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Vascular Endothelial Growth Factor Inhibits Apoptotic Death in Hematopoietic Cells after Exposure to Chemotherapeutic Drugs by Inducing MCL1 Acting as an Antiapoptotic Factor[1]

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ABSTRACT

We reported previously that vascular endothelial growth factor (VEGF) inhibits the apoptotic death of hematopoietic cells that is induced by exposure to ionizing radiation (O. Katoh *et al.*, Cancer Res., *55:* 5687–5692, 1995). In this study, we show that VEGF also inhibits apoptotic cell death that is induced by exposure to the chemotherapeutic drugs etoposide and doxorubicin. To elucidate the molecular mechanisms underlying this inhibitory effect of VEGF, we examined expression levels of BCL2 family proteins in CMK86, a human leukemia cell line, after treatment with VEGF. Northern blotting and immunoblotting analyses revealed that the expression level of MCL1, a member of the BCL2 family, was increased by VEGF. Moreover, to examine the effects of MCL1 on apoptotic cell death induced by exposure to etoposide, we generated a clonal U937 myeloid leukemia cell line transfected with vectors that promoted the constitutive expression of MCL1. MCL1 decreased the caspase 3 activity induced by exposure to etoposide and increased the viability of the transfected cells after etoposide exposure. Therefore, MCL1 may be involved in the inhibitory effect of VEGF on apoptotic cell death.

INTRODUCTION

VEGF[3] was originally cloned as a potent angiogenic factor that mediates developmental, physiological, and pathological neovascularization (1). VEGF has been reported to act as a survival factor, preventing the apoptotic death of microvascular endothelial cells (2, 3).

We reported previously that VEGF inhibits the apoptotic death of hematopoietic cells induced by exposure to ionizing radiation (4). However, it remains unknown whether VEGF can inhibit the apoptotic cell death that is induced by other agents, such as chemotherapeutic drugs, and the molecular mechanisms underlying this inhibitory effect of VEGF remain to be elucidated.

Here, we describe the inhibitory effect of VEGF on apoptotic cell death in CMK86, a human leukemia cell line, induced by exposure to the chemotherapeutic drugs etoposide and doxorubicin. In addition, to elucidate the molecular mechanisms behind this inhibitory effect of VEGF, we examined expression levels of the growing family of apoptosis-regulating factors, the BCL2 family (5). This family includes antagonists of cell death, such as BCL2, BCL-XL, MCL1, and so on (6–8), and agonists of cell death, such as BAX, BCL-XS, BAD, and so on (9, 10). We found that the expression level of MCL1 was increased in CMK86 cells treated with VEGF and that MCL1 decreased caspase 3 activity and increased the viability of U937 myeloid leukemia cells after exposure to etoposide.

MATERIALS AND METHODS

Cell Lines and Human Bone Marrow Hematopoietic Cells. CMK86 and U937 cells were grown in RPMI 1640 supplemented with 10% FCS. Human bone marrow cells were aspirated from the posterior iliac crest of a healthy adult volunteer after informed consent was obtained. $CD34^+$ cells were purified from the bone marrow according to previously described methods (11). Mononuclear cells were isolated by Ficoll-Hypaque centrifugation, incubated with FITC-labeled monoclonal anti-CD34 antibody (HPCA-2; Becton Dickinson Immunocytometry Systems, San Jose, CA) for 30 min at 4°C, and then washed with PBS. The cells were sorted using the FACS Vantage system (Becton Dickinson Immunocytometry Systems) equipped with a 4-W argon laser. The CMK86 and $CD34^+$ cells were incubated with RPMI 1640 with 10% FCS containing human recombinant VEGF (165 amino acids) purchased from Toyobo (Tokyo, Japan) at a final concentration of 20–50 ng/ml.

RNA Extraction, Northern Blot Hybridization Analysis, and Quantitative RT-PCR. Extraction of total RNA and poly(A)+ RNA was performed as described previously (12). Northern blots of RNA extracted from each of the cell lines were hybridized with the cDNAs of human *MCL1*, *BCL2*, *BCL-X*, and β-actin. The radioactivity level was measured with a laser imaging analyzer (BAS-2000; Fuji Photo Film, Tokyo, Japan). The radioactivity associated with gene expression in each sample was expressed as the yield of the *MCL1* gene relative to that of the β-actin gene. The reverse-transcribed cDNA from each sample was subjected to PCR amplification using primers based on the *MCL1* and β-actin gene sequences. The PCR mixture has been described previously (13), and the sequences of the *MCL1* primers used were: forward primer, 5′-TCTCTCGGTACCTTCGGG-3′ (nucleotides 605–622); and reverse primer, 5′-GCACTTACAGTAAGGCTATC-3′ (nucleotides 1128–1109). The reaction mixtures were amplified for 28 cycles in a thermal cycler (Geneamp PCR System 2400; Perkin-Elmer Applied Biosystems Division, Norwalk, CT) under the following conditions: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final incubation at 72°C for 7 min. We used the β-actin gene as an internal control, and the sequences of its primers, amplification cycles, and PCR products were as described previously (13). The PCR products were then electrophoresed using 2% (w/v) agarose gels, transferred to nylon membranes, and subjected to hybridization analysis with ^{32}P-labeled cDNA probes. After washing each filter, the radioactivity level was measured with a laser imaging analyzer as described above.

Immunoblotting Analysis. Immunoblotting analysis was performed as described previously (14). Cell lysates were isolated from $\sim 1 \times 10^7$ cells, and aliquots containing 20 μg of proteins were subjected to electrophoresis in 7.5% SDS-polyacrylamide gel, then transferred onto a polyvinylidene difluoride membrane (Immobilon PVDF; Millipore Co., Bedford, MA). The membranes were blocked for 1 h in PBS-T containing 5% fat-free dried milk. Specific antibodies to BCL2 family proteins were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; anti-MCL1, anti-BCL-X, and anti-BAX polyclonal antibodies), DAKO Japan Inc. (Kyoto, Japan; anti-BCL2 monoclonal antibody), or MBL Inc.(Nagoya Japan; anti-BAD polyclonal antibody). After incubation for 45 min at room temperature in PBS-T containing 0.05–0.2% (v/v) anti-BCL2 family antibodies, the membranes were washed with PBS-T

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; RT-PCR, reverse transcriptase-PCR; PBS-T, PBS with 0.1% Tween 20; CMV, cytomegalovirus.

and then incubated for a further hour at room temperature in PBS-T containing 0.025% (v/v) goat antirabbit IgG antibody (Biosource, Camarillo, CA) or 0.05% (v/v) sheep antimouse antibody conjugated to horseradish peroxidase (Amersham, Buckinghamshire, United Kingdom). After a final wash with PBS-T, the BCL2 family proteins on the membrane were visualized with an enhanced chemiluminescence Western blotting kit (Amersham) according to the manufacturer's protocol.

Establishment of Stable Transfectants. The pBK-CMV phagemid was used to promote constitutive expression of human MCL1. Human *MCL1* cDNA containing the entire protein coding region (nucleotides 37–1549; Ref. 8) was ligated into pBK-CMV in the sense orientation. This clone, designated pBK-CMV-MCL1, was used for the transfection assay. A human myeloid leukemia cell line, U937, was used for cloning of the transfectant. Approximately 5×10^6 U937 cells were washed twice with PBS and resuspended in 0.5 ml of PBS containing 20 μg pBK-CMV-MCL1 or 20 μg of empty pBK-CMV vector. Electroporation was performed on a Genepulser (Bio-Rad, Hercules, CA) using settings of 240 V and 960 μF. The electroporated cells were incubated on ice for 10 min and then cultured in RPMI 1640 with 10% FCS at 37°C. After 48 h, G418 was added to a final concentration of 0.8 mg/ml. After 7 days, the transfectants were subjected to limiting dilution in 96-well plates, and stable transfectants were obtained after a further 7 days of incubation. To confirm the expression of MCL1 protein, Western blot analysis using anti-MCL1 antibody was performed.

Cell Survival Assay after Exposure to Chemotherapeutic Drugs. CMK86 cells in the logarithmic growth phase after incubation in growth medium for 3 h with or without 50 ng/ml VEGF were washed three times with the medium and then incubated in growth medium for 1 h with or without etoposide or doxorubicin. The cells were then washed again three times with the medium and suspended in growth medium at 4×10^5 cells/ml for 24 h. The numbers of viable cells were determined by trypan blue staining. Cell counts were performed in duplicate for each sample. U937 cells and MCL1 transfectants in the logarithmic growth phase were also used for the cell survival assay after exposure to etoposide as described above. Each experiment was performed five times.

Analysis of Apoptotic Cell Death. Cells incubated with 50 μM etoposide were fixed with 70% ethanol. Following treatment with RNase (1 mg/ml in PBS), the cells were stained with propidium iodide solution (50 μg/ml) and analyzed using the Cytoron-absolute flow cytometry system (Ortho Diagnostic System).

Analysis of Caspase 3 Activity. The cells were washed, and the pellets were resuspended in lysis buffer (50 mM HEPES, 250 mM NaCl, and 0.1% NP40) supplemented with 100 mM NaF, 200 μM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A. Caspase 3 activity was measured using fluorogenic peptides, as described previously (15). The cell extracts (30 μg of protein) were incubated with 100 μM Ac-DEVD-MCA (Peptide Institute) in reaction buffer [50 mM Tris-HCl (pH 7.5), 1 mM DTT, and 0.1 mM EDTA) for 30 min at 30°C. Fluorescence was measured at 380 nm for excitation and 460 nm for emission.

RESULTS

VEGF Inhibits Apoptotic Cell Death after Exposure to Chemotherapeutic Drugs. We performed a cell survival assay to investigate the effects of VEGF on apoptotic cell death in CMK86, a human leukemia cell line, after exposure to the chemotherapeutic drugs etoposide or doxorubicin. Numbers of viable CMK86 cells remaining after exposure to these chemotherapeutic drugs were significantly higher in cultures preincubated with VEGF than in those preincubated without VEGF (Fig. 1). We further analyzed the cell cycle distribution to elucidate the mechanism by which these drugs induces cell death. As shown in Fig. 2, apoptotic cells began to appear in the CMK86 cultures from 4 h after continuous exposure to etoposide was begun (50 μM). By treatment with VEGF, the fraction of apoptotic cells was decreased to 70–80% of the control level. These data suggest that VEGF inhibits apoptotic death in CMK86 cells induced by exposure to chemotherapeutic drugs.

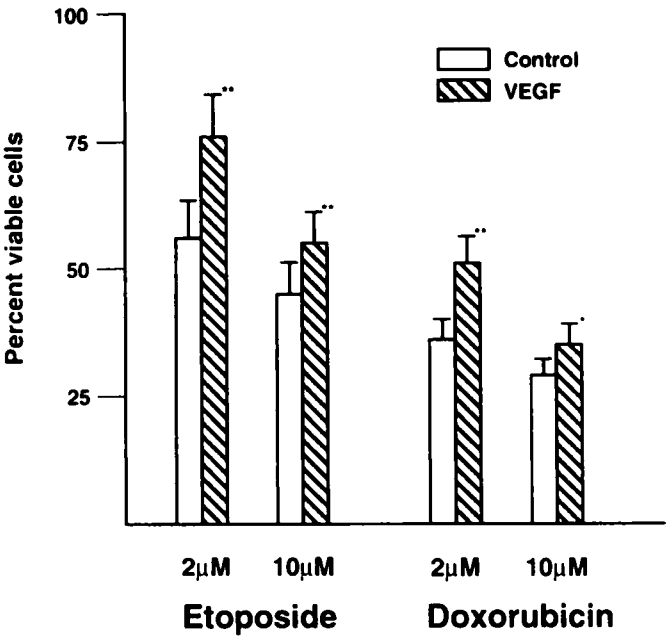

Fig. 1. The effect of VEGF on the survival rate of CMK86 after exposure to the chemotherapeutic drugs etoposide and doxorubicin. *Columns*, percentages of viable cells preincubated with (■) or without (□) VEGF. *, $P < 0.05$; and **, $P < 0.01$, statistically significant difference by Student's *t* test compared with untreated controls.

VEGF Induces MCL1 Expression in CMK86 Cells and Human Bone Marrow Hematopoietic Cells. The BCL2 family contains both antiapoptotic proteins (such as BCL2, BCL-XL, MCL1, and so on) and apoptosis-inducing proteins (such as BAX, BAD, BCL-XS, and so on). These proteins act on the outer mitochondrial membrane and function upstream of caspase 3 protease in the signaling pathway of apoptosis (16). We examined expression levels of the BCL2 family genes *BCL2*, *BCL-XL*, *BCL-XS*, *MCL1*, *BAX*, and *BAD* in CMK86 cells stimulated with VEGF. Northern blot hybridization analysis revealed that the expression levels of *MCL1* and *BCL-X* mRNA were higher than the *BCL2* mRNA expression level in CMK86 cells but that only the *MCL1* mRNA level was increased by stimulation with VEGF (Fig. 3*A*). An increase in MCL1 protein was confirmed by immunoblotting analysis (Fig. 3*B*). In contrast, the expression levels of BCL2 and BCL-XL (M_r 26,000) proteins were not changed by VEGF treatment. BCL-XS protein (M_r 18,000) was not detected in the CMK86 cells during this experiment. BAX and BAD proteins were detected by immunoblotting analysis, but their expression levels were not changed by VEGF treatment (data not shown). These data demonstrate that, of all of the BCL2 family proteins we tested, only MCL1 protein was induced in CMK86 cells by treatment with VEGF.

Northern blot hybridization analysis revealed that induction of *MCL1* mRNA occurred 4 h after starting incubation with VEGF (20 ng/ml) and continued for 8 h (Fig. 4). Incubation with a higher dose of VEGF (50 ng/ml) induced greater *MCL1* mRNA expression. The expression levels of the *MCL1* mRNA by treatment with 20 and 50 ng/ml VEGF increased ~2- and 3-fold over that of the nontreated control, respectively, by the measurement of radioactivity levels with a laser imaging analyzer.

We further examined the expression level of *MCL1* mRNA in human bone marrow $CD34^+$ cells derived from a normal healthy volunteer. A signal for *MCL1* cDNA was detected by quantitative RT-PCR, and this was increased by VEGF treatment in a time-dependent manner (Fig. 5*A*). The ratio of *MCL1* mRNA to β-actin mRNA (acting as an internal control) was increased 2–3-fold by VEGF treatment (Fig. 5*B*). These data indicate that VEGF induced the

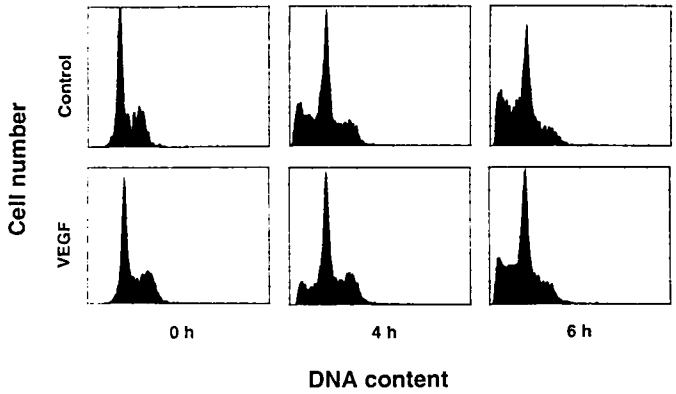

Fig. 2. Cell cycle distribution of CMK86 cells with or without VEGF pretreatment after exposure to etoposide.

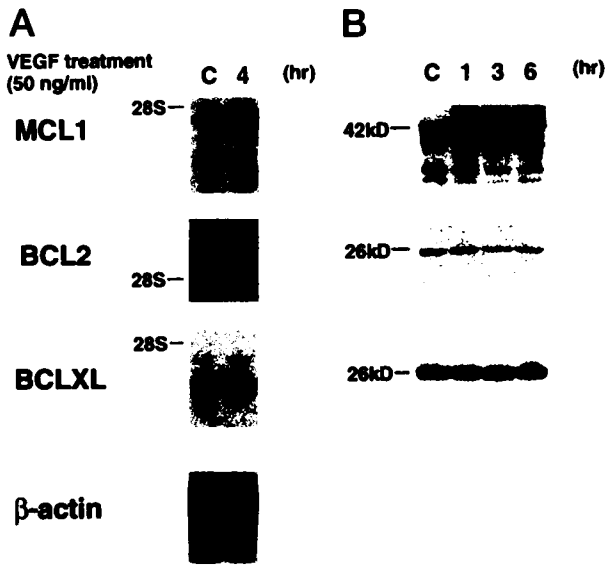

Fig. 3. Expression of the BCL2 family members MCL1, BCL2, and BCL-XL in CMK86 cells after VEGF treatment. *A*, Northern blots containing ~2 μg of poly(A)$^+$ RNA were hybridized with the cDNAs of BCL2 family genes or β-actin cDNA as probes. *B*, BCL2 family proteins were visualized by Western immunoblotting as described in "Materials and Methods."

expression of *MCL1* mRNA in both the human leukemia cell line, CMK86, and normal human hematopoietic stem cells in a time- and dose-dependent manner.

MCL1 Expression Inhibits Apoptotic Cell Death and Caspase 3 Activity. We generated a clonal U937 cell line transfected with pBK-CMV-MCL1, designated U937-pBK-MCL1, to investigate the effects of MCL1 on apoptotic cell death induced by exposure to etoposide. In this transfectant, the CMV promoter conferred constitutive expression of MCL1. Unfortunately, we could not generate CMK86 transfectants with the pBK-CMV expression vector. However, because apoptotic cell death induced by exposure to etoposide has been reported to occur in U937 cells as well as in CMK86 cells (17), we used U937 cells as substitutes for CMK86 cells in the transfection analysis. Immunoblotting analysis revealed that the MCL1 protein expression level in the transfectant U937-pBK-MCL1 was several times higher than the levels in U937 parent cells or cells transfected with the empty pBK-CMV vector, U937-pBK (Fig. 6*A*). We also performed a cell survival assay using these cell lines to examine the inhibitory effect of MCL1 on apoptotic cell death after exposure to etoposide (10 μM). Under the conditions of this experiment, larger numbers of U937-pBK-MCL1 cells than U937 or U937-pBK cells remained viable (Fig. 6*B*). We further analyzed caspase 3 activity in these cell lines after continuous exposure to etoposide (10 μM). Caspase 3 activities were increased in both U937-pBK-MCL1 and U937 cells from 6 to 10 h after exposure to etoposide (Fig. 7), and caspase 3 activities in U937-pBK-MCL1 cells were 60–70% of those in U937 cells at each point. We also analyzed the cell cycle distribution in these cell lines. The proportion of apoptotic U937-pBK-MCL1 cells was reduced when compared with U937 cells at a similar level of reduction in caspase 3 activity (data not shown). We generated other two clonal U937 cell lines transfected with pBK-CMV-MCL1, performed the cell survival assay, and measured caspase 3 activity using these clones. These transfectants had similar characteristics to the original clone (data not shown). Therefore, we propose that the results of these experiments were not due to clonal variation of the transfectants. These data suggest that MCL1 acts as an antiapoptotic factor after exposure to etoposide and that the inhibitory effect of MCL1 on caspase 3 activity may be involved in its antiapoptotic function.

DISCUSSION

VEGF inhibits apoptotic death in hematopoietic cells induced by various stresses, such as ionizing radiation (4) or chemotherapeutic drugs. In this study, we showed that MCL1, a member of the BCL2 family that acts as an antiapoptotic factor, was induced in hematopoi-

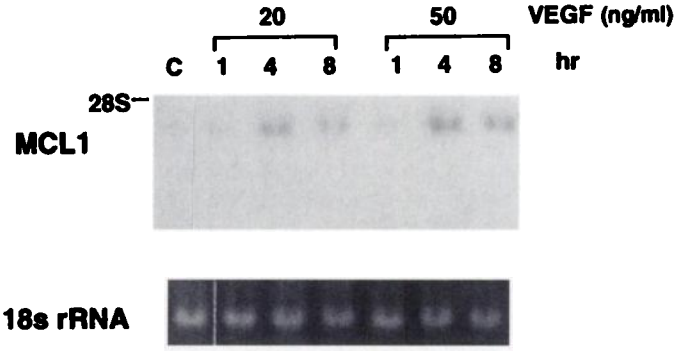

Fig. 4. Effect of VEGF on the expression of *MCL1* mRNA in CMK86 cells. Northern blots containing 10 μg of total RNA were hybridized with the *MCL1* cDNA probe. The control was 18S rRNA.

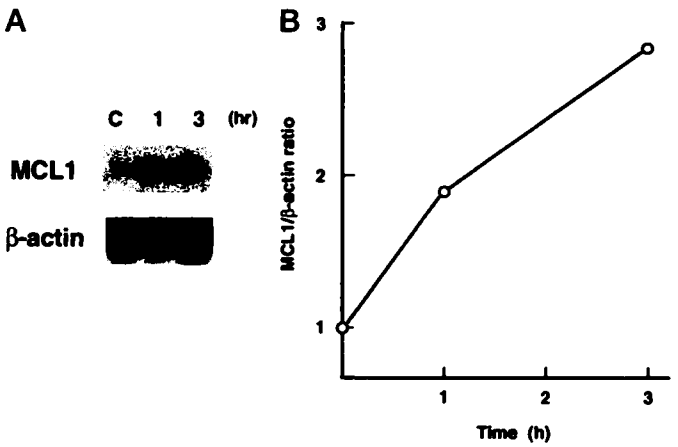

Fig. 5. Effect of VEGF on the expression of *MCL1* mRNA in human bone marrow CD34$^+$ cells as determined by quantitative RT-PCR. *A*, Southern blots containing the PCR products were hybridized with *MCL1* cDNA or β-actin cDNA probes. *B*, ratio of the yield of *MCL1* mRNA to that of β-actin mRNA in CD34$^+$ cells treated with VEGF is shown compared to the ratio in untreated cells.

etic cells by VEGF. MCL1 may, thus, be involved in the inhibitory effect of VEGF on apoptotic cell death.

MCL1 was originally cloned in ML-1, a human myeloid leukemia cell line, during phorbol ester-induced differentiation along the monocyte/macrophage pathway (8). CMK86 cells can differentiate into cells of the megakaryocytic lineage after the induction with 12-*O*-tetradecanoylphorbol-13-acetate (18, 19). However, we found no morphological change or expression of cell surface markers associated with megakaryocytes, such as CD61, in CMK86 cells after incubation with VEGF (data not shown). Therefore, VEGF did not have any effect on the differentiation of this cell line into megakaryocytes, and MCL1 may not be involved in the differentiation of CMK86 cells. MCL1 has been reported to be induced not only by differentiation-inducing agents such as phorbol esters but also by cytotoxic agents such as colchicine, vinblastine, or ionizing radiation (20, 21). MCL1, together with BCL2 and BCL-XL, is one of the negative regulators of apoptotic cell death in the BCL2 family (22). These proteins interact with apoptosis-inducing proteins such as BAX on the outer mitochondrial membrane, and BCL2 and BCL-XL suppress downstream signals, such as the release of cytochrome *c* from mitochondria (23) or the activation of caspase 3, that induce cells to undergo apoptotic cell death (16). In this study, we showed that MCL1 can also suppress caspase 3 activity induced by exposure to etoposide.

The MCL1 protein is larger than other members of the BCL2 family and contains a nonhomologous NH_2-terminal region rich in proline, glutamic acid, serine, and threonine sequences and pairs of arginine residues (8). Such sequences are typical of proteins that have a rapid turnover, and the half-life of the MCL1 protein is only 1 h, in contrast to that of the BCL2 protein, which is ~10 h in B cells (24, 25). In this study, of all of the BCL2 family members investigated, only MCL1 was induced by VEGF, whereas the expression levels of BCL2 or BCL-XL remained unchanged. These data suggest that BCL2 and BCL-XL are expressed constitutionally as antiapoptotic factors in hematopoietic cells and that if these cells are exposed to various stresses or stimulation by growth factors, MCL1 expression is induced and may reinforce the antiapoptotic functions of BCL2 and BCL-XL. The pattern of MCL1 expression *in vivo* differs from those of BCL-2 and BCL-XL (26). The cells that express MCL1 are more differentiated than those expressing BCL2. The mechanisms underlying the transcriptional regulation of the MCL1 gene may therefore

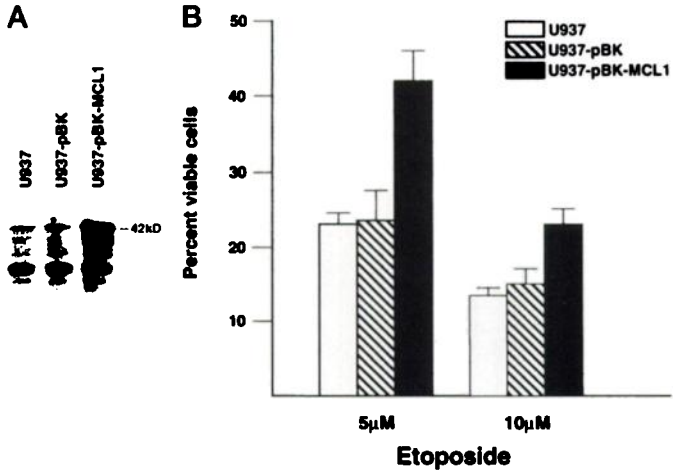

Fig. 6. Effect of MCL1 on survival rates of U937 cells after exposure to etoposide. *A*, MCL1 proteins levels in the transfectants U937-pBK-MCL1 and U937-pBK and in parent U937 cells were visualized by Western immunoblotting, as described in "Materials and Methods." *B*, *columns*, percentages of viable U937 (□), U937-pBK (▨), and U937-pBK-MCL1 (■) cells after exposure to etoposide.

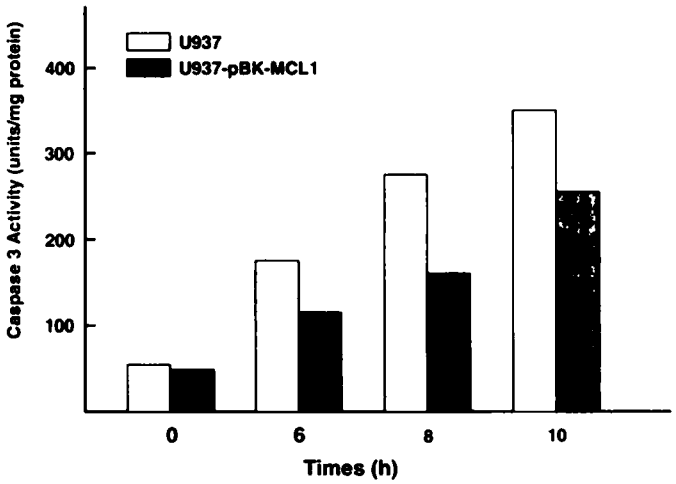

Fig. 7. Effect of MCL1 on caspase 3 activity in U937 cells after exposure to etoposide. *Columns*, caspase 3 activities in U937 (□) and U937-pBK-MCL1 (■) cells.

be different from those involved in the regulation of BCL2 and BCL-XL.

Mechanisms underlying transcriptional up-regulation of the *MCL1* gene caused by VEGF remain unknown. Phospholipase Cγ-1 or mitogen-activated protein have been reported to be involved in the signal transduction pathway following activation of VEGF receptors (27, 28). These molecules may be concerned with the transcriptional up-regulation of the *MCL1* gene by VEGF. However, transcription factors involved in transcriptional regulation of the *MCL1* gene remain unknown, and studies such as reporter assays on transcriptional control region of the *MCL1* gene have not been performed yet.

VEGF was originally cloned as a potent angiogenic factor. We have reported previously that KDR/Flk1, a receptor for VEGF, is expressed in normal human hematopoietic precursor cells and leukemia cells (4). A recent gene targeting study revealed that Flk1 is required for primitive and definitive hematopoiesis (29), and a study using an *in vitro* embryonic stem cell differentiation system showed that VEGF stimulated the formation of immature blasts expressing marker genes characteristic of hematopoietic precursors from embryonic stem cells (30). Therefore, VEGF may play an important role in primitive and definitive hematopoiesis. In addition, we have reported previously that VEGF is expressed in normal human hematopoietic precursor cells and leukemia cells (4). VEGF may act on hematopoietic precursor cells in an autocrine or paracrine manner. Moreover, leukemia cells that produce VEGF may have some ability to confer resistance against chemotherapy or radiotherapy in an autocrine or paracrine manner. MCL1 may be involved not only in the inhibitory effect of VEGF on apoptotic cell death but also in the development of the primitive and definitive hematopoietic system stimulated by VEGF. However, the effect of MCL1 on primitive and definitive hematopoiesis remains to be elucidated.

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