Leukocytic-Vascular Endothelial Growth Factor and Integrin $\alpha_v\beta_3$ in Acute Myeloid Leukemia: Relation to Clinical Outcome

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Different signaling routes seem to be simultaneously triggered in leukemia, with distinct and overlapping activities. Different reports emphasize the interaction between vascular endothelial growth factor (VEGF) and integrin $\alpha_v\beta_3$ as a key control system of angiogenesis, oncogenes, and metastasis. The current study was undertaken to investigate leukocytic-VEGF and integrin $\alpha_v\beta_3$ as correlated with clinical outcome in patients with acute myeloid leukemia (AML). The study groups included 10 newly diagnosed AML patients before the start of any chemotherapeutic medication and 10 normal healthy control subjects. The level of VEGF was estimated in culture supernatant of peripheral blood mononuclear cells (PBMC) of both groups using commercially available ELISA kit. The degree of integrin $\alpha_v\beta_3$ expression on PBMC was estimated by indirect immunofluorescence. Obtained results showed that the level of VEGF and degree of expression of integrin $\alpha_v\beta_3$ were significantly higher in AML patients than in normal healthy subjects. However, no significant correlation was observed between the levels of VEGF and the degree of expression of integrin $\alpha_v\beta_3$. When clinical findings were concerned, there was a significant positive correlation between VEGF and the percentage of blasts, both in peripheral blood and bone marrow. On the other hand, such correlations were not observed in case of integrin $\alpha_v\beta_3$. In conclusion, our results proved the importance of VEGF and integrin $\alpha_v\beta_3$ in the pathogenesis of AML. However, the per se increased production or/and secretion of VEGF and integrin $\alpha_v\beta_3$ by leukemic PBMC cells, respectively can not be used as independent predictor(s) for clinical outcome in AML patients. It is more comprehensive to study changes of intracellular signaling pathways when such critically interacting factors are concerned in the leukemic process.

Acute myelocytic leukemia (AML) is a malignant neoplasm of hematopoietic cells characterized by an abnormal proliferation of myeloid precursor cells, decreased rate of self-destruction and an arrest in cellular differentiation. The leukemic cells have an abnormal survival advantage. Thus, the bone marrow and peripheral blood are characterized by leukocytosis with a predominance of immature cells, primarily blasts. As the immature cells accumulate in the bone marrow, they replace the normal myelocytic cells, megakaryocytes, and erythrocytic cells. This leads to a loss of normal bone marrow function and associated complications of bleeding, anemia, and infection ( Löwenberg et al., 1999).

The endothelium forms a network of vasculature spanning the whole cavity of the bone marrow, thus providing a blood supply like in solid organs (Mazo et al., 1999). Accordingly, increased bone marrow angiogenesis, the process of new blood vessel formation from endothelial precursors, is crucial for the pathogenesis and progression of AML and other hematological malignancies similar to solid invasive tumors (Aguayo et al., 2000). A critical event in the regulation of angiogenesis is the signaling cascade involving vascular endothelium growth factor (VEGF-A, VEGF), which is a multifunctional, secreted cytokine that stimulates endothelial cells to proliferate, to migrate, and to increase their permeability to plasma proteins, (Karamysheva, 2008). The ability of this factor to enhance vascular permeability defines its important role in tumor cell penetration into vascular networks.
Leukocytic-VEGF and Integrin αvβ3 in AM

and metastasis (Sierra, 2005). VEGF exists as five different isoforms of 121, 145, 165, 189 and 206 amino acids (Neufeld et al., 1996). On adult endothelial cells it exhibits high-affinity binding sites corresponding to two distinct tyrosine kinase receptors, the VEGF receptor (VEGFR)-1 encoded by Flt-1 and VEGFR-2 encoded by KDR/Flk-1 with much greater binding affinity to VEGFR1. (Robinson et al., 2001). It now appears that VEGF also has autocrine functions acting as a survival factor for tumour cells protecting them from stresses such as hypoxia, chemotherapy and radiotherapy. The mechanisms of action of VEGF are still being investigated with emerging insights into overlapping pathways and cross-talk with other receptors (Byrne et al., 2005).

Adhesion molecules, including integrins, mediate critical cytosolic signaling events that regulate both physiologic and pathologic events, including complex processes such as leucocyte migration, angiogenesis, tumor growth, and metastasis (Moschos et al., 2007). Several examples of crosstalk between integrins and growth factor receptors indicate that integrin ligation is required for growth factor–induced biological processes. Furthermore, integrins can directly associate with growth factor receptors, thereby regulating the capacity of integrin/growth factor receptor complexes to propagate downstream signaling (reviewed in Eliceiri, 2001). Integrin αvβ3 (leukocyte response integrin) is the cell surface receptor for vitronectin, fibronectin, fibrinogen, and denatured collagen and is widely distributed on monocytes, vascular endothelial cells and tumor cells (Capo et al., 1999; Kerr et al., 2002; Kumar, 2003). It plays an important role in regulation of cell growth, differentiation, migration, and angiogenesis. The adhesion of integrin αvβ3 to the ECM proteins induces focal adhesion contacts which generate the cascade of phosphorylation of many signal transduction molecules, including tyrosine kinases (FAK, pp60src), cytoskeletal proteins (vinculin, paxillin), Grb-2, and MAP kinase (reviewed in Hodivala-Dilke K. 2008). Previous reports have provided evidences for the interaction between VEGF and integrin αvβ3 as regards tumor progression (De et al., 2005; Soldi et al., 1999). So, in the current study we investigated leukocytic-VEGF and integrin αvβ3 as related to clinical outcome in patients with AML.

**Subjects and Methods**

The present study was conducted on ten newly diagnosed AML patients before the start of any chemotherapeutic medication. Ten normal healthy subjects were included as a normal control group. Patients were obtained from Hematology Department, Medical Research Institute, University of Alexandria. Patients were subjected to detailed history taking and thorough clinical examination with special emphasis on lymph nodes, liver and spleen.

Diagnosis of AML based on standard morphology and cytochemistry of peripheral blood and bone marrow films according to the French-American-British (FAB) criteria (Bennett et al., 1985). Diagnosis was confirmed by immunophenotyping using a comprehensive panel of monoclonal antibodies against myeloid associated antigens as proposed by the EGIL group (Bene et al., 1995).

**Routine Hematological Investigations**

Complete blood picture including hemoglobin concentration, platelet count as well as total and differential leukocytic counts was done using automated blood cell counter (Hema Star II). Films from fresh blood samples and bone marrow aspirate were stained by Leishmann stain and cytochemical staining to confirm the differential blood leukocytic count and detect abnormal leukocyte morphology and characteristic abnormalities in the bone marrow (Bain et al., 2001).

**Cell Culture**

Peripheral blood mononuclear cells (PBMC) and leukemic blast cells were isolated from normal control subjects and AML patients, respectively. Simply, heparinized venous blood samples were centrifuged over ficoll-hypaque density gradient (Sigma) at 1800 rpm for 45 min., washed three times with saline (NaCl 0.9%), and resuspended in RPMI-1640 medium (Gibco, UK) supplemented with 10% fetal calf serum,
100 IU/mL penicillin, 100 mg/mL streptomycin and 2 mM glutamine. Viability of the isolated cells was tested using dye exclusion technique which is based on the impermeability of viable cells to trypan blue. Resuspended cells were cultured in 96 wells flat bottom polystyrene culture plates (2x10^5 cells/well) and stimulated with 20µg/ml phytohaemagglutinin (PHA; Sigma). After incubation for 72 hrs in a humidified 5% CO_2 incubator at 37°C, culture supernatant was collected and stored at -70°C till time of assay for VEGF (Fiedler et al., 1997).

Measurement of Leukocytic-VEGF-A (VEGF)

The level of VEGF was measured in culture supernatant using commercially available enzyme linked immunosorbent assay [ELISA] kit [Human VEGF-A ELISA Kit, Bender MedSystems, Austria] and following manufacturer recommendations. The sensitivity of the applied ELISA kit is 20 pg/ml. Concentrations were deduced from a manually constructed standard curve using supplied standard concentrations and corresponding optical densities.

Expression of Leukocytic-Integrin α_β_3

The degree of expression of integrin α_β_3 on PBMN was assessed by indirect immunofluorescent technique as previously described (Yläne et al., 1990) with mild modification. Briefly, PBMN cells were attached to glass slides by cytoospin centrifugation and fixed in 100% ethanol. PBMN monolayers were washed in PBS (pH 7.4) containing 0.2% bovine serum albumin and then incubated with the first monoclonal antibody [1/100, mouse anti-human integrin alphavbeta3, Chemicon International, Austria] for 45 min at room temperature. After washing with PBS, pH 7.4, samples were incubated with goat anti-mouse IgG coupled to fluorescein isothiocyanate [1/50, Goat-anti-mouse IgG (Fc specific), FITC conjugate, Sigma] for 45 min at room temperature in the dark. Slides were then washed in PBS (pH 7.4) and mounted in glycerol-PBS (9–1). Expression was scored blindly [(+), (++), and (+++) by fluorescent microscopic examination considering intensity and density of fluorescence on the periphery of the cells.

Statistical Analysis

Statistical analysis was done using Statistical Package for Social Sciences (SPSS/version 15) software. Chi-square test and student t-test were used for comparing qualitative and quantitative data of each two groups, respectively. Person correlation coefficient was used to detect the correlation between different variables. The level of significant was 0.05. Numerical values are presented as mean ± SD.

Results

Subjects under study included ten newly diagnosed AML patients (2 males and 8 females, mean age 43.5±13.52 years) before the start of any chemotherapeutic medication and ten normal healthy control subjects (4 males and 6 females, mean age 41.6±10.33 years). No significant difference was observed between the two groups as regards age and sex.

Clinical data

All control subjects were completely healthy with normal complete blood picture. Total leukocytic count (TLC) and percentage of leukemic blasts in peripheral blood (PB) and bone marrow (BM) in AML patients at presentation are presented in Table (1). According to FAB classification, AML patients were either M1, M2, or M4 (number = 4, 2, and 4, respectively).

<table>
<thead>
<tr>
<th>Min.</th>
<th>Max.</th>
<th>Mean</th>
<th>S.D.</th>
</tr>
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<tbody>
<tr>
<td>TLC(10^9 / L)</td>
<td>8600.0</td>
<td>75000.0</td>
<td>34551.0</td>
</tr>
<tr>
<td>Blasts in PB (%)</td>
<td>12.0</td>
<td>80.0</td>
<td>51.30</td>
</tr>
<tr>
<td>Blasts in BM (%)</td>
<td>22.0</td>
<td>95.0</td>
<td>60.8</td>
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Leucocytic-VEGF

The level of VEGF in culture supernatant of PBMN from AML patients was significantly higher than corresponding level in normal control subjects, \(253.8 \pm 121.49\) vs \(143.5 \pm 20.94\) pg/ml, respectively, \(P = 0.0093\). (Table 2, Figure 1). In AML patients, estimated level of VEGF had a significant positive correlation with the percentage of blasts, both in PB & BM. \((P = .033 & .011,\) respectively). However, no significant correlation was observed when clinical staging, age, and sex were concerned in relation to VEGF.

Leucocytic- integrin \(\alpha_\beta_3\)

The intensity of expression of integrin \(\alpha_\beta_3\) on PBMN from AML patients was significantly higher than that in normal control subjects, \(P= 0.0006\). (Table 2, Figure 2, 3). However, there was no significant correlation between leukocytic- VEGF and integrin \(\alpha_\beta_3\). Similarly, no significant correlation was observed when percentage of blasts, clinical staging, age, and sex were concerned in relation to integrin \(\alpha_\beta_3\) in AML patients.

Figure 1. Leukocytic-VEGF in AML patients and normal control subjects.
Table 2. Leukocytic-VEGF and integrin αvβ3 in AML patients and normal control subjects.

<table>
<thead>
<tr>
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<th>AML patients</th>
<th>Normal control</th>
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<tbody>
<tr>
<td></td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>VEGF(pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>68 – 480</td>
<td>120 – 184</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>253.8±121.49</td>
<td>143.5±20.94</td>
</tr>
<tr>
<td>P</td>
<td>0.0093*</td>
<td></td>
</tr>
<tr>
<td>Integrin αvβ3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>0 (0.0%)</td>
<td>6 (60.0%)</td>
</tr>
<tr>
<td>++</td>
<td>2 (20.0%)</td>
<td>4 (40.0%)</td>
</tr>
<tr>
<td>+++</td>
<td>8 (80.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>P</td>
<td>0.0006*</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05 is significant.

Figure 2. Leukocytic- integrin αvβ3 in AML patients and normal control subjects.
Discussion

AML is a rapid progressive disease characterized by high proliferation of leukemic blasts with consequent repression of normal hematopoiesis in the bone marrow. AML shows the characteristic malignant behavior through the ability of immature cells to circulate in blood and to invade peripheral tissues. In fact, the leukemic process is very complicated where it is "fed" by intensively...
interacting leukemic cells, endothelial cells and extracellular matrix. Among the important candidates of this interplay are angiogenic growth factors and adhesion molecules (McKenzie, 2005; Wood, 2007). In the current study we are concerned with two important candidates; VEGF and integrin \( \alpha_v \beta_3 \).

Our results revealed that leukemic PBMN produce significantly higher levels of VEGF than normal PBMN. Such data support pervious evidences for the importance of leukocyte-associated VEGF in the pathogenesis of AML. It was previously observed that leukemic blasts from AML patients express and produce significantly increased levels of VEGF (Aguayo et al., 2000; Dias et al., 2000; Fiedler et al., 1997; Hussong et al., 2000; Loges et al., 2006).

It is now quite evidenced that acute leukemia cells use angiogenic growth factor signaling pathways, namely those activated by VEGF in autocrine and paracrine fashions. The presence of VEGFR-1 and -2 on many of leukemic blasts implies that expansion of the leukemic population may be facilitated by an autocrine loop (Bellamy et al., 2001). Such autocrine action of VEGF was supported by other more recent studies (Fragoso et al., 2007, Vales et al., 2007). Moreover, the leukemia-derived VEGF can also stimulate the production of growth factors, including interleukin-6 [IL-6] and granulocyte-macrophage colony stimulating factor [GM-CSF], by human endothelial cells, which in turn further promotes the growth of leukemia cells (the paracrine loop). In a system where leukemia cells are co-cultured with endothelial cells, IMC-2C6, a fully human anti-VEGFR2 antibody, inhibits both the production of IL-6 and GM-CSF by endothelial cells and the growth of leukemia cells. In addition, IMC-2C6 effectively blocks VEGF-induced migration of KDR\(^+\) human leukemia cells, and when administered in vivo, significantly prolonged survival of mice inoculated with KDR\(^+\) human leukemia cells (Zhang et al., 2004). Autocrine and paracrine actions of VEGF results in leukemic cell proliferation, increased survival and migration. Paracrine growth stimulation may not only be restricted to the bone marrow microenvironment, but may also take place at extramedullary sites. Circulating AML blasts may profit from paracrine provision of growth factors in various capillary beds. This may result in their expansion in peripheral blood (Fragoso et al., 2007).

On the contrary, Litwin et al., (2002); showed a discrepancy between bone marrow vascularity and VEGF expression in vivo and VEGF expression and angiogenesis from 2-day conditioned medium ex vivo. They suggested that angiogenesis in AML likely represents a response to microenvironmental factors in vivo, rather than being an intrinsic property of leukemic cells. Actually, the control of angiogenesis involves not only changes in the profiles of environmental angiogenic cues, such as the increase in VEGFR2 in response to VEGF, but also changes in the adhesive capacity of the endothelial cells. In particular, the changes in one family of adhesion molecules, integrins, are thought to regulate several of these angiogenic steps. In this respect the interaction of co-expressed endothelial VEGFR2 and integrin \( \alpha_\beta_3 \) is suggested to play an important role (Hodivala-Dilke, 2008). Recent studies have identified a significant increase in activated \( \alpha_\beta_3 \) on angiogenic vessels, and these cells also appear to express high levels of VEGFR2 (Mahabeleshwar et al., 2008). It was previously shown that VEGF increases the expression of \( \alpha_\beta_3 \) by human microvascular endothelial cells and also enhances cell adhesion and migration mediated by \( \alpha_\beta_3 \) (Byzova et al., 2000; Senger et al., 1996).

Current results also showed that the expression of leucocytic- integrin \( \alpha_\beta_3 \) was significantly higher in AML patients than in
normal control subjects. Up to our knowledge, current study is the first report looking for the expression of integrin αvβ3 by peripheral blood blast cells in AML patients. It was previously observed that integrin αvβ3 is expressed by leukemic cell lines (Aswald et al., 2004, Atkins et al., 1998, Benedetto et al., 2006) and other metastatic malignant cells (Felding-Habermann et al., 2001, 2002).

In cancer growth, the modification of integrin structure is often associated with both quantitative and qualitative alterations in integrin cell surface patterns (Mizejewski, 1999). A characteristic feature of integrins, including αvβ3, is the capacity to transmit signals bidirectionally, both inside-out and outside-in. (Byzova et al., 2000). Integrin activation, or inside-out signaling, is a tightly governed process involving conformational changes within the highly conserved cytoplasmic tail of integrin receptor β subunits (Hughes et al., 1996) and provides a mechanism of integrin regulation. This process occurs when an agonist binds to a traditional receptor that ultimately changes the activation state of an integrin (Banno et al., 2008). Previous studies emphasize the role of integrin αvβ3 as a “gatekeeper” of VEGF-mediated processes (Borges et al., 2000, Reynolds et al., 2004, Soldi et al., 1999). More recently, De S. et al., (2005), have defined a mechanism where by integrin αvβ3, through activation, clustering, and signaling, regulates the production of VEGF in tumor cells expressing this integrin. Tumors with “activatable” but not “inactive” β3 integrin secrete high levels of VEGF, which in turn promotes extensive neovascularization and augments tumor growth in vivo. This stimulation of VEGF expression depends upon the ability of αvβ3 integrin to cluster and promote phosphorylation of p66 Shc (Src homology 2 domain containing).

We could not find a significant correlation between released VEGF and expressed integrin αvβ3. When clinical and hematological findings were concerned, our data revealed a significant positive correlation between VEGF and the percentage of blasts, both in PB & BM. On the other hand, such correlations were not observed in case of integrin αvβ3. In addition, no significant correlation was observed between either VEGF or integrin αvβ3 and clinical staging, age, and sex. Several reports pointed to the correlation between increased VEGF and worse prognosis (Aguayo et al, 1999, 2002; de Bont et al., 2002). In addition, increased expression of integrin αvβ3 was associated with poor prognosis and increased metastasis in solid tumors (Gasparini et al., 1998; Kageshita et al., 2000). However, our results suggested that it is not just the quantitative analysis which reflects the importance of VEGF and integrin αvβ3 in the leukemic process. Looking for qualitative changes as well as intracellular signaling pathways is more indicative for the ultimate interaction between these two importantly cooperating factors.

In conclusion, obtained data confirm the importance of VEGF and integrin αvβ3 VEGF in the pathogenesis of AML. The above mentioned discussion reflects the complex nature of interaction between VEGF and integrin αvβ3 in controlling the leukemic process. So, it is better to rely on related qualitative rather than just quantitative changes. The intimate link between integrin αvβ3 and VEGF in tumor growth and angiogenesis may influence anti-integrin as well as anti-VEGF therapeutic strategies.

References


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