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Expression of the Vascular Endothelial Growth Factor (VEGF) Receptor Gene, *KDR*, in Hematopoietic Cells and Inhibitory Effect of VEGF on Apoptotic Cell Death Caused by Ionizing Radiation

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ABSTRACT

Vascular endothelial growth factor (VEGF) has been identified as a peptide growth factor specific for vascular endothelial cells. In this study, we demonstrated the expression of the *KDR* gene transcript, which encodes a cell surface receptor for VEGF, in normal human hematopoietic stem cells, megakaryocytes, and platelets as well as in human leukemia cell lines, HEL and CMK86. Moreover, we showed the expression of *VEGF* gene transcript in these normal fresh cells and cell lines. To elucidate biological functions of VEGF on hematopoiesis, we determined whether this growth factor has mitogenic activity to hematopoietic cells or the ability to suppress apoptotic cell death. The liquid culture and colony-formation assay revealed that VEGF suppressed apoptotic cell death of both CMK86 cells and normal hematopoietic stem cells caused by gamma-ray irradiation, although mitogenic activity of VEGF was not detected. The ability of VEGF to suppress apoptotic cell death was independent of the change of cell cycle distribution. These data suggest that VEGF may play an important role in survival or maintenance of hematopoietic stem cells due to the prevention of apoptotic cell death caused by some stresses such as ionizing radiation and that VEGF may give leukemia cells some abilities of resistance against radiotherapy in an autocrine or paracrine manner.

INTRODUCTION

Many cytokines and peptide growth factors are involved in hematopoiesis. The receptors for the majority of the hematopoietic cytokines belong to the cytokine receptor family or hematopoietin receptor family (1). These receptors consisting of multiple subunits do not contain an intrinsic enzymatic activity. On the other hand, the receptors for macrophage colony-stimulating factor and stem cell factor, encoded by *FMS* and *KIT*, belong to the tyrosine kinase family (2, 3). These receptors as well as the receptors for platelet-derived growth factor or FGF[2] family contain a tyrosine kinase motif in the cytoplasmic domain and contain a unique kinase insert domain intervening into the middle of the tyrosine kinase motif (4–7). Although many hematopoietic cytokines and growth factors have been identified, molecular mechanisms of hematopoietic system have not been fully understood yet. We paid attention to growth factor receptor-type tyrosine kinases expressed in hematopoietic cells to identify a peptide growth factor that binds and activates its receptor expressed in hematopoietic cells, since other peptide growth factors, including unknown factors, may be involved in the regulation of hematopoiesis.

We describe here the cloning of gene fragments of growth factor receptor-type tyrosine kinase expressed in a human leukemia cell line, CMK86 (8), $CD34^+$ cells, and megakaryocytes to identify peptide growth factors regulating hematopoiesis, including megakaryocytopoiesis. Several tyrosine kinase genes including *KDR*, *FMS*, *KIT*, and *TIE* were detected by RT-PCR using degenerated primers corresponding to the catalytic domains of the tyrosine kinase family (2, 3, 9, 10). We also report here mRNA expression of the *FLT-1* and *VEGF* genes (11, 12) in human leukemia cell lines, hematopoietic stem cells, and megakaryocytes. The *KDR* gene product and the *FLT-1* gene product belong to the VEGF receptor family (9, 13, 14).

VEGF has been identified as a peptide growth factor specific for vascular endothelial cells (11). cDNA of VEGF was originally isolated from a cDNA library prepared from bovine pituitary folliculostellate cells. VEGF not only has mitogenic activity for vascular endothelial cells but also has permeability-enhancing activity for blood vessels (15). This growth factor is known to have four molecular forms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) generated by alternative splicing of its RNA transcript (16). The two shorter isoforms have both the mitogenic and permeability-enhancing activity, whereas the other two longer isoforms have only the permeability-enhancing activity (16). Human VEGF cDNA was cloned from a cDNA library prepared from leukemia cell lines HL-60 and U937 (11, 15). Biological functions of VEGF on hematopoietic cells, however, remain unknown. To elucidate this question, we analyzed the mitogenic and colony-stimulating activities of VEGF and the ability of VEGF to suppress apoptotic cell death using CMK86 cells and human hematopoietic progenitor cells in cord blood, and we discovered the ability of VEGF to suppress apoptotic cell death caused by γ-rays.

MATERIALS AND METHODS

Isolation of Human Bone Marrow and Cord Blood Hematopoietic Cells. Human bone marrow MNC and cord blood MNC were isolated by Ficoll-Hypaque centrifugation from normal healthy volunteers or umbilical cords after delivery. Informed consent was obtained from all cases. $CD34^+$ cells and megakaryocytes in bone marrow were isolated by using immunomagnetic beads (DYNABEADS M-450; Dynal, Oslo, Norway) coupled with antibodies against CD34 (QBEND10) and human platelet GPIIb/IIIa monoclonal antibody (TP80; Seikagaku-Kogyo, Tokyo, Japan) as described previously (17). Human platelets were isolated from whole blood as described previously (18). Human monocytes, T cells, and B cells in peripheral blood were purified by sorting with flow cytometer (Epics Elite; Coulter, Miami, Florida) after labeling with monoclonal antibodies against CD14 (MY4), CD2 (OKT11), and CD20 (OKB20), respectively.

Cell Lines. The CMK86 and HEL cells were grown in RPMI 1640 supplemented with 10% FCS in a humid atmosphere with 5% CO_2 at 37°C (8).

RNA Extraction, PCR, and Cloning of cDNAs. RNA extraction and cDNA synthesis were performed as described previously (19). PCR was carried out for 40 cycles using an upstream degenerated primer TK1 (5′-GT (CGT)-AA(CT)CT(ACGT)CT(GT)GG(ACG)GC(AC)TG(CT)AC-3′; corresponding to the conserved subdomain IV, VNLLGACT, in the catalytic domain of human tyrosine kinase family) and a downstream antisense primer TK-2 (5′-AT(AG)TC-(CGT)C(GT)(AG)GC(ACT)AG(ACGT)CC(AG)AA(AG)TC-3′; the conserved subdomain VII, DFGLARD). The thermal cycle profile consisted of denaturing at 94°C for 40 s, annealing at 42°C for 3 min, and extension at 72°C for 3 min. PCR products were subcloned into plasmid vectors, and nucleotide sequences were determined by the dideoxy chain termination method. The PCR for detection of

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[2] The abbreviations used are: FGF, fibroblast growth factor; RT, reverse transcription; VEGF, vascular endothelial growth factor; IL-3, interleukin-3; MNC, mononuclear cells; GM-CSF, granulocyte/macrophage-colony-stimulating factor; BFU-E, burst-forming units-erythroid; CFU-GM, colony-forming units-granulocyte/macrophage; CFU-GEMM, colony-forming units-mixed.

KDR and *VEGF* mRNA was carried out for 30 cycles using a sense (5′-CAGATCTACGTTTGAGAACCTC-3′) and an antisense primer (5′-TCCATTGGCCCGCTTAACGGT) for *KDR* (660-bp product; amino acid number 576–795), a sense (5′-GAGTCCTTTATCCTGGATGC-3′) and an antisense primer (5′-ACAGAGCCCTTCTGGTTGGT-3′) for *FLT-1* (758-bp product; amino acid number 486–740), and a sense (5′-TCGGGCCTCCGAAACCATGA-3′) and an antisense primer (5′-CCTGGTGAGAGATCTGGTTC-3′) for *VEGF* (corresponding to the 5′-untranslated region and 3′-untranslated region, respectively; Ref. 20). The thermal cycle profile consisted of denaturing at 94°C for 40 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min 30 s. PCR products were electrophoresed with 3% agarose gel, followed by ethidium bromide staining and Southern blot hybridization analysis using ECL detection system (Amersham, Buckinghamshire, United Kingdom). These PCR products were subcloned into plasmid vectors at once and confirmed the nucleotide sequences, and then these products were used as probes.

Irradiation Procedure. A ^{60}Co γ-ray apparatus (111TBq; Shimadzu, Kyoto, Japan) was used as the radiation source. Exponentially growing CMK86 cells (5×10^5/ml) or cord blood MNC in the medium were exposed to ^{60}Co γ-rays (55 cGy/min) at 37°C.

Colony-forming Assay. Irradiated CMK86 cells were cultured in RPMI 1640 containing 15% FCS and 0.14% agar. Irradiated cord blood MNC were cultured in Iscove's modified Dulbecco's medium containing 30% FCS, 1% deionized BSA, 5×10^{-5} M 2-mercaptoethanol, 1 unit/ml erythropoietin, 100 ng/ml IL-3, 100 ng/ml stem cell factor, 100 ng/ml GM-CSF, and 0.9% methyl cellulose. After 14 days of culture in 10-mm tissue culture dishes at 37°C and 5% CO_2 in a fully humidified atmosphere, total colony number, including BFU-E, CFU-GM, and CFU-GEMM, was counted.

Treatment with VEGF. To analyze effects of VEGF on hematopoietic cells, cells were incubated with the medium containing various concentrations of human recombinant VEGF (165 amino acids) purchased from Toyobo (Tokyo, Japan). After 3 h incubation, the cells were washed with medium and suspended in the medium without VEGF. This treatment with VEGF was performed before or after irradiation to cells, and then cells were used for colony-forming assay, analysis of apoptosis, or cell cycle analysis. The cells for the mock experiments without irradiation or the VEGF treatment were handled in the same way as the cells with these treatments.

Analysis of Apoptotic Cell Death. Irradiated CMK86 cells were cultured in RPMI 1640 with 10% FCS for 7 days, and cell density was maintained at $2–5 \times 10^5$ cells/ml by appropriate dilution with the medium. The cultured cells after irradiation at days 0–7 were collected, fixed with methanol:acetic acid (3:1), and stained with orcein-acetic acid. The stained cells were classified into intact cells, normal mitotic cells, or apoptotic cells under light microscope as described previously (21). More than 2000 cells were counted and classified at each point.

Analysis of DNA Fragmentation. DNA was extracted from CMK86 cells 3 days after irradiation as described previously (21). DNA was electrophoresed in 2.4% agarose gels and stained with ethidium bromide.

Cell Cycle Analysis. CMK86 cells cultured with or without 50 ng/ml VEGF in RPMI 1640 containing 10% FCS for 3 h were fixed with 70% ethanol. Following treatment with RNase (1 mg/ml in PBS), cells were stained with propidium iodide solution (50 μg/ml). Cells were analyzed by using the Cytoron-absolute flow cytometry system (Ortho Diagnostic System).

Analysis of Cell Growth. After incubation with RPMI 1640 containing 0.5% FCS for 48 h, CMK86 cells were cultured with various concentrations of VEGF, IL-3, and FCS. After 3 days of culture in 10-mm culture dishes, the total cell number was counted with a Coulter counter Z_{BI} (Coulter Electronics).

Statistics. All data represent the means ± SE of at least three experiments. Statistical significance was determined by Student's *t* test.

RESULTS

Identification of Tyrosine Kinase Genes Expressed in Hematopoietic Cells. RT-PCR using degenerated primers, TK1 and TK2, was performed from mRNA of CMK86 cells and human megakaryocytes. Some growth factor receptors, such as FGF receptors, the FMS and KIT proteins, have a unique kinase insert domain intervening into the middle of the tyrosine kinase domain (7). The degenerated primers, TK1 and TK2, were constructed for cloning of cDNA fragments corresponding to the unique kinase insert domains, and their sequences are less conserved than those of the catalytic domain of tyrosine kinase family. After subcloning into plasmid vectors of PCR products, the nucleotide sequences of 20 clones were determined. Four receptor-type tyrosine kinase genes (*KDR, FMS, KIT,* and *TIE*) with two nonreceptor-type tyrosine kinase genes (*LCK* and *ABL*) were included among them. The others had no homology to tyrosine kinase family (data not shown).

Expression of *VEGF* Gene Transcript in Hematopoietic Cells. Among these growth factor receptor-type tyrosine kinase genes, we further studied *KDR*, which encodes a cell membrane receptor for VEGF (9, 14), and *FLT-1*, which encodes another receptor for VEGF (13). To confirm the expression of the *KDR* and *FLT-1* gene transcripts in hematopoietic cells, RT-PCR was performed. The 660-bp band derived from *KDR* mRNA and the 758-bp band derived from *FLT-1* mRNA were detected in human CD34$^+$ cells, megakaryocytes, and platelets as well as HEL and CMK86 cells, while they were not detected in either CD14$^+$ cells (monocyte), CD2$^+$ cells (T cell), or CD20$^+$ cells (B cell) from human peripheral blood (Fig. 1, *A, B, C,* and *D*). We also examined for the presence of *VEGF* mRNA. RT-PCR analysis by *VEGF*-specific primers revealed the 648- and 516-bp bands in HEL, CMK86, CD34$^+$ cells, megakaryocytes, and CD14$^+$ cells, while only the 516-bp band was detected in platelets (Fig. 1, *E* and *F*). In addition, two additional bands larger than 771 bp in HEL and CMK86 cells were observed. Origins of these two additional bands, however, were not determined yet.

Biological Effects of VEGF on CMK86 Cells. To elucidate biological functions of VEGF on hematopoiesis, we determined whether or not VEGF has mitogenic activity to CMK86 cells. CMK86 cells were cultured with medium containing 0.5–5% FCS or 0–10 ng/ml

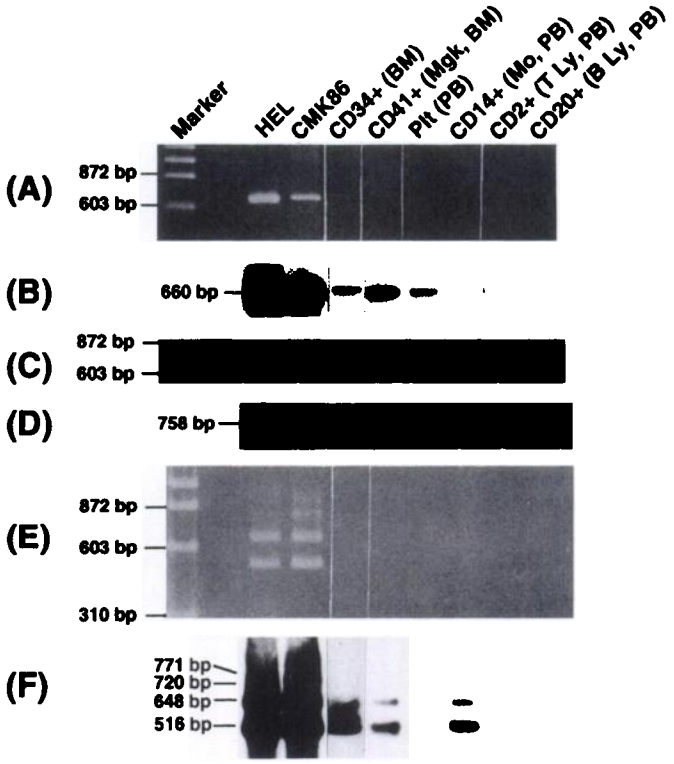

Fig. 1. RT-PCR analysis for the transcripts of *KDR*, *FLT-1*, and *VEGF* genes in human hematopoietic cells. The transcripts of *KDR* gene (*A* and *B*), *FLT-1* gene (*C* and *D*), and *VEGF* gene (*E* and *F*) were analyzed by RT-PCR. PCR products were electrophoresed with 3% agarose gel and stained with ethidium bromide (*A, C,* and *E*), followed by Southern blot hybridization analysis (*B, D,* and *F*). Fragments of *φX174/Hae*III were used as markers.

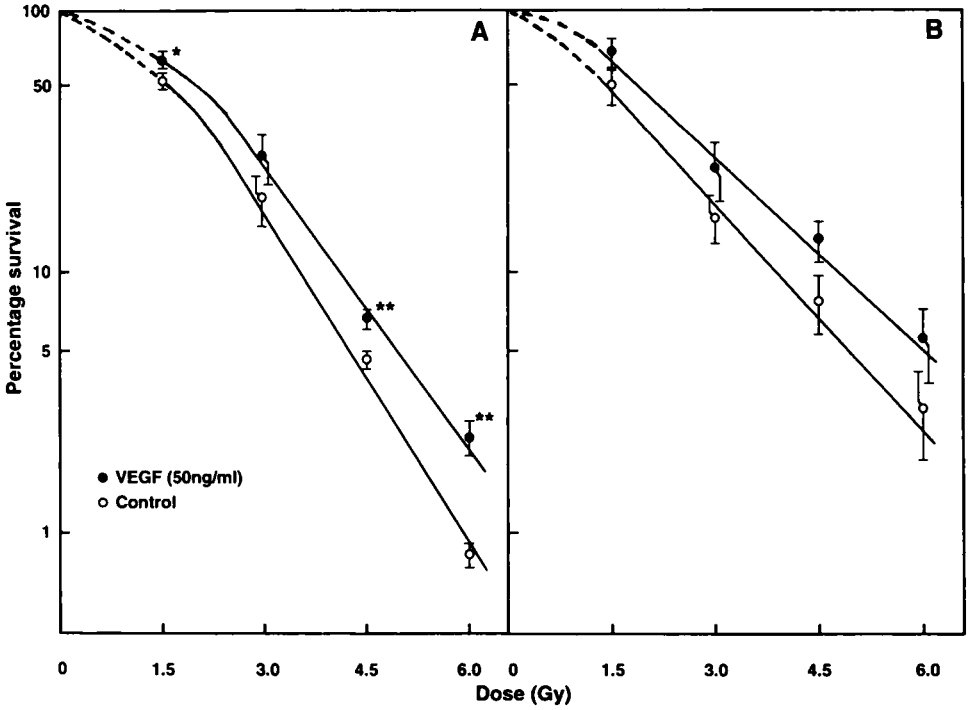

Fig. 2. The effect of VEGF on the clonogenic survival of CMK86 cells treated before (*A*) or after (*B*) irradiation. ○, untreated cells; ●, cells treated with VEGF (50 ng/ml) for 3 h. *, $P < 0.05$ and **, $P < 0.01$: statistical significance by Student's *t* test compared to each untreated control.

IL-3 in the presence of 0.5–100 ng/ml VEGF. With any combination of them, however, any mitogenic activity of VEGF to CMK86 cells was not observed (data not shown).

We then determined whether VEGF can promote survival of CMK86 cells exposed to gamma-rays. The soft agar colony-forming ability was examined after irradiation with 1.5–6 Gy of ^{60}Co gamma-rays. CMK86 cells were cultured with the medium containing 50 ng/ml VEGF for 3 h before or after irradiation, and then cells were plated. The plating efficiency was 5–10% in each experiment and was not changed by the treatment with VEGF. Thus, VEGF does not have colony-stimulating activity to unirradiated CMK86 cells. The surviving fractions of the cells treated with VEGF before or after irradiation, however, were increased (Fig. 2). The surviving fraction of CMK86 cells treated with VEGF before 6 Gy irradiation was twice higher than that without the treatment with VEGF, and increments of surviving fraction by the treatment before irradiation at 1.5, 4.5, and 6.0 Gy were statistically significant. To define the ultimate form of cell death caused by irradiation, we examined morphological changes of CMK86 cells after irradiation. When the cells were cultured for 3 days after exposure at 3.0 Gy, typical morphological changes for apoptotic cell death with chromatin condensation or apoptotic body were observed in more than 90% of dying cells (Fig. 3). Moreover, agarose electrophoresis of DNA extracted from the irradiated cells showed the ladder pattern caused by internucleosomal DNA fragmentation (data not shown).

The irradiated cells were maintained in the appropriate cell density

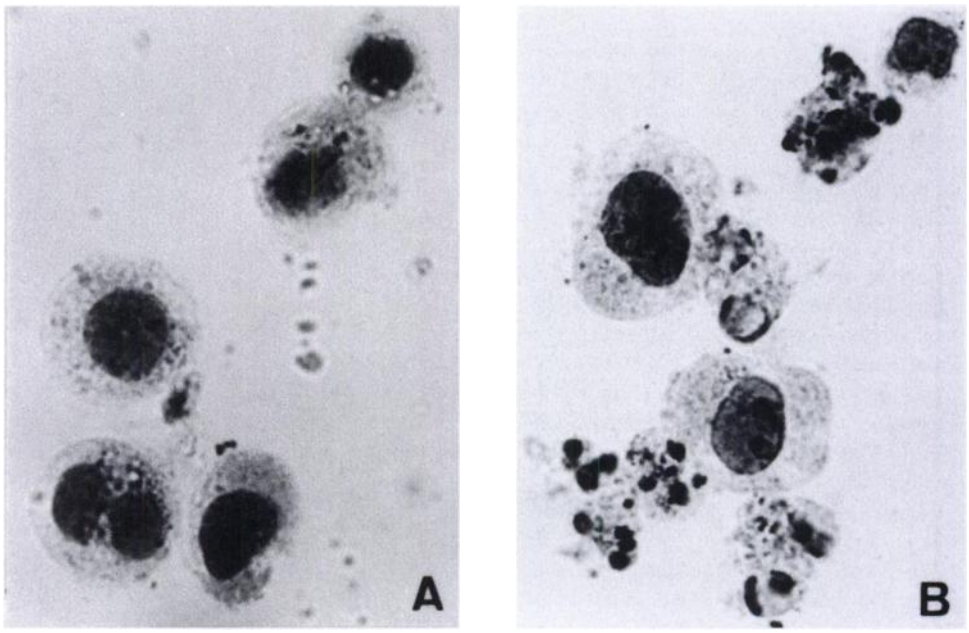

Fig. 3. Morphological changes associated with radiation-induced apoptotic cell death in CMK86 cells. The cells without irradiation (*A*) and the cells incubated for 3 days after 3 Gy irradiation (*B*) were stained with orcein-acetic acid.

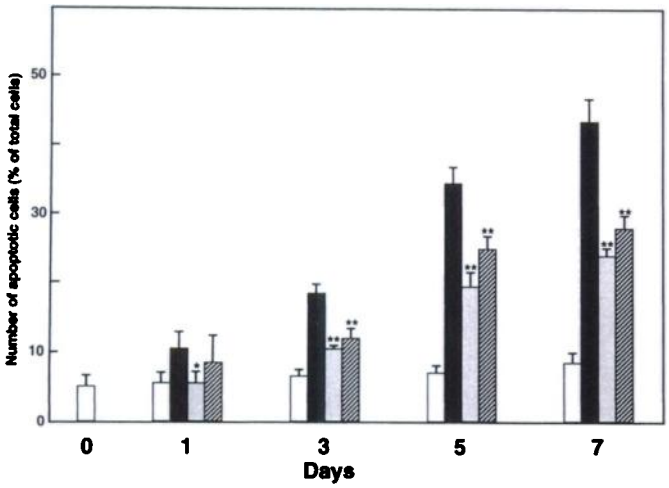

Fig. 4. The effect of VEGF on apoptosis in CMK86 cells. The cells irradiated at 3 Gy were cultured, and apoptotic cells were counted after the staining with orcein-acetic acid. Shown is the percentage of the apoptotic cell number in CMK86 cells without irradiation (□), the irradiated cells without the VEGF treatment (■), and the irradiated cells treated with VEGF (50 ng/ml) before (▨) and after (▨) irradiation. *, $P < 0.05$ and **, $P < 0.01$: statistical significance by Student's t test compared to each untreated control.

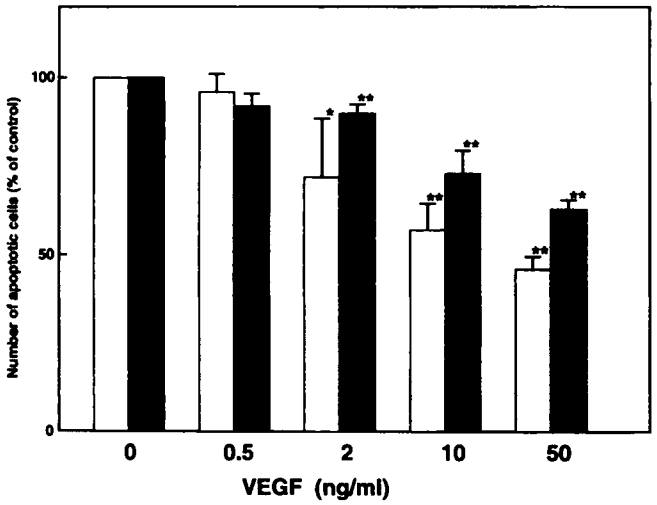

Fig. 5. Dose effect of VEGF to suppress apoptosis in CMK86 cells. The cells were treated with VEGF at various concentration before (□) or after (▨) irradiation at 3 Gy. After 5 days of culture, apoptotic cells were counted. The percentage of apoptotic cell number treated with VEGF against the mock treatment were shown. *, $P < 0.05$ and **, $P < 0.01$: statistical significance by Student's t test compared to the control without VEGF.

by dilution with the medium, and the number of apoptotic cells was counted every day. The percentage of apoptotic cells after irradiation at 3.0 Gy was increased gradually (Fig. 4). At 7 days of culture, the percentage of apoptotic cells was maximum in all cases. When the culture was continued longer than 7 days, the percentage of apoptotic cells decreased gradually because the survived cells increased. Treatment with VEGF before or after irradiation significantly suppressed the increment of apoptotic cell death. As shown in Fig. 5, VEGF suppressed the apoptotic cell death dose dependently. The suppressive effect of the treatment before irradiation was larger than that after irradiation.

To evaluate the effect of VEGF treatment on cell cycle, cell cycle distribution was analyzed by flow cytometry. The percentage of CMK86 cells in each phase after incubation with 50 ng/ml VEGF for 3 h was as follows: 47.1% in G_1 phase, 40.8% in S phase, and 12.1% in G_2-M phase, while that of the cells incubated without VEGF was 43.8% in G_1 phase, 42.8% in S phase, and 13.4% in G_2-M phase. Thus, there was no apparent difference in cell cycle distribution between them.

Suppressive Effect of VEGF on the Cell Death of Normal Hematopoietic Progenitor Cells. To analyze this suppressive effect of VEGF on apoptotic cell death of normal hematopoietic progenitor cells, we performed colony-forming assay using cord blood. Total colony number including BFU-E, CFU-GM, and CFU-GEMM was approximately 0.08–0.1% of total cord blood MNC. The treatment with VEGF did not change total colony number, the colony number of each component (BFU-E, CFU-GM and CFU-GEMM), or the size of each colony. Thus, VEGF has no colony-stimulating activity to normal human hematopoietic progenitor cells. As shown in Fig. 6, however, the treatment with 50 ng/ml VEGF before or after irradiation increased the survival rates of colony-forming cells. Statistical significance ($P < 0.05$) was seen on the survival rates of the VEGF-treated cells compared to those of the untreated cells, except for the cases treated after irradiation at 3.0 and 4.5 Gy.

DISCUSSION

VEGF is a peptide growth factor specific for vascular endothelial cells (11). In this study, the mRNAs of both *KDR* and *FLT-1* genes encoding receptors for VEGF and the mRNAs of the *VEGF* gene were detected in $CD34^+$ cells, megakaryocytes, and platelets by RT-PCR. Since the $CD34^+$ fraction contains hematopoietic stem cells and progenitor cells, these data suggest that VEGF may function as a regulator of survival, growth, or differentiation of hematopoietic stem cells and progenitor cells committed to megakaryocytic lineage. By PCR primers for VEGF used in our study, the mRNA forms were recognized as PCR products with 516, 648, 720, and 771 bp in length, which represent four molecular forms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$), respectively (20). Our detection of the two shorter bands in $CD34^+$ cells, megakaryocytes, and monocytes indicates the production of $VEGF_{121}$ and $VEGF_{165}$ in these cells. Since it has been reported that these two shorter forms can be efficiently secreted from cells and have the activity for vascular permeability as well as the mitogenic activity for vascular endothelial cells (16), VEGF produced in hematopoietic cells may be secreted into bone

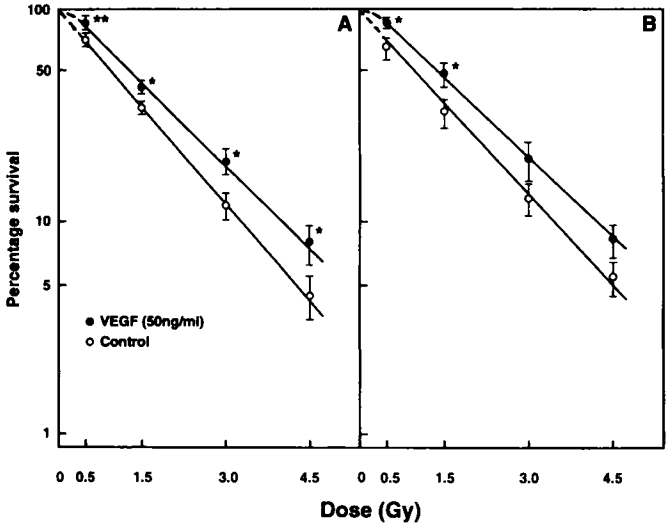

Fig. 6. The effect of VEGF on the clonogenic survival of normal human hematopoietic stem cells in cord blood. The cells were treated with VEGF (50 ng/ml) before (*A*) or after (*B*) irradiation. *, $P < 0.05$ and **, $P < 0.01$: statistical significance by Student's t test compared to each untreated control.

marrow microenvironment and may regulate the functions of marrow endothelial cells.

Although we could not find mitogenic activity or colony-stimulating activity of VEGF to hematopoietic cells in this study, we found that VEGF suppressed cell death of both CMK86 cells and normal hematopoietic progenitor cells caused by gamma-ray irradiation. The maximum percentage of apoptotic cell number (Fig. 4) looks lower than the cell killing rate at 3 Gy on colony-forming assay in Fig. 2. However, the cell killing detected by colony-forming assay represents the total accumulation of dead cells during the incubation for 2 weeks. On the other hand, the living cells after irradiation on the liquid culture system (Fig. 4) increased during the incubation period. Thus, the percentage of apoptotic cells on each day does not imply directly the accumulation of apoptotic cell death. Therefore, the cell killing rate of colony-forming assay does not correspond to the percentage of apoptotic cells in liquid culture. Since, however, the morphological feature of cell death by radiation was proved to be apoptotic cell death, VEGF was likely to suppress apoptotic cell death of hematopoietic cells in any case. These data suggest that VEGF can protect hematopoietic cells from some exogenously added stress such as ionizing irradiation. Because $CD34^+$ cells were shown to express the VEGF mRNA, VEGF may play an important role in survival or maintenance of hematopoietic stem cells in an autocrine or a paracrine manner. In a case of leukemia, however, VEGF may protect leukemia cells from apoptotic cell death induced by exposure to ionizing irradiation or anti-leukemia drugs. Thus, leukemia cells producing VEGF may have some ability of resistance against radiotherapy or chemotherapy in an autocrine or paracrine manner.

Recently, it has been reported that some hematopoietic cytokines or growth factors such as IL-3, GM-CSF, and SCF are capable of suppressing apoptotic cell death of cytokine-dependent cell lines or mast cells as well as proliferating these cells (22–24). In our study, however, we could not detect any mitogenic activity or colony-stimulating activity of VEGF to hematopoietic cells. Thus, molecular mechanisms of the suppressive effect of VEGF on the apoptotic cell death of hematopoietic cells may be distinct from those of other cytokines or growth factors. Signal transduction pathways of VEGF receptors remain unknown at the present time. Since, however, it has been reported that protein kinase C mediates basic FGF protection of apoptosis in endothelial cells (25), protein kinase C or some other protein kinases may be involved in signal transduction pathways of VEGF receptors.

It is well known that sensitivity for cell death against gamma-ray exposure is cell cycle dependent, *i.e.*, cells in late S phase are less sensitive, while cells in G_2-M phase are more sensitive to radiation. The treatment of CMK86 cells with VEGF in this study, however, made no apparent change in the cell cycle distribution as far as we examined by flow cytometry. Thus, the ability of VEGF to suppress apoptotic cell death was independent of the change of cell cycle distribution.

The mRNAs of both VEGF and VEGF receptor genes were detected in not only $CD34^+$ cells but also megakaryocytes and platelets. However, any effect of VEGF on progenitor cells with megakaryocytic lineage in cord blood was not detected by colony-forming assay. To elucidate the functions of VEGF on megakaryocytopoiesis, some other serum-free culture systems may be needed. We have demonstrated recently the mRNA expression of the FGF receptor gene family in human megakaryocytes (18). It has been reported that acidic FGF (FGF1) and basic FGF (FGF2) were able to stimulate megakaryocyte colony formation and that *in vivo* administration of FGF4 increased platelet count in peripheral blood in mice (26, 27). These reports, together with our results in this study, indicate that the FGF family and VEGF, well known as an angiogenic factor (11, 28), may function as a hematopoietic factor, especially on megakaryocytopoiesis. Megakaryocytes and endothelial cells synthesize and express similar membrane glycoproteins (GPIIIa, P-selectin, and others) and cytoplasmic factors (von Willebrand factor and others), as well as FGF receptors and a VEGF receptor (29). We also cloned in this study the cDNA fragment of *TIE* gene, which encodes an orphan receptor-type tyrosine kinase (data not shown). This gene was originally cloned from human vascular endothelial cell cDNA library (10). These observations may be supported by the fact that vascular endothelial cells and hematopoietic cells have a common origin of mesoderm in embryogenesis.

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