Vascular Endothelial Growth Factor and Cellular Chemotaxis: A Possible Autocrine Pathway in Adult T-Cell Leukemia Cell Invasion

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ABSTRACT

Our previous report (T. Hayashibara *et al.*, Leukemia, *13:* 1634–1635, 1999) revealed a possible link between high plasma vascular endothelial growth factor (VEGF) concentration and leukemic cell invasion in adult T-cell leukemia (ATL). However, the biological mechanism of this link has not been elucidated. The purpose of this study was to address that mechanism. Our present observations showed that VEGF mRNA was expressed in ATL cell lines. The corresponding protein was secreted into the extracellular environment, which suggested that the major source of plasma VEGF is ATL cells themselves. More interestingly, all of the cell lines examined were found to express the mRNA and protein for fms-like tyrosine kinase-1 (Flt-1), which is one of the receptors for VEGF. Cytofluorometric analysis demonstrated the VEGF binding potency of these cells. In clinical specimens, expression of VEGF and Flt-1 mRNAs was detected in all (100%) of 11 and 8 (73%) of 11 ATL patients, respectively. Cytofluorometric analysis revealed that VEGF effectively bound only to Flt-1-expressing cells. These findings are highly suggestive of an autocrine pathway involving VEGF operating in ATL. The proliferation of ATL cell lines was not affected by treatment with an anti-VEGF antibody or exogenous VEGF, which indicated that VEGF has no mitogenic effect on ATL cells. In contrast, we made the interesting finding that treatment with exogenous VEGF enhanced the chemotactic activities of some ATL cell lines, which may play a key role in ATL cell invasion. Collectively, these data lead us to propose a possible autocrine mechanism involving VEGF operating by way of Flt-1, in which ATL cells up-regulate their own chemotaxis to facilitate their invasion into various organs.

INTRODUCTION

ATL[2] is defined as mature $CD4^+$ T-cell leukemia (1, 2) that is caused by HTLV-I (3–5). The clinical characteristics of ATL show that there are at least four different subtypes, *i.e.*, acute, chronic, lymphoma, and smoldering, which differ with respect to the aggressiveness of the disease, as indicated by features including the serum LDH and calcium levels (6, 7). Clinically, ATL is a very progressive, lethal disease (with a mean survival of only 8 months), and has diverse manifestations. In addition, ATL is characterized by an invasion of leukemic cells into various organs such as liver, spleen, lung, skin, and intestinal tract (7–10). The mechanism of these movements remains to be elucidated.

Angiogenesis, the formation of new capillaries from preexisting ones, plays an essential role in tumor growth (11–13). During tumorigenesis, neovascularization can be stimulated by appropriate factors (14). One such factor is VEGF, a vascular permeability factor that is a dimeric glycoprotein with a M_r of ~45,000 (15–17). VEGF exerts its biological function through VEGF receptors that are high-affinity tyrosine kinase receptors on the cellular membrane, namely, KDR and Flt-1 (18–21). The expression of VEGF has been widely demonstrated in various human malignant tumors including hematological malignancies (22–26), and the expression levels often correlate positively with tumor progression (27, 28). Clinical studies have revealed that both the number and the density of microvessels in several human solid cancers may be directly associated with invasion and metastasis (13, 29), which suggests an effector role of VEGF in tumor cell invasion.

Cell migration plays a central role in a wide variety of biological phenomena. In metastasis, tumor cells migrate from the initial tumor mass into the circulatory system, which they subsequently leave, and then migrate into a new site. To migrate, cells must acquire a spatial asymmetry enabling them to turn intracellularly generated forces into net cell body translo-

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[2] The abbreviations used are: ATL, adult T-cell leukemia; HTLV-I, human T-cell leukemia virus type I; VEGF, vascular endothelial growth factor; KDR, kinase insert domain-containing receptor; Flt-1, fms-like tyrosine kinase-1; FBS, fetal bovine serum; rIL-2, recombinant interleukin-2; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription-PCR; PMA, phorbol-12-myristate 13-acetate; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; rhVEGF, recombinant human VEGF.

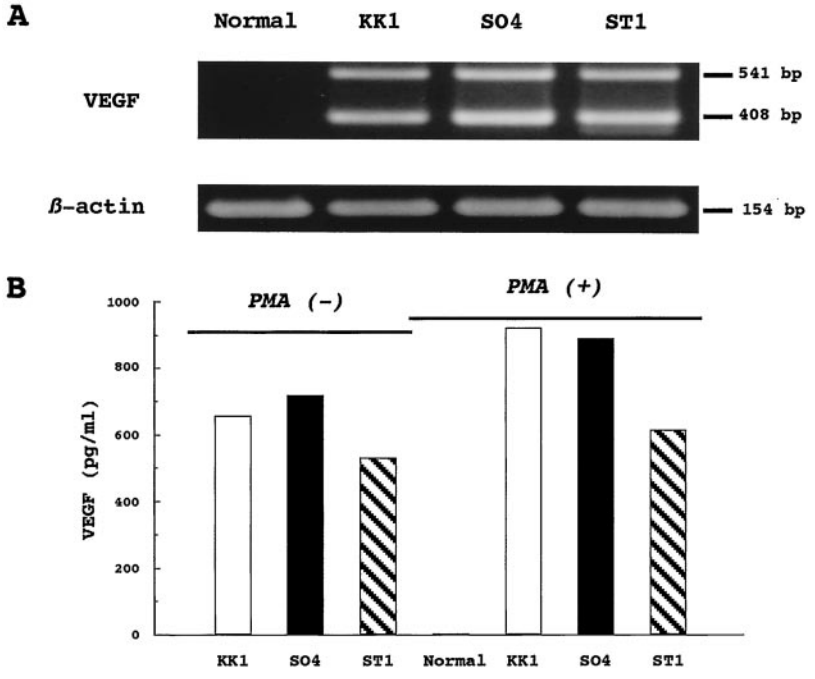

Fig. 1 *A*, RT-PCR analysis of VEGF transcripts in ATL cell lines. VEGF-121 (*408 bp*) and VEGF-165 (*541 bp*) were detected in all of the ATL cell lines examined. *Normal*, normal PBMCs representative of all of the four normal subjects studied. β-actin transcripts (*154 bp*) were used as an internal control. *B*, ELISA assay results demonstrating VEGF concentrations in conditioned media collected from ATL cell lines and normal PBMCs (*Normal*) 24 h after the cells were seeded into a culture flask in the presence [*PMA*(+)] or absence [*PMA*(-)] of PMA (4.8 nM). *Normal*, mean VEGF value from medium of normal PBMCs ($n = 5$). The absorbance of each well was measured spectrophotometrically at 450 nm and plotted against a standard curve with VEGF levels expressed as pg/ml. Each column shows the average of triplicate wells.

cation. One manifestation of this asymmetry is polarized morphology. An early event in polarization after stimulation of round cells by chemoattractant ligands is a change in filamentous, F-actin distribution from azimuthal symmetry around the cell rim to concentration in a particular region (30, 31). Kanno *et al.* recently showed that the Flt-1-mediated signal elicits cell migration by actin reorganization (32).

Although our previous study demonstrated a close correlation between high plasma VEGF levels and ATL cellular invasion (33), the biological significance of this finding has not yet been explored. The purpose of the present study was to clarify the mechanism of ATL cell invasion. Our results suggest an autocrine pathway involving VEGF production and up-regulation of chemotaxis, which facilitates the invasion of ATL cells into various organs.

MATERIALS AND METHODS

Clinical Specimens and Cell Lines. Peripheral blood was drawn from ATL patients and healthy volunteers, and the mononuclear cells were collected by centrifugation of the blood through a Ficoll gradient and used as primary ATL cells and normal mononuclear cells, respectively. All of the materials were obtained after informed consent. Cell lines KK1, SO4, and ST1 are of true ATL cell origin, as confirmed by the concordance of the integration site(s) of the HTLV-I proviral genome and/or the T-cell receptor β-chain gene rearrangement profiles with those of the respective original leukemia cells (34). These ATL cell lines were maintained in RPMI 1640 containing 10% FBS and 0.25 units/ml rIL-2 (kindly provided by Takeda Chemical Industries, Osaka, Japan). The megakaryoblastic cell line MEG-01 (35) was cultured in RPMI 1640 supplemented with 10% FBS. UT-7 (36), a megakaryoblastic cell line, was kindly provided by N. Komatsu (Jichi Medical School, Tochigi, Japan) and was maintained in Iscove's modified Dulbecco's medium containing 20% FBS and 2 ng/ml of granulocyte-macrophage colony-stimulating factor. All of the cells were maintained at 37°C in a 5% CO_2-95% air atmosphere.

RT-PCR. Total cellular RNA was extracted from these cell lines, fresh ATL cells, and PBMCs of healthy volunteers using ISOGEN kits (Nippon GENE, Toyama, Japan) and treated with DNase (Message Clean Kit; GenHunter, Nashville, TN) to remove contaminating DNA. RT-PCR was performed according to the manufacturer's directions (GeneAmp RNA PCR Kit; Perkin-Elmer, Foster, CA). One μg of RNA was used for cDNA synthesis, which was carried out using Moloney murine leukemia virus RT (2.5 units/μl) and 2.5 μM oligo $d(T)_{16}$ as primer for 20 min at 42°C. The reaction was stopped by heating the sample to 99°C for 5 min. Different aliquots of c-DNA were amplified with the specific primers for VEGF, flt-1, KDR, and β-actin as a control for successful c-DNA synthesis. The PCR primers used for VEGF, Flt-1, KDR, and β-actin were, respectively: 5′-GAAGTGGTGAAGTTCATGGATGTC-3′ (forward) and 5′-CGATCGTTCTGTATCAGTCTTTCC-3′ (reverse; Ref. 23); 5′-GAGAATTCACTATGGAAGATCTGATTTCTTACAGT-3′ (forward) and 5′-GAGCATGCGGTAAAATACACATGTGCTTCTAG-3′ (reverse; Ref. 23); 5′-GTCGACAGTACCCTTGTTATCCAAGC-3′ (forward) and 5′-TGGTCGACCATGACGATGGACAAGTA-3′ (reverse; Ref. 37): and 5′-TCATCACCATTGGCAATGAG-3′ (forward) and 5′-CAGTGTGTTGGCGTACAGGT-3′ (reverse). The reaction was performed using TaKaRa Taq DNA polymerase (Takara Shuzo Co., Shiga, Japan) and cycles as follows: 32 cycles at 94°C for 1 min, 64°C for 1.5 min (last two cycles, 2 min), 72°C for 2 min (last two cycles, 5 min) for VEGF; 38 cycles at 94°C for 40 s (last three cycles, 90 s), 54°C for 78 s (last three cycles, 90 s), 72°C for 72 s (last three cycles, 120 s) for

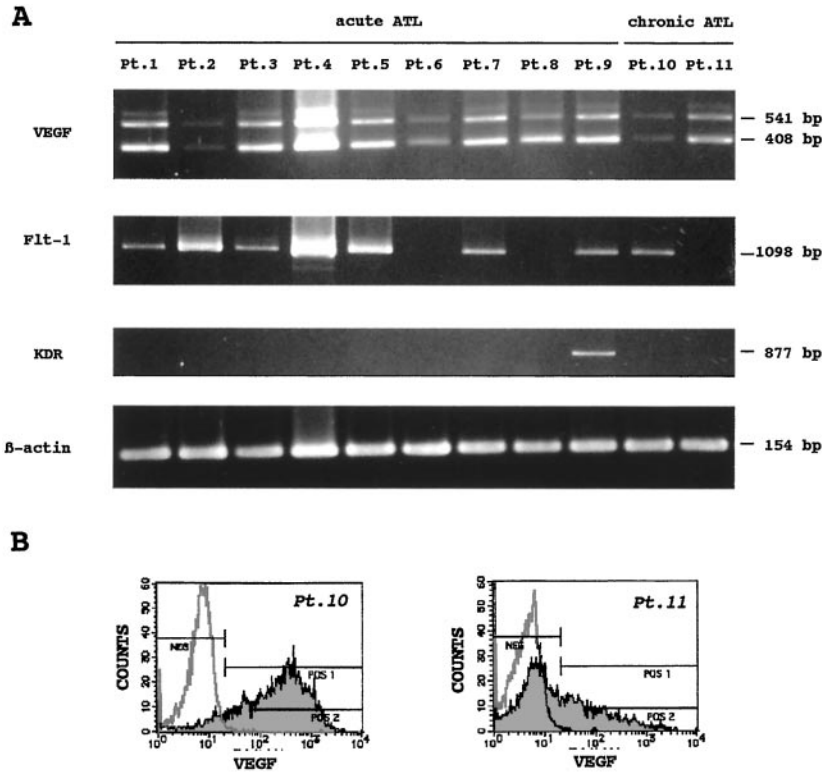

Fig. 2 A, RT-PCR analysis of VEGF, Flt-1, and KDR transcripts in ATL patients. VEGF-121 (*408 bp*) and VEGF-165 (*541 bp*) were detected in all of the ATL patients examined. Flt-1 expression (*1098 bp*) was detected in 8 of 11 ATL patients examined. KDR expression (*877 bp*) was detectable in only 1 of 11 ATL patients examined after 35 PCR cycles. β-actin transcripts (*154 bp*) were used as an internal control. *Patients (Pts.) 1–9*, acute ATL patients; *Pts. 10* and *11*, chronic ATL patients. *B*, representative cytofluorometric analysis of binding of VEGF to ATL primary cells. Cells from Pt. 10 (with Flt-1 expression) bound to VEGF; however, cells from Pt. 11 (without Flt-1 expression) did not bind to VEGF.

Flt-1; 35–41 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 2 min (last cycle, 12 min) for KDR; and 30 cycles at 95°C for 1 min, 54°C for 1 min, 72°C for 1 min (last cycle, 6 min) for β-actin. The expected amplification product sizes were 408 bp (VEGF-121) and 541 bp (VEGF-165; Ref. 23), 1098 bp (Flt-1; Ref. 23), 877 bp (KDR; Ref. 37), and 154 bp (β-actin). PCR products were separated on 2% agarose gels, stained with ethidium bromide, and visualized under UV light.

VEGF Protein Quantitation. ATL cell lines (2×10^6 cells/ml) were grown in RPMI 1640 containing 10% FBS and 0.25 units/ml rIL-2 in the presence or absence of 4.8 nM PMA (Sigma Chemical Co., Saint Louis, MO) for 24 h, and then VEGF in the supernatants was measured using a solid-phase ELISA kit for human VEGF (R&D Systems, Minneapolis, MN). One hundred μl of each supernatant as well as standard recombinant VEGF (15.6–1000.0 pg/ml) were added with assay diluent to each well of the assay microplate coated with a murine monoclonal antibody against VEGF and then were incubated with a polyclonal antibody against VEGF conjugated to horseradish peroxidase. After color development, absorbance at 450 nm was measured using a Multiskan (Labsystems Oy, Helsinki, Finland). The VEGF assay had a minimum detectable concentration of 5.0 pg/ml and did not cross-react with other known cytokines. VEGF in the conditioned medium from normal PBMCs (2×10^6 cells/ml) as a control was measured by the same method in the presence of 4.8 nM PMA.

Western Blotting. Cell pellets (1×10^6 cells) washed with PBS were suspended in 100 μl of 1× SDS-PAGE sample buffer [50 mM Tris buffer (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromphenol blue] and boiled for 5 min. Then 10-μl aliquots of these total cell lysates were subjected to SDS-PAGE on a 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Clear Blot Membrane-p; ATTO Co., Tokyo, Japan). Flt-1, bound to the membrane, was detected using an immunoassay kit (The Protein Detector Western Blot Kit BCIP/NBT System; KPL, Gaithersburg, MD). After transfer, the membrane was blocked in blocking buffer (included in the kit) for 1 h at room temperature. After the membrane was washed with PBS, it was incubated with 0.5 μg/ml of a goat antihuman Flt-1 antibody (R&D Systems) in the blocking buffer for 1 h at room temperature. The membrane was then washed in Wash Solution (included in the kit). After immunoblots were incubated with 0.4 μg/ml antigoat IgG antibody conjugated with alkaline phosphatase (KPL) for 1 h at room temperature and washed with the Wash Solution, immunoreacted proteins were visualized by the addition of BCIP/NBT substrate (included in the kit). When a suitable color intensity was observed, the reaction was stopped by immersing the membrane in reagent-quality water for 1–2 min. After the immunoblots were examined, the membranes were further stained with Coomassie Blue to confirm transfer efficiency.

Cytofluorometry of VEGF Receptor. The binding of VEGF was examined using an indirect immunofluorescence assay kit according to the manufacturer's instructions (FluorokineTM Biotinylated Human VEGF; R&D Systems). PBS-washed cells (4×10^6 cells/ml in PBS) were incubated with biotinylated VEGF for 60 min at 2–8°C. After this incubation, an avidin-FITC reagent was added to the reaction mixture. After an additional 30-min incubation at 2–8°C in the dark, the cells were washed twice using RDF1 buffer (included in the kit) to

remove unreacted avidin-fluorescein and were resuspended in RDF1 buffer for the final flow cytometric analysis. As a negative control, an identical sample of cells was stained with biotinylated soybean trypsin inhibitor.

Cell Proliferation Assay. To examine the effect of anti-VEGF antibody on the proliferation of ATL cell lines, the cell lines (1×10^6 cells/ml) were cultured in RPMI 1640 containing 10% FBS and 0.25 units/ml rIL-2 in 96-well tissue culture plates for 48 h in the presence or absence of 0.5 μg/ml antihuman VEGF antibody (R&D Systems), and the proliferation status of each cell line was then estimated by measuring the conversion of MTS into water-soluble formazan (CellTiter 96AQueous; Promega, Madison, WI). After color development, absorbance at 490 nm (indicating the number of living cells) was measured using a Benchmark Microplate Reader (Bio-Rad, Fukuoka, Japan). The effect of exogenous rhVEGF (0–400 ng/ml; R&D Systems) on the proliferation of these cell lines (5×10^5 cells/ml for 2 days and 2×10^5 cells/ml for 5 days) was also examined by the same method.

Chemotaxis Assay. Cell migration of ATL cell lines *in vitro* was measured using 12-well Transwell cell culture chambers with 5.0-μm-pore polycarbonate filter inserts (Costar, Cambridge, MA). After cells were washed twice with RPMI 1640, 200 μl of cell suspension (2×10^6 cells/ml) in RPMI 1640 containing 10% FBS and 0.25 units/ml rIL-2 was seeded in each upper well. In the lower chamber, 400 μl of RPMI 1640 containing 10% FBS and 0.25 units/ml rIL-2 plus various concentrations of rhVEGF (0–600 ng/ml) was added. After a 3-h incubation at 37°C in 5% CO_2-95% air, the cells that passed through the filter into the lower wells were quantified with CellTiter 96AQueous (as detailed above), and were expressed as a percentage of the control (in the absence of VEGF).

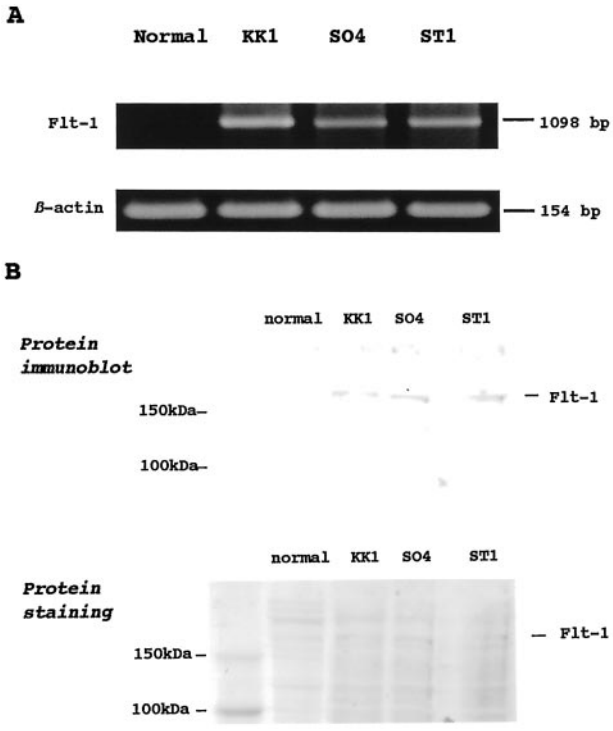

Fig. 3 *A*, RT-PCR analysis of Flt-1 transcripts in ATL cell lines. Flt-1 expression (*1098 bp*) was detected in all of the ATL cell lines examined. *Normal*, normal PBMCs representative of all of the 4 normal subjects studied. *B*, Western blot analysis of Flt-1 expression in ATL cell lines. All of the ATL cell lines examined showed Flt-1 (the M_r deduced from the amino acid sequence is about 150,000) expression. *Top*, Flt-1-immunoblots; *bottom*, Coomassie-Blue staining of the same membrane after the immunoblotting, showing the transfer efficiency. *Normal*, normal PBMCs representative of all 4 of the normal subjects studied.

RESULTS

Expression of VEGF in ATL Cells. We previously reported that plasma VEGF is elevated in certain populations of ATL patients (33); however, the origin of the VEGF has remained unknown. To address this question, we first examined the VEGF mRNA expression by RT-PCR analysis in three ATL cell lines (KK1, SO4, ST1) and also examined PBMCs from healthy donors. As shown in Fig. 1*A*, all three cell lines were found to express the mRNAs corresponding to the 121 and the 165 isoforms of VEGF, but PBMCs from healthy donors did not express these mRNAs under our VEGF RT-PCR conditions. To assess whether the VEGF protein was being secreted, ATL cell lines were cultured and supernatants were collected for VEGF determination by ELISA assays. Secreted VEGF was remarkably high in the conditioned medium from all of the ATL cell lines (Fig. 1*B*). In contrast, although PMA has been reported to up-regulate VEGF expression in a variety of cell types (38), the mean VEGF concentration in the conditioned medium of normal PBMCs treated with PMA was under the minimum detectable level (5 pg/ml). Furthermore, the amount of VEGF secreted by ATL cell lines seemed to be increased by the addition of PMA (Fig. 1*B*). Next, we examined the expression of VEGF mRNA in PBMCs from a total of 11 ATL patients (acute ATL, 9 patients; chronic ATL, 2 patients). Morphological and marker analyses indicated that the proportions of ATL cells in patients' PBMCs ranged from 53 to 95%. Using RT-PCR, VEGF mRNA expression was observed in all of the 11 patients (Fig. 2*A*). These data indicated that ATL cells are capable of synthesizing and secreting VEGF.

Expression of VEGF Receptors on ATL Cells. VEGF is thought to perform its functions by interacting with two high-affinity tyrosine kinase receptors, Flt-1 and KDR (18–21). Using RT-PCR, we first checked the expression of Flt-1 in ATL cell lines. As shown in Fig. 3*A*, we detected Flt-1 mRNA in all of the cell lines examined, but not in PBMCs from healthy donors under our Flt-1 RT-PCR conditions. Expression of Flt-1 was confirmed by Western blot analysis (Fig. 3*B*).

KDR expression in megakaryocytic cell lines has recently been reported (39); therefore, we used megakaryocytic cell lines MEG-01 and UT-7 as positive controls for KDR expression. As shown in Fig. 4, KDR mRNA was detectable in MEG-01 and UT-7 after more than 35 PCR cycles. In contrast, no KDR mRNA could be detected in ATL cell lines examined or in PBMCs from healthy donors even after 41 PCR cycles (Fig. 4). We then examined the expression of VEGF receptors in primary ATL cells. As shown in Fig. 2*A*, expression of Flt-1 and KDR mRNAs (35 PCR cycles) was seen in 8 (73%) of 11 and 1 (9%) of 11 ATL clinical specimens, respectively. With 41 PCR cy-

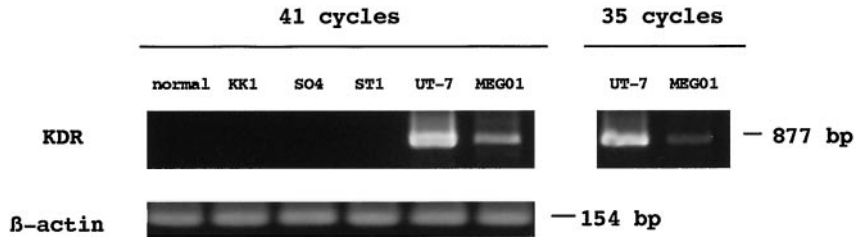

Fig. 4 RT-PCR analysis of KDR transcripts in ATL cell lines. KDR expression (*877 bp*) was detected in both of the megakaryocytic cell lines (*UT-7* and *MEG-01*), as positive controls, after a 35-cycle amplification. In contrast, KDR was not detected in normal PBMCs or in any of the ATL cell lines examined, even after 41 cycles of amplification. *Normal*, normal PBMCs representative of all four of the normal subjects studied.

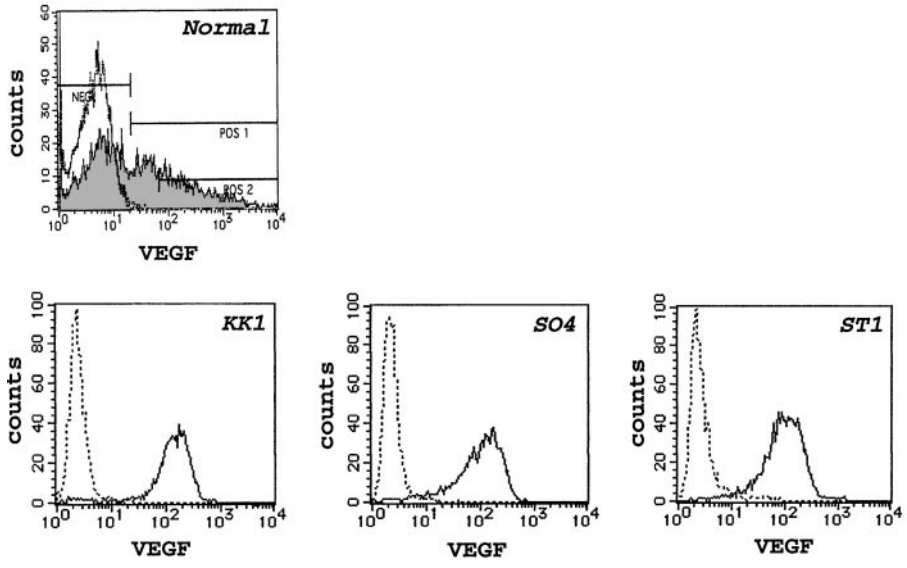

Fig. 5 Cytofluorometric analysis of the binding of VEGF to ATL cell lines. All of the ATL cell lines examined showed the ability to bind VEGF on their surface. *Normal*, normal PBMCs representative of all three of the normal subjects studied.

cles, KDR remained negative in all of the primary ATL cells examined except those from patient 9 (data not shown).

To determine whether VEGF could bind to the cell surface, cells were incubated with biotinylated rhVEGF, and the cell-surface-bound VEGF was visualized by flow cytometry. As shown in Fig. 5, VEGF bound to all three ATL cell lines but not to PBMCs from healthy donors. In ATL patients, only the ATL cells expressing Flt-1 effectively bound VEGF (Fig. 2*B*).

Effect of VEGF on ATL Cell Proliferation. To investigate the possibility that the ATL cells stimulate their own proliferation by VEGF secretion, the effect of VEGF on cellular proliferation was examined. Because VEGF was already secreted into the medium of the cell lines (Fig. 1*B*), we first examined the cell proliferation status in the presence and absence of anti-VEGF antibody. As shown in Table 1, anti-VEGF antibody had no significant effect on the proliferation. We next examined the effect of exogenous VEGF on the proliferation. The cell proliferation was not affected by rhVEGF (0–400 ng/ml) treatment (Fig. 6), which indicated that VEGF has no mitogenic effect on ATL cells.

Effect of VEGF on Chemotactic Activity of ATL Cell Lines. It has been shown that Flt-1 plays a role in VEGF-stimulated migration of monocytes/macrophages (40, 41). Therefore, it was of interest to examine the effect of VEGF on the chemotactic activity of ATL cell lines. We examined the effect of exogenous rhVEGF on the chemotaxis. As shown in Fig. 7, KK1 cells were not affected by rhVEGF treatment (0–600 ng/ml). In contrast, exogenous rhVEGF enhanced the chemotaxis of both SO4 and ST1 in a dose-dependent manner. The maximal response was obtained at a concentration of 400 ng/ml At this concentration, the percentages of migrated SO4 and ST cells relative to the controls were 159.6 and 168.4%, respectively, both of which were significant increases ($P < 0.05$, Student's t test). These results indicate that VEGF is able to elicit a migratory response in some ATL cells.

Table 1 The effect of anti-VEGF antibody on proliferation of ATL cell lines

One $\times 10^6$ cells/ml were cultured in the presence or absence of anti-VEGF antibody in 96-well tissue-culture plate for 48 h, and the proliferation status of each cell line was examined as described in "Materials and Methods." There was a linear relationship between the number of living cells and absorbance at 490 nm. The results are presented as mean ± SE ($n = 3$).

Cell line	Absorbance at 490 nm	
	− antibody	+ antibody
KK1	0.963 ± 0.015	0.973 ± 0.019
SO4	1.205 ± 0.021	1.144 ± 0.022
ST1	1.691 ± 0.022	1.760 ± 0.020

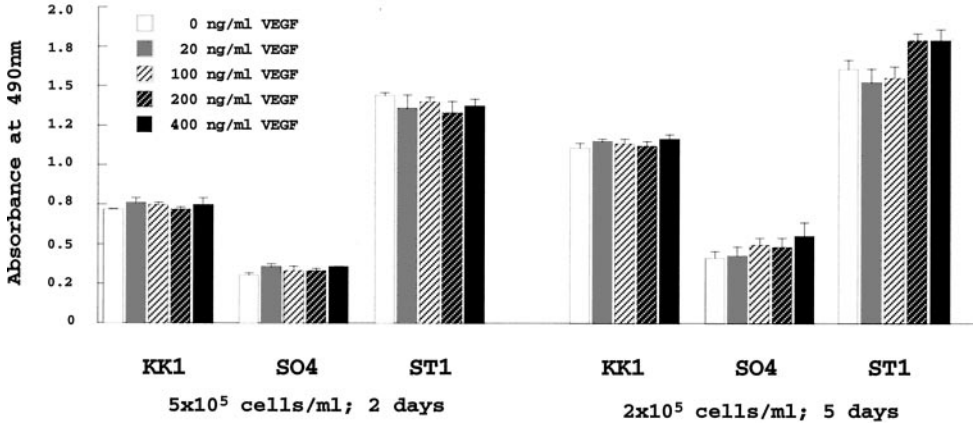

Fig. 6 Effect of VEGF on the proliferation of ATL cell lines. Cells were incubated with various concentrations of VEGF (0–400 ng/ml), and the proliferation status was measured by the same method as described in Table 1. The cellular proliferation was not affected by VEGF treatment. *Left-hand columns*, 5×10^5 cells/ml incubated for 2 days. *Right-hand columns*, 2×10^5 cells/ml incubated for 5 days. Data are presented as mean ± SE from three experiments.

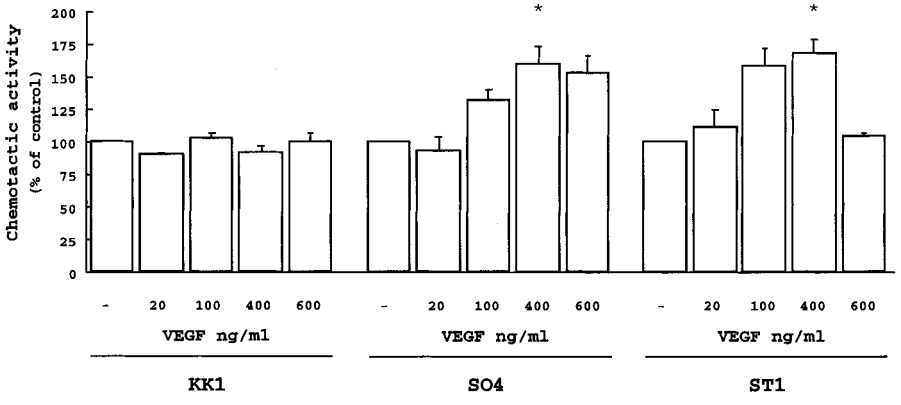

Fig. 7 Chemotactic activity of VEGF on ATL cell lines. Different concentrations of rhVEGF (0–600 ng/ml) were placed in the lower compartment of the chemotaxis chamber. The number of cells that migrated was quantified by the MTS assay (described as "Materials and Methods") and is expressed as a percentage of control (in the absence of VEGF). VEGF was found to up-regulate the chemotactic activity of some ATL cell lines. Results are shown as the mean ± SE of triple replicates. *, $P < 0.05$ *versus* control.

DISCUSSION

Patients with ATL often manifest leukemic cell infiltration into various organs such as skin, bone, liver, spleen, pleural effusion, ascites, and so forth; however, the mechanisms of the cellular invasion remain to be elucidated. We previously demonstrated that a high incidence of ATL cell infiltration is closely related to a high plasma VEGF level (33). This finding may provide key insights into the mechanism of cellular invasion. In the present study, all of the ATL cell lines examined showed high levels of expression of both VEGF mRNA and VEGF protein. Clinical samples from ATL patients also showed expression of VEGF mRNA, which indicated the VEGF secreting potential of ATL cells. VEGF stimulates the proliferation and migration of endothelial cells, which results in the enhancement of angiogenesis (42). A variety of malignant human tumors are known to secret VEGF (22–26), and clinical studies have demonstrated that tumor angiogenesis may be directly correlated with invasion and metastasis (13, 29). Those previous reports imply a paracrine effector role of VEGF in tumor invasion. Our previous report showed that the plasma VEGF levels were 7.0–253.1 pg/ml (median, 62.8 pg/ml) in acute-type-ATL patients (33). Because a VEGF concentration of 50 pg/ml has been demonstrated to stimulate endothelial cells *in vitro* (43, 44), plasma VEGF should act as an inducer of ATL cell invasion in a paracrine fashion.

An important finding of our study is that Flt-1, one of the VEGF receptors, was expressed in all of the ATL cell lines examined and in the majority of ATL patients. Moreover, VEGF was found to bind only to ATL cells expressing Flt-1 message, as shown by cytofluorometric analysis. On the other hand, KDR was not detected in any of the ATL cell lines examined and was detected only in a minority of ATL patients, which indicated that Flt-1 may be the key VEGF receptor in ATL cells. This was an expected finding in light of the report that only Flt-1, and not KDR, was expressed in a series of lymphoid cell lines (25). Collectively, these data suggest the possibility of an autocrine pathway involving VEGF operating in ATL through Flt-1.

Several previous studies have established that the activation of KDR by VEGF in cells devoid of Flt-1 results in a mitogenic response, whereas the activation of Flt-1 by VEGF in cells lacking KDR does not induce cell proliferation (45, 46). In accordance with this view, our present study showed that VEGF had no mitogenic effect on ATL cell lines expressing only Flt-1. We previously observed no significant correlation between plasma VEGF levels and several prognostic factors, including the percentage of Ki-67-positive cells in ATL patients (33). Because the percentage of Ki-67-positive cells directly reflects the cellular proliferation activity (47), the possibility that VEGF may have mitogenic effect on primary ATL cells seems to be

not great, even with other angiogenic factors included in the plasma of ATL patients.

Cell migration is a complex phenomenon that requires cytoskeleton-regulated cell motility (30) and is essential for tumor cell invasion. We showed here the potency of VEGF to induce migration activities in some ATL cell lines. This is in good agreement with a recent report that the Flt-1-mediated signal regulates cell migration through modulating actin reorganization and leads to biological responses distinguishable from those of KDR (32). We observed maximal enhancement of the migration of these cell lines at 400 ng/ml rhVEGF, which is higher than the concentration reported to be effective in the case of monocyte/macrophages (40, 41). It may be difficult for exogenous rhVEGF to affect the ATL cell lines *in vitro* because of the fact that VEGF is already secreted into the medium of these cell lines.

Taking these findings together, we propose an autocrine mechanism in which ATL cells that secrete VEGF up-regulate their own chemotactic activity through Flt-1 to facilitate their cellular invasion into various organs.

Because ATL is resistant to conventional chemotherapies, new therapeutic strategies are required to improve the prognosis. Both a neutralizing monoclonal antibody to human VEGF and inhibitors of the tyrosine kinase activity of the VEGF receptor have been shown to be effective inhibitors in various models of tumor invasion, and some of these reagents are currently being used in clinical studies on human cancers (48–51). Expression of VEGF receptors is low in normal tissue and is only up-regulated during the development of these pathological states when neovascularization occurs (24, 52). Thus, it seems very promising to use these novel compounds for treatment of ATL, in which VEGF appears to play an important role.

Keeping these considerations in mind, further effort should be directed toward clarifying the detailed molecular mechanisms of action of VEGF and its receptors in ATL patients. Knowledge about the signal transduction in this system will shed more light on the leukemogenesis of ATL, and possibly open new avenues toward therapeutic strategies for this currently incurable disease.

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