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Identification of a vascular endothelial growth factor (VEGF) antagonist, sFlt-1, from a human hematopoietic cell line NALM-16

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Abstract An antagonistic activity against vascular endothelial growth factor (VEGF) was identified in the culture supernatants of certain human hematopoietic cell lines and the antagonistic protein was purified from NALM-16 (B cell) culture supernatant. Amino acid sequencing of the N-terminus and Western blot analysis confirmed that the antagonist was identical to a soluble truncated form of Flt-1 (sFlt-1). Seventeen of 52 leukemia and lymphoma cell lines investigated expressed sFlt-1 mRNA, and 16 of the sFlt-1 expressing cells also expressed VEGF and membrane-bound Flt-1 (mFlt-1). This report is the first showing that sFlt-1 can be produced by malignant hematopoietic cells, suggesting that the production of VEGF antagonist by hematopoietic cells may play some role in the regulation of VEGF activity in normal and malignant hematopoietic cell proliferation.

Key words: Vascular endothelial growth factor; Flt-1; Antagonist; Hematopoietic cell

1. Introduction

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a multifunctional cytokine that regulates angiogenesis, vasculogenesis and inflammation [1]. VEGF is widely expressed in normal tissues as well as in cultured human umbilical vein endothelial cells (HUVEC) [2]. A variety of solid tumors are also known to secrete VEGF as an important mediator of angiogenesis to support their growth in vivo and up-regulation of VEGF production is generally observed in tumor cells under hypoxic conditions [3]. VEGF exerts its biological function through high affinity tyrosine kinase receptors such as Flt-1 (fms-like tyrosine kinase 1) [4], and Flk-1 [5]/KDR [6] (fetal liver kinase 1, or kinase domain-containing receptor). Recent studies showed that a number of hematopoietic cells expressed VEGF as well as its receptors, indicating autocrine or paracrine growth mechanisms for hematopoietic cells [7,8].

Kendall et al. [9] reported that the mRNA for a soluble truncated form of Flt-1 (sFlt-1) was generated by alternative splicing in HUVEC and that recombinant sFlt-1 inhibited the mitogenic activity of VEGF on HUVEC by functioning as a competitive inhibitor. They also showed sFlt-1 to be a naturally produced antagonist after purifying the endogenous sFlt-1 protein from HUVEC cultures. When expressed in tumor cells by gene transfection, sFlt-1 was also shown to have in vivo antitumor activity [10]. Recombinant soluble Flk-1/KDR was also examined for its antitumor activity and was shown to be effective by functioning as a dominant-negative inhibitor for VEGF-induced angiogenesis [11]. However, a natural form of soluble Flk-1/KDR has not yet been identified.

In this study, we investigated the possibility that hematopoietic malignant cell lines produce a factor antagonistic to VEGF and found that sFlt-1 was produced by NALM-16, a B cell line derived from a patient with acute lymphoblastic leukemia [12]. Production of sFlt-1 protein has been reported in HUVEC cultures, but not in malignant hematopoietic cells or other cell types. To the best of our knowledge, our study is the first to show that functional sFlt-1 is produced by hematopoietic cells.

2. Materials and methods

2.1. Preparation of cell culture supernatants

Cell culture supernatants were prepared from 52 hematopoietic cell lines maintained in our center. Cells were cultured in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, New York, USA), 100 U/ml of penicillin and 50 μg/ml of streptomycin at 37°C in a humidified 5% CO_2/air mixture. The cells were harvested and seeded at a density of 2×10^6 cells/ml in 10 ml of fresh medium. After 3 days of culture under the same conditions, the supernatants were collected, and used as samples for screening VEGF antagonist producing cells.

2.2. VEGF inhibition assay

The human gastric cancer cell line KATO-III, which produces a high level of VEGF under hypoxic conditions, was used in our VEGF inhibition assay. The cells cultured in 10% FBS–RPMI 1640 medium at 37°C in a humidified 5% CO_2/air mixture were harvested and suspended in fresh medium at a density of 5×10^4cells/ml. 200 μl of the cell suspension and 50 μl of the sample for screening were mixed in the wells of 96 well plates and incubated at 37°C in a multiple gas incubator (MCO-175M, Sanyo Electric, Tokyo, Japan) with humidified nitrogen gas containing 2% O_2 and 5% CO_2. After 24 h, the supernatant was harvested and its VEGF content was measured by ELISA (R&D Systems, Minnesota, USA). The inhibition of the rate of VEGF production was calculated by the following equation: Inhibition (%) = (1−VEGF production with sample/VEGF production without sample)×100.

2.3. Purification of VEGF antagonist

To purify the VEGF antagonist, culture supernatants of NALM-16 cells were prepared on a large scale. The cells were grown in 10% FBS–RPMI 1640 medium and were transferred to 2 l of FBS-free RPMI 1640 medium at a density of 2×10^6 cells/ml in a 5 l spinner bottle. After 3 days of culture at 120 rpm, the supernatant was collected and used as the starting material for the purification procedure.

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A total of 20 l of NALM-16 cell culture supernatant was concentrated 40-fold by UF module (AIL1010, Asahi Kasei, Tokyo, Japan) and dialyzed against 10 mM phosphate buffer (pH 7.4) containing 0.1% polyethylene glycol (PEG) and 0.05% NaN_3 using the same module. As a primary step, the activity was purified on a Macro prep ceramic hydroxyapatite type 1 column (26 mm diameter × 300 mm, bed volume 160 ml, Bio-Rad, Hercules, CA, USA). Fractions with the desired activity were pooled, concentrated, and dialyzed against 20 mM Tris–HCl buffer (pH 7.4) containing 0.1% PEG and 0.05% NaN_3. As the next step, pooled activity-containing fractions from hydroxyapatite column chromatography were further purified by Heparin-5PW column chromatography (7.5 mm diameter × 75 mm, bed volume 3.3 ml, Tosoh, Tokyo, Japan). The fractions exhibiting antagonist activity were again pooled, concentrated, and dialyzed against PBS containing 0.1% PEG and 0.05% NaN_3. Furthermore, culture supernatant of P30/OHKUBO cells, a B cell line derived from a patient with acute lymphoblastic leukemia [13] and positive for VEGF antagonist activity, was purified on the same hydroxyapatite column under conditions similar to that of the NALM-16 supernatant.

2.4. Molecular weight estimation by SDS–PAGE gel extraction assay

The purified sample obtained after Heparin-5PW column chromatography was separated in 7% SDS–PAGE gel under non-reducing conditions and the antagonist activity was monitored after elution from the gel.

2.5. N-terminal amino acid sequence analysis

The purified sample was applied to an SDS–PAGE gel, and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane (0.2 μm; Bio-Rad). The blotting was carried out at 25 V for 2 h at 4°C in a 10 mM CAPS, 10% methanol buffer (pH 11). The protein was stained with Coomassie brilliant blue (CBB-1, Wako, Osaka, Japan), and the protein bands were cut out and subjected to amino acid sequence analysis on a gas phase protein sequencer (Applied Biosystems, California, USA).

2.6. Western blotting analysis

The culture supernatants of NALM-16 and P30/OHKUBO cells purified by hydroxyapatite column chromatography were applied to SDS–PAGE, and blotted onto a PVDF membrane. These were probed with a polyclonal goat anti-human Flt-1 antibody (R&D Systems) to detect Flt-1. A secondary antibody conjugated to horseradish peroxidase and an enhanced chemoluminescence (ECL) kit (Amersham, Buckinghamshire, UK) were used for detection.

2.7. Scatchard analysis of VEGF binding to sFlt-1

The specific binding of VEGF to sFlt-1 was evaluated by Scatchard analysis. Purified sFlt-1 was prepared from both NALM-16 cells and HUVEC (Kurabo Biomedical, Osaka, Japan) culture supernatants by two-step chromatography, and were coated onto the surface of 96 well plates (20 ng/well) for 16 h at 4°C. After blocking the remaining protein binding sites of the wells with 0.5% gelatin, 50 μl of an increasing concentration of ^{125}I-labeled VEGF (Amersham) was added to the wells. After 2 h incubation at room temperature, the free VEGF was washed out by rinsing with PBS. Each well was then individually counted for the binding radioactivities by γ counter (Aloka, Tokyo, Japan). Non-specific binding was calculated as the VEGF binding to a well with no sFlt-1 coating.

2.8. Reverse transcription polymerase chain reaction (RT-PCR)

The expression of VEGF, sFlt-1, and mFlt-1 was analyzed at the mRNA level by RT-PCR. Cytoplasmic RNA was extracted from cultured cells using Ultraspec RNA isolation system (Biotecx Laboratories, Texas, USA). 1 μg of purified total RNA was used to synthesize the first strand cDNA applying reverse transcriptase preamplification using random primer, 100 pmol in 30 μl. The RT was carried out in a final volume of 30 μl of RT buffer (50 mM Tris–HCl, pH 8.4, 75 mM KCl, 3 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin). The mixture was incubated at 65°C for 15 min. Then 200 U of RT, 1 μl of 25 mM dNTP mix, 6.7 mM DTT and 20 U of RNase inhibitor were added to the reaction, which was incubated at 37°C for 60 min. 2 μl of the first strand cDNA was diluted with PCR buffer (10 × 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris–HCl, pH 8.8, 0.01% gelatin) containing 30 pmol sense and antisense primer, 15 nmol of dNTP mix and 1.5 U of Taq DNA polymerase. The primers were prepared according to published data and were:

VEGF: sense 5′-TTCTGTATCAGTCTTTCCTGGTGAG-3′
antisense 5′-CGAAGTGGTGAAGTTCATGGATG-3′
sFlt-1: sense 5′-GCACCTTGGTTGTGGCTGAC-3′
antisense 5′-CAACAAACACAGAGAAGG-3′
mFlt-1: sense 5′-GCACCTTGGTTGTGGCTGAC-3′
antisense 5′-CGTGCTGCTTCCTGGTCC-3′

Antisense primers for sFlt-1 and mFlt-1 were designed to target alternatively spliced sFlt-1 mRNA and mFlt-1 mRNA, respectively.

PCR was performed under the following conditions: VEGF: 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, extension for 1 min at 72°C; sFlt-1 and mFlt-1: 35 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 58°C, extension for 4 min at 72°C.

3. Results and discussion

3.1. Antagonistic activity detected in culture supernatants

From the 52 hematopoietic cell lines tested, the supernatants of two cell lines, NALM-16 and P30/OHKUBO, were shown to reduce VEGF production in KATO-III cell cultures by 34 and 30% respectively. Furthermore, these supernatants

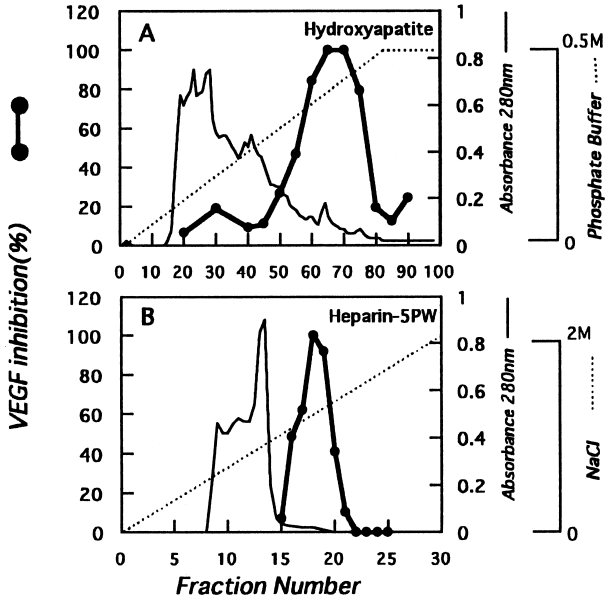

Fig. 1. Purification of VEGF antagonist by two-step chromatography. A: Concentrated NALM-16 cell culture supernatant was applied to a hydroxyapatite column (26 mm diameter × 300 mm, bed volume 160 ml) previously equilibrated with 10 mM phosphate buffer (pH 7.4) containing 0.1% PEG and 0.05% NaN_3. The column was washed with the same buffer, and eluted with a linear gradient of 10–500 mM phosphate buffer (pH 7.4) containing 0.1% PEG and 0.05% NaN_3 at a flow rate of 8 ml/min. Sample with VEGF antagonist activity was eluted at phosphate concentrations between 340 to 480 mM. These were pooled, concentrated, and dialyzed against 20 mM Tris–HCl buffer (pH 7.4) containing 0.1% PEG and 0.05% NaN_3. B: Pooled fractions with antagonist activity after hydroxyapatite column chromatography were applied to a Heparin-5PW column (7.5 mm diameter × 75 mm, bed volume 3.3 ml) previously equilibrated with 20 mM Tris–HCl buffer (pH 7.4) containing 0.1% PEG and 0.05% NaN_3. The column was washed with the same buffer, and eluted with a linear gradient of 0–2 M of NaCl in the same buffer at a flow rate of 0.5 ml/min. One active peak detected between NaCl concentrations of 1.12 and 1.30 M separated from major protein peaks.

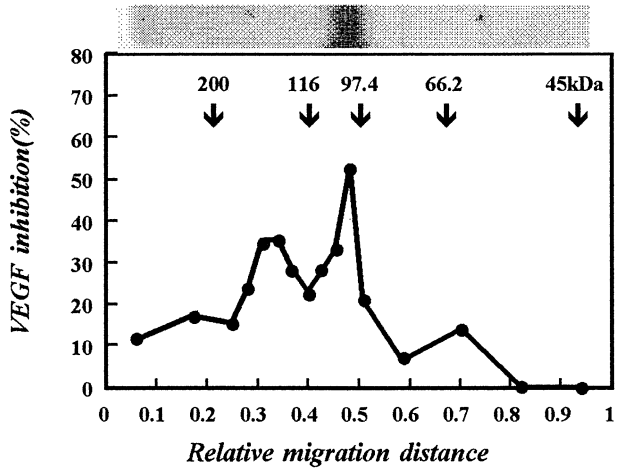

Fig. 2. Molecular weight estimation of VEGF antagonist by gel extraction analysis. The protein sample obtained after two-step column chromatography was separated on 7% SDS–PAGE gel under non-reducing conditions. The gel was sliced into 2 mm pieces, and soaked in 200 μl of 10% FBS–RPMI 1640 medium for 18 h at 4°C. The gel extracts were dialyzed against the RPMI 1640 medium, and were examined for VEGF antagonist activity. The molecular weight of the fraction showing activity was estimated against mobility of protein standards. The positions of molecular weight standards are indicated by arrows. The gel after silver staining is shown above the activity profile. One activity peak at 100 kDa corresponded to a major protein band present in the gel.

inhibited VEGF-dependent growth of HUVEC (data not shown). However, it was still not clear whether the supernatants contained an inhibitor of VEGF production by the KATO-III cells or they contained a VEGF antagonist. When the supernatants were mixed with recombinant VEGF, the amount of VEGF detected by ELISA was found to decrease significantly, indicating the presence of VEGF-specific antagonist in the culture supernatant. Therefore, we used a direct inhibition assay based on ELISA instead of an inhibition assay based on VEGF production by KATO-III cells for further purification of the activity.

3.2. Purification of VEGF antagonist

Approximately 20 l of FBS-free culture supernatant of NALM-16 cells was used for purification of the VEGF antagonist. Two-step column chromatography using hydroxyapatite and Heparin-5PW was performed as shown in Fig. 1. The specific activity increased 3.8 times after hydroxyapatite chromatography and another 86 times after Heparin-5PW chromatography. Purified proteins with high specific activity in the Heparin-5PW fractions amounted to 815 μg and a pooled sample was used for molecular weight determination

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                1       10        20        30        40      47
Flt-1(human)---MVSYWDTGVL LCALLSCLLL TGSSSGSKLK DPELSLKGTQ HIMQAGQ
                                                ?::: ::::?::??: ::::::?
VEGF antagonist(NALM-16)-----------------XKLK DPELXLKXXQ HIMQAGX
                                          1  4         14      21
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Fig. 3. Amino acid sequence comparison between VEGF antagonist isolated from NALM-16 cells and human Flt-1. The protein showing up as a 100 kDa band obtained as described in the legend for Fig. 2 was used for N-terminal amino acid sequence analysis. Twenty-one amino acids from the N-terminus were analyzed and are shown in alignment with a sequence from human Flt-1. The human Flt-1 sequence was obtained from GenBank database (NP_002010). X designates unidentified amino acid residues.

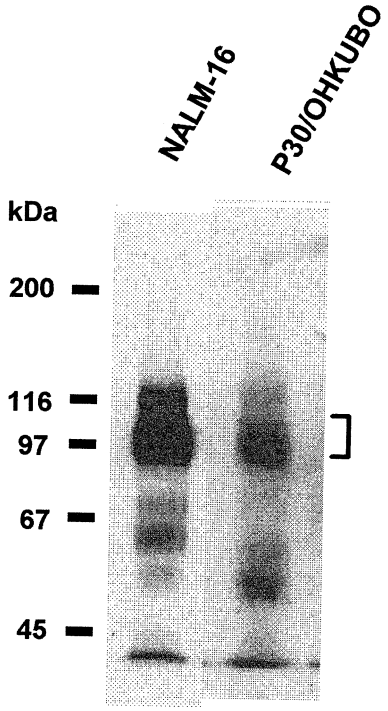

Fig. 4. Detection of sFlt-1 by Western blotting. VEGF antagonists purified from the culture supernatant of NALM-16 and P30/OHKUBO cells were blotted onto a PVDF membrane, and incubated with polyclonal anti-human Flt-1 antibody. The antibody recognizes the extracellular portion of Flt-1. The strong Flt-1 bands derived from NALM-16 and P30/OHKUBO cells corresponded to a molecular weight between 90 and 110 kDa (indicated by rectangular bar).

by SDS–PAGE. The protein staining pattern of an SDS–PAGE gel is shown in Fig. 2 together with the antagonist activity profile of protein extracts from 2 mm gel slices. A major activity band was located at a molecular weight of 100 kDa. At the corresponding position, a broad protein band was identified in the gel and this was used for further N-terminal sequence analysis. A fraction with minor activity was also identified between 130 and 160 kDa. Since we could not detect any protein band at the corresponding position, we did not analyze this fraction any further.

3.3. Identification of VEGF antagonist as sFlt-1

We next tried to identify the VEGF antagonist by N-terminal amino acid analysis and Western blotting. Although we did not determine the C-terminal sequence of the VEGF antagonist protein in NALM-16 cell culture supernatants, we concluded that this protein was sFlt-1 on the basis of the following. (1) Of 21 analyzed N-terminal amino acids, except for five unidentified amino acid residues, 16 amino acid residues completely matched those of a sequence in human Flt-1 [9] (Fig. 3). (2) An antibody recognizing the extracellular domain of human Flt-1 reacted with a 100 kDa band in the protein purified from NALM-16 culture supernatants (Fig. 4), but an antibody recognizing the cytoplasmic region did not react with the antagonist (data not shown). (3) The molecular mass is in good agreement with that of human sFlt-1 as described previously. (4) Alternatively spliced sFlt-1 mRNA was detected by RT-PCR in NALM-16 cells (Fig. 5). (5) The antagonist was detected in the culture supernatant.

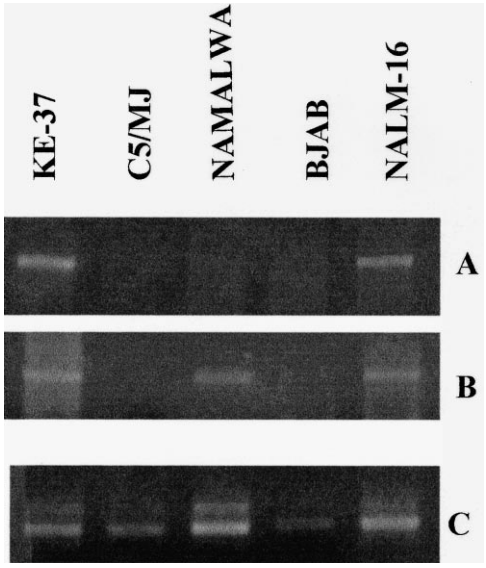

Fig. 5. RT-PCR detection of sFlt-1, mFlt-1 and VEGF mRNA in hematopoietic cell lines. Expression of sFlt-1 (A), mFlt-1 (B) and VEGF (C) was analyzed at mRNA level by RT-PCR. Results of five different cell lines are shown. NALM-16 and KE-37 expressed both sFlt-1 and mFlt-1 mRNA (lanes 1 and 5). NAMALWA expressed mFlt-1 but not sFlt-1 (lane 3). C5/MJ and BJAB were negative for both sFlt-1 and mFlt-1 mRNA (lane 2 and 4). VEGF mRNA was detected in all five cell lines. Another 47 cell lines were also examined in the same way and results are summarized in Table 1.

3.4. Scatchard analysis

We further analyzed whether sFlt-1 derived from NALM-16 cells could really bind to VEGF. The sFlt-1 proteins from NALM-16 cell and HUVEC culture supernatants were compared by Scatchard analysis using ^{125}I-labeled VEGF. As shown in Fig. 6, sFlt-1 derived from NALM-16 bound to VEGF with high affinity as in the case of HUVEC. Therefore, we concluded that sFlt-1 obtained from NALM-16 cells can act as a VEGF antagonist by directly binding to VEGF.

3.5. Expression of Flt-1 and VEGF in other hematopoietic cell lines

We next examined sFlt-1 expression in other hematopoietic cell lines. sFlt-1 protein was detected in P30/OHKUBO culture supernatant by Western blotting analysis (Fig. 4). As for other cell lines, we investigated sFlt-1 mRNA expression as well as that of mFlt-1 and VEGF by RT-PCR. Fifty-two cell lines representing the main hematopoietic lineages including T, B, myeloid, monocytoid, megakaryocytic, and erythroid cells were analyzed, and the results are summarized in Table 1. Seventeen cell lines expressed sFlt-1 while 32 expressed mFlt-1. As an example of mRNA expression profile, the expression of five representative cell lines is shown in Fig. 5. Nearly all cell lines (90%) expressed VEGF mRNA. There is no apparent correlation between the expression of sFlt-1 and a particular cell lineage. When cells expressed sFlt-1 mRNA, mFlt-1 mRNA was always simultaneously expressed.

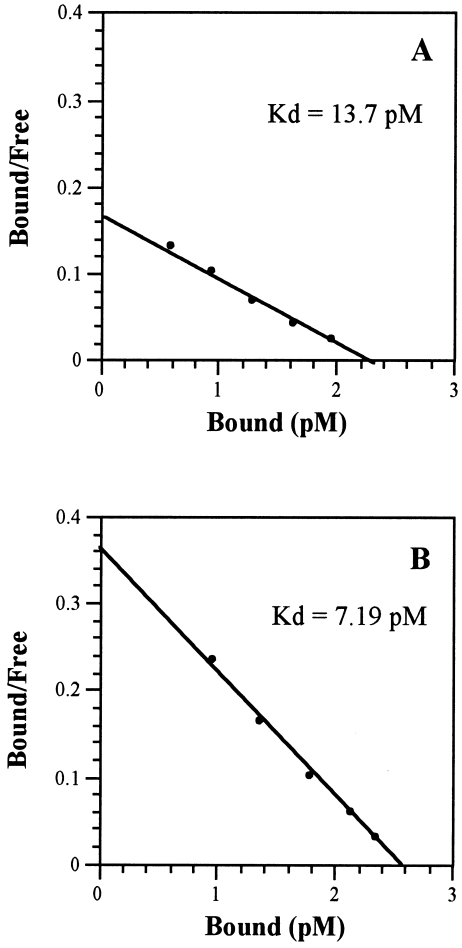

Fig. 6. Scatchard analysis. Purified sFlt-1 derived from NALM-16 cells (A) or HUVEC (B) were coated onto the surface of 96 well plates. Increasing concentrations of ^{125}I-labeled VEGF were added to each well and specific binding was counted by γ counter. The analysis showed that sFlt-1 derived from both NALM-16 cells and HUVEC acts as a high-affinity VEGF receptor. Dissociation constant are shown in the boxes.

Among the 17 cell lines expressing sFlt-1 mRNA, we identified only two cell lines with VEGF antagonist activity in the

Table 1
The expression of the mRNA for VEGF, its receptor sFlt-1, and mFlt-1 by representative hematopoietic cell lines

Cell phenotype	Number of cells tested	Number of cells positive for		
		VEGF	sFlt-1	mFlt-1
T cell	19	18(95%)	7(37%)	12(63%)
B cell	24	21(88%)	9(38%)	15(63%)
Myeloid	5	4(80%)	0(0%)	3(60%)
Monocytoid	2	2(100%)	1(50%)	1(50%)
Megakaryocytic	1	1(100%)	0(0%)	0(0%)
Erythroid	1	1(100%)	0(0%)	1(100%)
Total	52	47(90%)	17(33%)	32(62%)

Figures in parentheses show the percentage of positive cell lines representing that lineage.

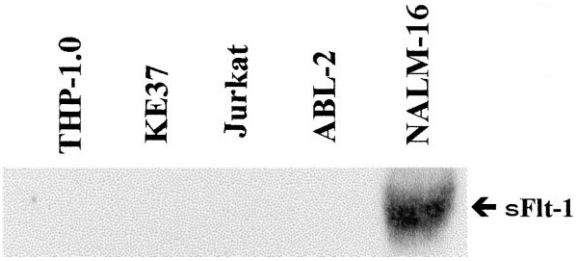

Fig. 7. Western blotting of sFlt-1. Supernatants of NALM-16 and four other cell lines which express sFlt-1 mRNA but did not show VEGF antagonist activity were used for partial purification of sFlt-1. Hydroxyapatite column fractions corresponding to the active fraction of NALM-16 culture supernatants were pooled, concentrated, and dialyzed against PBS. Detection of sFlt-1 was performed by Western blotting as described in Fig. 4. None of these four cell lines with no VEGF antagonist activity expressed detectable amounts of sFlt-1 protein.

culture supernatants. This may possibly be explained partly by absence of expression of sFlt-1 at the protein level in the sFlt-1 mRNA-positive but antagonist activity-negative cell lines. However, there is another possible explanation based on the above finding that most of the sFlt-1 expressing cell lines also expressed VEGF mRNA. The simultaneous production of sFlt-1 and VEGF would probably result in neutralization of antagonist activity in the cell culture supernatants. To examine this hypothesis, we performed Western blotting using four of the 15 sFlt-1 mRNA-positive cell lines whose supernatant showed negative antagonistic activity. As shown in Fig. 7, none of them expressed detectable amounts of sFlt-1 at the protein level, supporting the first hypothesis.

VEGF and its receptors play pivotal roles in endothelial cell functions, such as angiogenesis, vasculogenesis, and permeabilization of blood vessels. Furthermore, VEGF has been shown to have a number of effects on hematopoietic cells, such as regulation of early hematopoietic cell proliferation [8], autocrine proliferation of malignant cells [14], maturation of dendritic cells [15], and the protection of stem cells from apoptosis [16]. Our study suggests that the production of sFlt-1 as a VEGF antagonist by hematopoietic cells may play some regulatory roles in VEGF activity in normal and malignant hematopoietic cells.

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