to programmed cell death in this case does not involve p53 (data not shown).

The present study demonstrates for the first time the positive role of B-*myb* gene in the induction of apoptosis, and it provides another example whereby a gene involved in cell proliferation can trigger the alternative apoptotic pathway of cell self-destruction in the presence of growth arrest signals. The most likely mechanism by which B-*myb* may accelerate the programmed cell death would be through the positive or negative regulation of the transcription of genes directly involved in the apoptotic process. This is supported by studies of B-*myb* from different species that demonstrated its DNA-binding capacity and nuclear localization of protein product (21–23). In an attempt to explain the increased sensitivity of M1 cells overexpressing murine B-*myb* gene we analyzed the expression of genes such as c-*myc*, c-*myb*, *bcl-2*, *bax*, and *p53* genes, the protein products of which can modulate apoptotic cell death because they could be potential cellular targets for B-*myb*. Our results showed that none of the studied genes is involved in B-*myb*-accelerated programmed cell death. Perhaps future experiments aimed at identifying new physiological targets for B-*myb* will clarify the exact mechanism through which B-*myb* can modulate the apoptotic process.

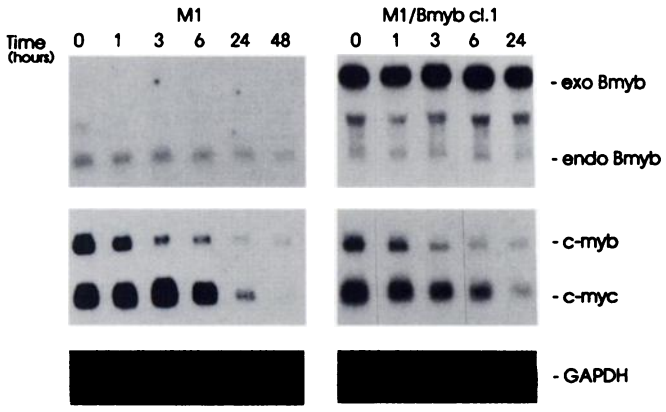

Fig. 3. Northern blot analysis of expression of B-*myb*, c-*myb*, and c-*myc* in parental M1 and M1/B-*myb* cl.1 cells during culturing in TGF-β1. Total RNAs were isolated from the cells, harvested at the indicated time, and analyzed by Northern blot hybridization with indicated probes. Data from cells overexpressing B-*myb* at the 48-h time point are not shown because rapid loss of viability caused massive degradation of RNA. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe was used to verify the equivalency in loading and RNA integrity.

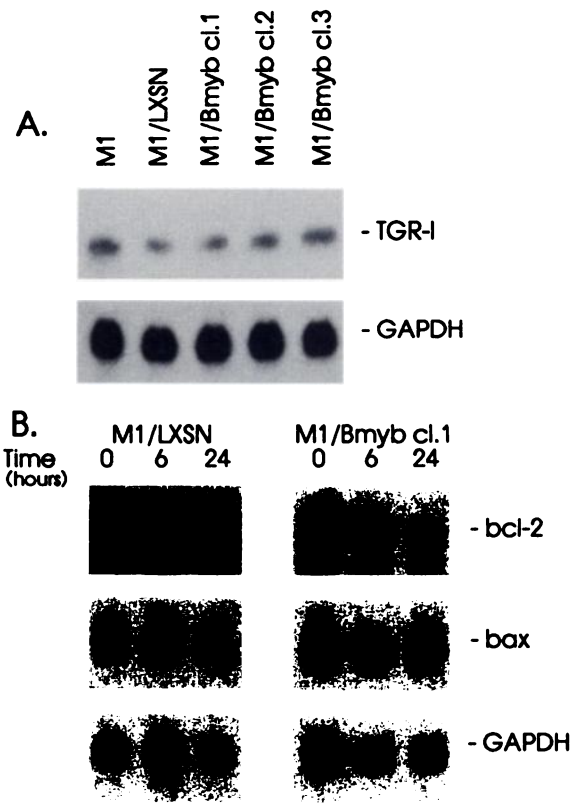

Fig. 4. *A*, levels of expression of receptor type I for TGF-β1 mRNA in M1 and M1/LXSN cells and M1 clones expressing deregulated B-*myb*. *B*, regulation of expression *bcl-2* and *bax* mRNAs in M1/LXSN and M1/B-*myb* cl.1 cells during TGF-β1 treatment. The total RNAs isolated from the cells cultured in the presence of TGF-β1 for the indicated time were analyzed by Northern blot hybridization. Autoradiograms are all from the same blot, which was stripped and rehybridized with different probes. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

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