Original Article
Up-regulation of VEGF and its receptor in refractory leukemia cells

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Abstract: Objective: To analyze the causative mechanisms in refractory leukemia cells. Methods: Vascular endothelial growth factor (VEGF) blood plasma concentrations in 35 de novo, 6 relapse, 20 remission leukemia patients and 10 healthy kids were determined via ELISA analyses. Transcription levels of the VEGF receptors (VEGFR) Fms-like tyrosine kinase-1 (Flt-1) and kinase-domain insert containing receptor (KDR) were determined in participants’ leucocytes with RT-PCR. Apoptosis rates as well as Cyt-C and Caspase-3 expression was determined in Jurkat, JurkatBcl-2, healthy and recurrent leukemia leukocytes with and without VP-16 applications via flow cytometry. Total Akt (t-Akt) expression and its phosphorylation (p-AKT) status in leucocytes of the participants were analyzed with western blots. Results: Healthy children and the remission group had the lowest blood plasma VEGF concentrations (91.16 ± 41.34 vs. 135.80 ± 111.28 pg/ml), followed by de novo leukemia patients (362.49 ± 195.68 pg/ml-494.19 ± 186.23 pg/ml) and relapse patients (574.37 ± 278.45 pg/ml) (P< 0.01). The same trend was statistically significant visible for Flt-1 and KDR expressions in leucocytes of the participants. Stable Bcl-2 overexpression led to reduced apoptosis rates as well as Cyt-C and Caspase-3 expressions in Jurkat cells after VP-16 application, which was similar in leucocytes of remission patients. In contrast to no phosphorylation in healthy children, Akt was phosphorylated in 10% remission samples, 30% de novo leukemia samples and in 67% of recurrent leukemia leucocytes. Conclusion: High VEGF plus VEGFR expression and AKT phosphorylation are highest in leucocytes of remission patients, suggesting VEGF signaling as a cause of reduced apoptosis susceptibility upon treatments.

Keywords: VEGF, PI3K/AKT, Flt-1, KDR, leukemia, apoptosis

Introduction
Therapies of 20 to 30% acute lymphoblastic leukemia and 50% acute non-lymphoblastic leukemia do not lead to long term disease-free survival and progress to refractory leukemia [1, 2]. The pathogenesis varies from chromosome variation, gene mutation, inhibition of tumor cell apoptosis and drug resistance to abnormal cell signaling [3, 4]. Vascular endothelial growth factor (VEGF) is a cytokine that stimulates endothelial cells to proliferate and migrate. VEGF receptors are Flt-1 (Fms-like tyrosine kinase-1), Flt-4 and KDR (kinase-domain insert containing receptor). The Flt-1 manifests distinct blood vessel invigorating effects and vascular permeability. KDR is associated with tumor vessel differentiation and proliferation. Flt-4 can promote the growth of specific cell subsets and restrain the cell apoptosis caused by chemotherapeutic drugs [5]. VEGF expression has a certain correlation with the degree and the stage of tumor malignancy since VEGF and its receptor levels are lower in lymphoma I-II stages than in III-IV stages [5-8]. It also has been shown, that high VEGF secretion is necessary for the growth of leukemia cells, while VEGF inhibition led to apoptosis [9]. Other researchers noted that the Flk-1/KDR receptor and the PI3-kinase/Akt signal transduction pathway are crucial for endothelial cell survival induced by VEGF and inhibition of apoptosis may represent a major aspect of the regulatory activity of VEGF on the vascular endothelium [10]. It has been reported that PI3K/AKT can phosphorylate Bad and Caspase-9, both apoptotic up-regulating factors, thereby leaving them inactive in human prostate cancer, ovarian tumor, and breast cancer [11, 12].
In this study we investigated whether highly VEGF and its receptors Flt-1/KDR/Flt-4 expressing leukemia cells might become drug resistant, thereby leading to refractory leukemia and particularly focused on apoptosis related mechanisms.

Materials and methods

Patients

The study was carried out from September, 2012 to September, 2013, in Shanghai Children's Medical Centre. The study was approved by the ethical committee of the Hospital and all participants and their guardians were informed with the experimental purpose and process. All patients had no active infections, no immune system diseases and no other neoplastic diseases. Their liver and kidney functions were normal and no adrenocortical hormone was applied recently. Initially, 35 de novo patients including 21 acute lymphoblastic leukemia (ALL) B type and 8 ALL T type (19 males, 10 females, aged from 1-11 years old with a median age of 6.5 years) as well as 6 (4 males and 2 females, average age 6.7 years) acute non-lymphoblastic leukemia (ANLL) (M1, M2a, M2b, M3 and M4) patients were involved. All patients were diagnosed by FAB classification [13]. In addition, 6 refractory patients (3 males and 3 females, average age 6.8; median age 7.6 years) acute myeloblastic leukemia (NB4), acute promyelocytic leukemia (HL-60), acute erythroleukemia (HEL), chronic granulocytic leukemia (K562), and granulocytic leukemia (U937), which possessed the characteristic of spontaneous PI3K/Akt phosphorylation and served as a positive control, were purchased from American type culture collection (ATCC). The Jurkat + Bcl-2 which was stable transfected with Bcl-2 was a gift from Professor Debatin of the University of Ulm. The normal leukocytes were provided by the healthy children. All cell lines were suspended and cultivated in 1640 culture containing 10% fetal bovine serum at standard culture condition. Generally, the culture was replaced once every five to seven days (ratio of 1:19). 50 µg/ml Etoposide (V16) was administered to the cell cultures 6 h before analysis of Cyt-C and Caspase-3 expressions.

Collection of the participants' plasma and the cell culture medium

The anticoagulated blood (2 mL) of the patients in de novo and refractory groups before the following chemotherapy and in the remission group after chemotherapy was collected. The blood was centrifuged at room temperature at 2000 rpm/min for 10 min. The plasma was stored at -70°C. The cultured cells growing in the logarithmic phase were collected for further procedures.

Separation of bone marrow lymphocytes

4 mL lymphocyte separation liquid was added to a 15 mL centrifugation tube. The heparin sodium anticoagulant treated bone marrow (4 mL) was fully blended with 1640 culture in an equal volume. The mixture was horizontally centrifuged at 3000 rpm × for 30 min. After centrifugation, the liquid was divided into three layers. The plasma and 1640 culture were in the upper layer, the red blood cells and granulocytes were mainly in the lower layer, whereas the lymphocyte separation liquid was in the middle layer. In the upper and middle interface, there was a narrow band of white cloudy layer mainly containing mononuclear cells which included lymphocytes and monocytes. The capillary pipet was inserted into the cloudy layer and the mononuclear cells were drawn and placed in another centrifuge tube. A volume of five times 1640 culture was added and the mixture was centrifuged at 1500 rpm × for 10 min twice. After the final centrifugation, the super-
Table 1. Primer sequence of the exons of VEGF and its receptors

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence</th>
<th>Fragment length</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>Forward 5'-GAA GTG GTG AAG TTC ATG GAT GTC-3'</td>
<td>541/408 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CGA TCG TTC TCT ATG CTT TCC-3'</td>
<td></td>
</tr>
<tr>
<td>KDR</td>
<td>Forward 5'-GAG GCC CAC TCA TGG TGA TTG-3'</td>
<td>709 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TGC CAG CAG TCC AGC ATG AG-3'</td>
<td></td>
</tr>
<tr>
<td>Flt-1</td>
<td>Forward 5'-GAG AAT TCA CTA TGG TGA TTG-3'</td>
<td>498 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GAG CAT GCG GAT AAA TAC ACA TGT TCT AG-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward 5'-GAG AAT TCA CTA TGG AAG ATC TGA TTT CTT ACA GTG TCT GGT-3'</td>
<td>154 bp</td>
</tr>
</tbody>
</table>

Determination of VEGF concentrations in patients’ blood plasmas and leukemia cell supernatants by ELISA

The method of double antibody sandwiched ELISA was adopted. According to the kit instruction procedures (VEGF ELISA kit, Abcam, Cambridge, MA, USA), the samples were first diluted, after which VEGF monoclonal antibodies were added. Then the biotinylation antibody working liquid was added and finally the OD450 value was measured to obtain the VEGF concentration.

RT-PCR determination

Extraction of total RNA: After adding Trizol (Gibco-BRL; Life Technologies, Grand Island NY, USA) and chloroform to the cells in the logarithmic phase, the upper layer aqueous phase was drawn, then isopropanol in an equal volume was added, and finally RNA sediment was washed by 75% ethanol. The total RNA purity identification was determined by the ratio of A260/A280 and RNA gel electrophoresis results.

Reverse transcription-polymerase chain reaction (RT-PCR): According to the SuperScriptTMII kit (Invitrogen, Philadelphia, PA, USA) instruction, total RNA was transcribed into cDNA. TaKaRa Taq (Dalianbao Bioengineering Limited Company, Dalian, China) and the primers (Table 1) were used according to the manufacturer’s instruction to obtain the PCR products of VEGF, KDR, and Flt-1 with β-actin as a control.

Primer sequence design

Analysis of PCR products: A volume of 10 μl PCR products were taken and 1 μl buffer solution was added to perform gel electrophoreses with 1% agarose as the electrophoretic medium and ethidium bromide being added for staining at 100 V for 60 min. The length of the products was measured by standard molecular weight marker. The electrophoresis results were scanned by a computer imaging software analysis system (Quantity One, Bio-Rad, USA). β-actin was used as an internal reference and the ratio with the target genes was taken as the relative amount of gene transcription.

Detection of apoptotic factor expression with flow cytometry: 106 cells were placed into a 5 ml streaming standard tube and washed with 3 ml hank’s liquid (centrifugation at 1300 RPM, 4°C, 7 minutes). Then the cells were fixed with 4% PFA at 4°C for 20 minutes and washed with PBS, after which 50 μl 0.2% Saponin was added with slight oscillation for 5-10 minutes to rupture the cell membranes. Then 5 μl MOPC was added into the mixture at room temperature and incubated for 15 minutes.

Table 2. Plasma VEGF concentrations of patients in each group

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>VEGF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B type ALL de novo patients</td>
<td>21</td>
<td>362.49 ± 195.68a</td>
</tr>
<tr>
<td>T type ALL de novo patients</td>
<td>8</td>
<td>412.73 ± 178.25a</td>
</tr>
<tr>
<td>AML patients</td>
<td>6</td>
<td>494.19 ± 186.23a</td>
</tr>
<tr>
<td>Relapse (refractory) patients</td>
<td>6</td>
<td>574.37 ± 278.45a,b</td>
</tr>
<tr>
<td>Remission group</td>
<td>20</td>
<td>135.80 ± 111.28</td>
</tr>
<tr>
<td>Healthy children</td>
<td>10</td>
<td>91.16 ± 41.34</td>
</tr>
</tbody>
</table>

*aP < 0.01, when compared with the healthy children; ^P < 0.01, when compared with the remission group.
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for 5 minutes as reaction stopping agent. After two times washing with PBS, 20 μl anti-CytC: IgG2b (1:20) (Abcam, Cambridge, MA, USA) or 10 μl anti-Caspase 3-PE (Abcam, Cambridge, MA, USA) were added for 20 min at 4°C; after washing two times with PBS, then adding secondary antibody 20 μl (IgG2b-FITC) (Abcam, Cambridge, MA, USA) was added at 4°C for 20 min in a dark surrounding. After washing twice again, the cells were then fixed with 4% PFA to be processed by a LSR II flow cytometer (BD Biosciences, USA). The results were analyzed with BD Cellquest software (BD Biosciences, USA).

Detection of Akt expression and phosphorylation with western blotting: Briefly, after gel electrophoresis and protein transfer to the PDVF membranes the membranes were blocked, incubated with the primary polyclonal rabbit antibodies against Akt and p-Akt (1:500) (Abcam, Cambridge, MA, USA) and β-actin (1:4000) (Abcam, Cambridge, MA, USA) after which the secondary sheep anti rabbit HRP labeled antibodies (1:1000) (Abcam, Cambridge, MA, USA) were added.

Statistical analyses: The results of VEGF concentrations in various cell lines were expressed as mean ± SD. The expression difference between the groups was calculated by student t test. P-values < 0.05 were considered as statistically significant.

Results

VEGF concentrations in patients' blood plasma

The VEGF concentrations among groups are listed in Table 2. The plasma VEGF level in the relapse patients was the highest and was 6.3 fold higher than that in healthy children control plasmas, followed by the AML patients. VEGF blood plasma level in the remission group was similar to that in healthy donors.

The results of cell culture supernatant VEGF concentration from 6 kinds of leukemia cell lines are shown in Table 3. The VEGF concentrations in normal leukocyte culture medium was lowest and each tumor cell line secreted higher amount of VEGF, but in different degrees.

mRNA expression of Flt-1 and KDR in bone marrow lymphocytes of patients

Among the lymphocytes measured, relapse lymphocytes showed the highest expressions of Flt-1 and KDR, followed by AML lymphocytes and ALL de novo patients, whereas expressions in the remission group and healthy children were low (Table 4).

Analysis of apoptosis factor changes in leukemia cells after VP-16 treatment

We further explored anti-apoptosis effects of VP-16 on highly VEGF expressing Jurkat cells and Jurkat cells stably transfected with the Bcl-2 gene as reference and compared the results with VP-16 effects on leukocytes of healthy children and refractory leukemia patients. The expression of Cyt-C and Caspase-3, which are both apoptosis factors, in the two Jurkat cell groups was basically the same before addition of VP-16 to the medium, but differed after VP-16 treatment (Figure 1).

Taken together, the data in Figure 1 showed that the effectivity of VP-16 is essentially reduced when mechanisms related to apoptosis are hampered, since BCL2 is an anti-apoptotic gene. Next we compared apoptosis related Cyt-c and Caspase-3 expressions of leukocytes from healthy children and leukemia patients after VP-16 applications. In contrast to leukocytes from healthy children, leukemic cells did not express Caspase 3 and the apoptosis rate was essentially less (14.5 vs. 39.20%) (Figure 2).

Activation of the phosphatidylinositol-3/protein kinase B (PI3K)/Akt pathway

Next we analyzed, whether the PI3K/Akt pathway is changed in leukemic cells. The myelogenous leukemia cell line U937, which possesses

| Table 3. VEGF concentration in leukemia cell line supernatants |
|---------------|---------|----------|
| Cell lines    | Types   | Results (ng/ml) |
| HEL           | AEL     | 0.94 ± 0.11   |
| HL-60         | APL     | 0.81 ± 0.13   |
| Jurkat        | T type ALL | 1.55 ± 0.14   |
| K562          | CML     | 1.25 ± 0.08   |
| NB4           | AML     | 0.71 ± 0.09   |
| Normal leukocytes | Normal cells | 0.02 ± 0.005  |
| THP-1         | AML-M5b | 1.59 ± 0.12   |
the characteristic of spontaneous PI3K/Akt site phosphorylation served as positive control. Akt was expressed in peripheral blood mononuclear cells of all healthy children but it was not phosphorylated (p-Akt) (Figure 3A). Akt was expressed in 10 de novo patients (10/35) and was phosphorylated in 3 cases (Figure 3B). Akt was expressed in all relapse cases and phosphorylated in 4 of them (4/6) (Figure 3C). Akt was expressed in 10 of the 20 remission patients but only in 1 patient it was phosphorylated (Figure 3D).

Discussion

In our study we investigated VEGF and VEGFR expressions in the peripheral blood of de novo, relapse, remission patients and healthy children. The results showed that plasma VEGF levels were low in healthy children and the remission group, but significantly enhanced in 35 AML de novo and AML leukemia samples and highest in the relapse group (P < 0.01) (Table 1). Generally higher VEGF secretion of leukemia cells, though in different degrees, could also be shown for the leukemia cell lines HEL, HL-60, Jurkat, K562, NB4 and THP-1 (Table 2). The VEGFR Flt1 and KDR were over expressed in Leukemia patients leukocytes a similar pattern than VEGF. Both receptors were low expressed in the remission group and healthy children, enhanced expressed in ALL and AML leukocytes (P < 0.01) and highest expressed in the relapse group (P < 0.001).

VEGF can be secreted by the bone marrow stromal cells. After being recognized by the tumor cell receptors, VEGF can activate a series of cytokine reaction and promote the tumor cell proliferation [9, 13]. Our data showed that VEGF can also be secreted by leukemia cells, which can promote their germination, proliferation, and migration. The two high-affinity receptors of VEGF, Flt-1 and KDR, are known to be expressed in vascular endothelial cells, myometrial smooth muscle cells, and retinal endothelial cells. Our results showed that Flt-1 and KDR were highly expressed in leukemia cells with highest expression in relapse patients. The activated Flt-1 leads to cell migration, mononuclear cell chemotaxis regulation, and tissue factor expression. KDR activation leads to vascularization and hematopoietic cytopogenesis for cell proliferation and differentiation [14-16]. High VEGF secretion is necessary for the growth of leukemia cells and VEGF is a target for apoptosis induction [9]. Also other effective anti-tumor drugs achieve their treatment goal by inducing apoptosis of tumor cells [17, 18]. Meanwhile, plenty of researches also illustrate that if the apoptosis of tumor cells does not appear during chemotherapy, they are extremely prone to induce drug resistance. The drug induced apoptosis is realized by activation or inactivation of a series of apoptosis regulatory factors. After cytotoxic drugs entered the cells, apoptotic factors are expressed or inhibited (mainly Bcl-2 family members) and the changes of potential differences inside and outside the mitochondrial membrane occur. Cyt-C exists in the mitochondrial intermembrane space and AIF (Apoptosis Inducing Factor) are secreted to the endochylema and combine with Apaf-1 (Apoptotic protease activating factor-1) and Procaspase-9 to form the apoptosome and activated Caspase-9 is formed, which activates Caspase-3 and induces apoptosis in which Bcl-2 family members play a key regulatory role [19-24].

Since the VP-16 effect reduction was similar in Jurkat Bcl-2 and relapse cells, we hypothesized, that apoptosis might be blocked particularly in relapse leukemia cells (Figures 1, 2). VEGF receptor activated PI-3K/AKT signaling plays a vital role in the survival of leukemic lymphocytes (Tchaikovski et al., 2008) (Wang et al., 2000). PI-3K/Akt is a kind of fat protein kinase and its activation process is a series of phosphorylation reactions. Firstly, PI-3K is catalyzed

### Table 4. Relative mRNA transcription (%) of Flt1 and KDR in leukocytes

<table>
<thead>
<tr>
<th></th>
<th>B type ALL de novo patients</th>
<th>T type ALL de novo patients</th>
<th>AML patients</th>
<th>Relapse (refractory) patients</th>
<th>Remission group</th>
<th>Healthy children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flt-1</td>
<td>68.3 ± 16.1 ▲▲▲▲▲</td>
<td>59.1 ± 8.6 ▲▲▲▲▲</td>
<td>79.7 ± 25.7 ▲▲▲▲</td>
<td>100 ± 19.9 ▲▲▲▲▲</td>
<td>38.1 ± 13.1</td>
<td>31.9 ± 8.4</td>
</tr>
<tr>
<td>KDR</td>
<td>49.9 ± 11.9 ▲▲▲▲▲</td>
<td>44.9 ± 13.8 ▲▲▲▲▲</td>
<td>69.2 ± 31.6 ▲▲▲▲</td>
<td>100 ± 33.0 ▲▲▲▲▲</td>
<td>18.6 ± 4.8</td>
<td>24.2 ± 8.3</td>
</tr>
</tbody>
</table>

▲P < 0.05, ▲▲P < 0.01, compared to relapse patients, ▲▲▲P < 0.01, ▲▲▲▲P < 0.001, compared to remission group, ▲P < 0.05, ▲▲P < 0.01 compared to healthy children.
Figure 1. Flow cytometry results of Jurkat cells and Jurkat cells transfected with Bcl-2 survival rates as well as Cyt-C and Caspase-3 expressions before and after VP-16 treatment. On the left panels, before VP-16 treatment, Jurkat cells (A) and Jurkat cells transfected with Bcl-2 (C) appeared in the lower right corners, indicating that most of the cells were alive. Region 1 (R1) denotes that the cells showed no apoptosis morphology but were involved in the early apoptosis processes. Jurkat cells (C) and Jurkat cells transfected with Bcl-2 (D) did not express Cyt-C and Caspase-3 (the values are the ratio of positive cells). On the right panels, after VP-16 treatment, Jurkat cells (A) shifted into the upper left corner with an apoptosis rate of 57.25%. Cyt-C was expressed in all cells of R1 and the activation rate of Caspase-3 was 32.15%, showing that 32.15% cells were involved in the early apoptosis process (B). After VP-16 treatment, Jurkat cells transfected with Bcl-2 (C) appeared in the lower right corner, indicating that the majority of cells did not enter apoptosis. Very few R1 cells expressed Cyt-C with low activation of Caspase-3, showing that no cells were involved in the early apoptosis process (< 10 being regarded as negative).
Figure 2. Flow cytometry determining the changes of Cyt-C and Caspase-3 expressions in leukocytes of the control and relapse groups after treatment with VP-16. Data from healthy control children are shown in the left panels. A. The shift of cells into the upper right corner indicated apoptosis with a rate of 39.20%. B. Cyt-C was expressed in all R1 cells and the activation rate of Caspase-3 was 44.85%. Recurrent leukemia cells are shown in the right panels. A. 14.5% of the cells shifted into the upper left corner, indicating that 86.5% cells did enter apoptosis. B. Cyt-C was expressed in some of the R1 cells, but Caspase-3 was not activated, indicating that no cells were involved in the early apoptosis process (< 10 being regarded as negative).
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Figure 3. Western blot images of Akt expression and phosphorylation (p-Akt) in leukocytes. (A) Total Akt (t-Akt) and p-Akt in 8 peripheral blood samples of healthy children. (B) t-Akt and p-Akt in 10 de novo patients and (C) 6 relapsed patients. (D) t-Akt and p-Akt in 10 from 20 remission patients.

by activation signals to form phosphatidylinositol of diphosphonic acid and triphosphonic acid (PIP2, PIP3), which are subsequently adhered to Akt catalyzing region PH to cause Akt activation, then a series of downstream substrate kinases are activated in a cascade and finally the biological effect is accomplished [25, 26]. Our results showed that AKT was expressed in most of the cells, but the activation degree was different. AKT was not activated in the normal lymphocytes, but AKT was activated in 30% de novo leukemia patients, in 67% relapse leukemia patients and in 10% remission leukemia patients. It was obvious that activated Akt (p-Akt) occurred enhanced in relapse leukemia cells, which might explain their drug resistance. PI-3K/Akt activation can inhibit the apoptosis signal transferred from Bcl-associated protein (Bax) to the mitochondria, reduce Cyt-c translocation and restrain Caspase-9 activation, leaving the apoptosome unable to be formed and inhibiting Procaspase-3 to be associated to the activated Caspase-3.

In conclusion, significantly enhanced expression of VEGF and VEGF receptors Flt-1 and KDR appeared in all leukemia leukocytes and was highest in leukocytes of children with refractory leukemia. Further analysis revealed that Akt was expressed in all control leukocytes but without being phosphorylated, whereas the refractory leukemia group showed highest phosphorylation rates of Akt. We suggest, that particularly in relapse patients leukemic cells develop high VEGF combined with high Flt-1 and KDR expressions, which lead to increased PI3K Akt phosphorylation and concomitant apoptosis inhibition and drug resistance.

Acknowledgements

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Disclosure of conflict of interest

None.

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