Bone Marrow Angiogenesis in Patients with Hematological Malignancies: Role of VEGF

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ABSTRACT
The formation of new capillaries from pre-existing blood vessels is known as angiogenesis. This process is enhanced by several cytokines including basic fibroblastic growth factor (bFGF), IL-8, vascular endothelial growth factor (VEGF) and others. VEGF appears to be the most relevant cytokine in this respect, since its expression is closely correlated with vessel density in several human tumors and inhibition of VEGF by specific antibodies suppresses tumor growth. The aim of our work was to study BM angiogenesis in a variety of hematopoietic neoplasms and to investigate any possible role for VEGF in the growth of such neoplasms. A total of 80 patients with various hematological neoplasms were evaluated for BM angiogenesis. They were divided into 4 groups including 20 patients with non-Hodgkin's lymphoma and 20 patients with Hodgkin's disease, both with bone marrow infiltration, 20 patients with multiple myeloma and 20 patients with acute leukemia. Sera from patients and controls were collected for estimation of VEGF. Our results showed that both microvessel density in BM biopsies and serum VEGF levels in patients with hematological malignancies significantly exceeded those of the controls. Also, there was a significant positive correlation between the vessel count and VEGF levels in patients with both MM and NHL.

Key Words: Angiogenesis - Hematological malignancies - VEGF.

INTRODUCTION

The formation of new capillaries from existing blood vessels is known as angiogenesis. This process involves activation of endothelial cells of a mature vessel, localized degradation of the surrounding basement membrane, the movement of adjacent vascular endothelial cells through such a breach and their subsequent migration into the surrounding connective tissue stroma, where they eventually proliferate and form tubular structures. On joining with other similar structures, a network of new blood vessels is formed [5]. New vessel development is required for tumor cell proliferation, extracellular matrix invasion and haematogenous metastasis [26]. Although the importance of angiogenesis in the growth of solid tumours is well established, its role in haematopoietic malignancies is not clear yet.

The induction of angiogenesis is mediated by multiple molecules that are released by both tumor and host cells, including endothelial cells, epithelial cells, mesothelial cells and leukocytes. Among these, are members of the fibroblast growth factor (FGF) family, vascular endothelial cell growth factor (VEGF), IL-8, angiogenin, angiotropin, epidermal growth factor fibrin, platelet derived endothelial cell growth factor, tumor necrosis factor and others [6].

The extent of angiogenesis is determined by the balance between factors that stimulate and those that inhibit new blood vessel growth. In patients with Li-Fraumeni syndrome, neoplastic cells switch from an angiogenesis-inhibiting to an angiogenesis-stimulating phenotype. This switch coincides with the loss of the wild type allele of the p53 tumor suppressor gene which normally suppresses expression of both b FGF and VEGF [4,16]. In rodent and human colonic epithelial cells, transformation by activated ras oncogenes has been shown to upregulate the activity of the angiogenic molecule VEGF [21]. VEGF appears to be one of the most relevant
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Angiogenic factors, since its expression is closely correlated with vessel density in several human tumors and inhibition of VEGF by specific antibodies suppresses tumor growth [14].

VEGF is a homodimeric heparin-binding glycoprotein, which is a potent inducer of vascular permeability, thus allowing extravascular leakage of plasma proteins such as fibrinogen. This can then be converted into a cross-linked fibrin gel, thus inducing extracellular matrix, which may be critical for a sustained angiogenic response [22]. In addition, it serves as a specific endothelial cell mitogen due to the presence of two high-affinity cell surface receptors for VEGF on activated endothelial cells (KDR and Flt-1). VEGF expression is physiologically induced by hypoxia and plays a role in inflammatory diseases such as rheumatoid arthritis, wound healing and diabetic retinopathy [9].

The aim of this work is to study the role of VEGF in the process of angiogenesis in patients with some hematological malignancies.

PATIENTS AND METHODS

A total of 80 patients with various newly diagnosed haematological neoplasms with ages ranging from 16 to 70 years were included in our study, 46 of them were males and 34 were females. They were divided into 4 groups; group I consisted of 20 patients with multiple myeloma with a mean age of 60.2±8.1 years, group II consisted of 20 patients with acute leukemia, which were subdivided into group II a including 10 patients with acute lymphoblastic leukemia (ALL) with a mean age of 27.6±3.9 years and group II b including 10 patients with acute myeloid leukemia (AML) with a mean age of 31.6±5.2 years. Group III consisted of 20 patients with Hodgkin's disease (HD) with a mean age of 34.4±5.1 years and group IV consisted of 20 patients with non Hodgkin's lymphoma (NHL) with a mean age of 42.2±14.3 years.

To establish controls, we studied bone marrow biopsies from 15 lymphoma patients with no evidence of bone marrow infiltration, their mean age was 35.5±3.4 years. These were obtained as part of their clinical staging procedures. All biopsy specimens from patients and controls were evaluated for microvessel formation and sera from patients and controls were collected for estimation of VEGF levels.

Bone marrow trephine biopsies were obtained from the posterior superior iliac spine with Islam needle, immediately fixed in formol saline 10% and underwent conventional decalcification, paraffin embedding and stained with H & E and examined for morphological evaluation. To localize and count microvessels, B.M. sections were stained immunohistochemically by anti-von Willebrand factor (vWF) to highlight endothelial cells [19].

Four micrometer thick section from each paraffin embedded B.M biopsy was cut, de-waxed, rehydrated and subjected to vWF antigen retrieval using citrate buffer (pH6) and heating in a microwave oven at 1000 W for 3 successive cycles, 3 minutes each. The slides were left to cool for 15 minutes in the buffer, rinsed in distilled water twice and finally in phosphate buffered saline (PBS). Staining of the sections was done using Dako enVision+ system HRP (Dako-Denmark), in which any endogenous peroxidase activity is quenched by incubating the section for 5 seconds with a peroxidase block followed by staining with monoclonal mouse anti-human vWF diluted 50 times as a primary antibody (Dako-Denmark). After a washing step horseradish peroxidase (HRP) labeled polymer was added and then sections were incubated with 3,3’ diaminobenzidine DAB+Substrate chromogen which results in a brown colored precipitate at the vWF site thus identifying endothelial cells and microvessels (capillaries and small venules) [20].

Microvessels were counted per high power field using the same microscope (CH20 Olympus-Japan) applying a 10 X eyepiece and a 40 X objective lens.

VEGF was determined in the sera of the patients and controls using VEGF ELISA kit (Penninsula Inc. USA) [2].

The statistical analysis used in the present study included calculation of the mean, standard deviation (SD), analysis of variance (ANOVA) test followed by least significant difference (LSD) test if the F value was significant and the correlation coefficient test. A significant test was considered if the p value was below 5%.
RESULTS

Microvessels are identified as endothelial cells either single or clustered in nests or tubes and clearly separated from one another either with or without a lumen (not exceeding 10 microns). In the present work, the microvessels in patients with haematological malignancies were generally thin, winding, often without visible lumina with evident small lumenless endothelial sprouts as opposed to the straight vessels with no sprouts in control BM. Fig. (1) shows a high power view of HD (lymphocytic predominance) showing weak staining reaction around endothelial cells of a compact vessel. Fig. (2) a lower power magnification showing that the hemopoietic tissue has been replaced by a mass of small lymphocytic lymphoma cells with an increase in microvessel formation.

The mean microvessel counts reported as vessel per high power field (VC/HPF), in bone marrow (BM) biopsies from all groups of haematological neoplasms were found to exceed the control group. This difference was statistically significant in the multiple myeloma (MM) group (mean 11.7±4.2 VC/HPF) (p = 0.000), the acute lymphoblastic leukemia (ALL) group (5.7±5.4 VC/HPF) (p = 0.002), the Hodgkin’s disease (HD) group (9±3.9 VC/HPF) (p = 0.000) and the non Hodgkin’s lymphoma (NHL) group (4.7±3.4 VC/HPF) (p = 0.003). Although, the mean microvessel count in the acute myeloid leukemia group (AML) (3.4±1 VC/HPF) was higher than the control group (0.81±0.5 VC/HPF) this difference was not statistically significant (p = 0.083) (Table 1). On comparing the groups of haematological malignancies with each other, patients with MM were shown to have significantly higher microvessel counts than all the other haematological neoplasms studied. Also, HD patients were found to have significantly higher microvessel counts than NHL patients (F 19.580* & p < 0.01).

As regards serum VEGF levels, the mean values were significantly higher in all groups of patients with haematological malignancies when compared to the control group (Table 1).

Also, ALL patients had significantly higher serum VEGF levels than AML patients and among lymphoma patients, the NHL group had significantly higher serum VEGF levels than the HD group (F 22.884* & p < 0.01).

A significant positive correlation was found between the microvessel counts and the serum VEGF levels among both MM and NHL patients (r = 0.438, p < 0.05 & r = 0.556, p < 0.01 respectively) (Fig. 3).

Table (1): Mean microvessel counts and serum VEGF levels among various groups of hematological malignancies compared to the control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>VC/HPF</th>
<th>S-VEGF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I MM</td>
<td>11.7±4.2</td>
<td>559.75±152.3</td>
</tr>
<tr>
<td>Group IIa ALL</td>
<td>5.7±5.4</td>
<td>480.6±109.4</td>
</tr>
<tr>
<td>Group IIb AML</td>
<td>3.4±3</td>
<td>607.5±196.9</td>
</tr>
<tr>
<td>Group III HD</td>
<td>9±3.9</td>
<td>438.35±123.3</td>
</tr>
<tr>
<td>Group IV NHL</td>
<td>4.71±3.9</td>
<td>537.1±137.36</td>
</tr>
<tr>
<td>Control group</td>
<td>0.8133±0.54</td>
<td>139.8±74.74</td>
</tr>
<tr>
<td>F</td>
<td>19.58</td>
<td>22.884</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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Fig. (1): BM biopsy from a patient with HD (lymphocytic predominance) showing weak staining reaction around endothelial cells of a compact vessel.

Fig. (2): A lower power magnification of BM biopsy showing that the hemopoietic tissue has been replaced by a mass of small lymphocytic lymphoma cells with increase in microvessel formation.
DISCUSSION

For solid tumors to grow beyond 2 mm² in size, there is a strict requirement for angiogenesis [11]. A central role for VEGF in tumor neo-vascularization and growth in vivo was demonstrated by Kim et al., in 1993 who showed that the addition of neutralizing antibodies to VEGF resulted in a marked suppression of tumor growth [14]. In addition to its expression in solid tumors, VEGF is expressed in normal cells including activated macrophages, renal glomerular epithelial and mesangial cells, platelets and keratinocytes [2,27]. In a study conducted by Bellamy and colleagues, VEGF was found to be expressed in plasma cells in the bone marrow from patients with multiple myeloma, whereas VEGF receptors were observed to be markedly elevated in the normal bone marrow myeloid and monocytic cells surrounding the tumor. These data raise the possibility that VEGF may play a role in the growth of hematopoietic neoplasms such as multiple myeloma through either a paracrine or an autocrine mechanism [2].

In our study, we found that patients with hematological malignancies generally had significantly higher bone marrow microvessel counts than the controls. Our multiple myeloma patients had the highest bone marrow microvessel counts which significantly exceeded those of the other groups of studied patients, thus suggesting a higher angiogenic potential in such patients. These data support the hypothesis that hematopoietic neoplasms may be angiogenesis dependent, thus raising the possibility for a role of antiangiogenic drugs as adjuvant therapy in such conditions [25]. Vacca and his colleagues also demonstrated a significant increase of bone marrow angiogenesis (evaluated as microvessel area) in patients with active multiple myeloma compared with non-active myeloma and monoclonal gammopathies of unknown significance [26]. They also demonstrated that the angiogenic activity exerted by the culture media (CM) of plasma cells isolated from a given patient correlated significantly with the corresponding bone marrow microvessel area in all patients. Plasma cells may thus be a major source of angiogenic stimuli in the bone marrow microenvironment, although macrophages, T-cells and mast cells may also play a role [13].

It was also found that plasma cells isolated from the bone marrow of active multiple myeloma patients produce higher levels of basic fibroblast growth factor than in inactive myeloma, thus suggesting that this angiogenic factor plays a role in bone marrow neovascularization during multiple myeloma progression. On the other hand, neutralizing anti-FGF-2 antibodies caused only a partial decrease in the angiogenic activity exerted by the plasma cell CM in both in vivo and in vitro assays [27]. These data suggest that other factors secreted by plasma cells are able to induce angiogenesis directly or indirectly (via monocyte recruitment and activation), namely VEGF, tumor necrosis factor-α, macrophage colony stimulating factor, IL-1β and transforming growth factor β and these may act synergistically with b-FGF in myeloma [1].

On the other hand, among our lymphoma patients, we found that those with HD showed significantly higher microvessel counts than those with NHL. It is known that malignant lymphomas are heterogenous with respect to their microvasculature. Peripheral T-cell lymphomas characteristically display many vessels with the morphology of high endothelial venules and this feature is most prominent in peripheral T-cell lymphoma of angioimmunoblastic lymphadenopathy type. Vascularity is also prominent in Hodgkin’s disease, but not in most cases of low grade B-cell lymphomas [12]. Since more than 50% of our NHL patients belonged to the low grade B cell variety (small lymphocytic, lymphoplasmacytoid) and none exhibited the angioimmunoblastic lymphadenopathy-like pathology, angiogenesis was less prominent than in our HD patients, although it significantly exceeded the control group.

Similarly to peripheral T-cell lymphoma
high pretreatment s-VEGF in patients with NHL was associated with a poor outcome [23]. Also, elevated levels of both angiogenic factors have been detected in the serum and urine of patients with various types of cancer [15,17,24,28] and have been detected significantly less frequently in individuals with no sign of cancer as in ongoing inflammation or during wound healing [8].

Among both MM and NHL patients, a significant positive correlation was found between the microvessel count and serum VEGF levels. Why this positive correlation was not evident in other patients with hematological malignancies is not yet known but it is likely that other angiogenic factors may be operative in such patients.

In conclusion: Serum VEGF level and microvessel count in the B.M biopsies from patients with hematological malignancies significantly exceeded those of the control group reflecting an increased angiogenic potential in such patients. Neoplastic cells may produce or induce the secretion of VEGF in reactive cells with the enhancement of angiogenesis; anti-angiogenic drugs may thus have a role as adjuvant therapy in hematological neoplasms.

REFERENCES


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