



## Angiogenic factors in multiple myeloma: higher levels in bone marrow than in peripheral blood

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### ABSTRACT

**Background and Objectives.** To study the role of some soluble factors in the process of angiogenesis that accompanies multiple myeloma (MM).

**Design and Methods.** The concentrations of three well-known angiogenic peptides, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) were evaluated by an ELISA method. All of these factors were measured in the plasma obtained from peripheral blood (PB) and bone marrow (BM) aspirates of 34 patients affected by plasma cell disorders. This series included one patient with a solitary extramedullary plasmacytoma, 17 patients with MM at diagnosis, and 16 with previously treated MM.

**Results.** In all the patients, the concentration of each angiogenic factor was higher in bone marrow than in peripheral blood. Mean values of the three angiogenic factors in BM or in PB were lower in stage I than stage II-III. One patient with extramedullary solitary myeloma had high levels of VEGF and bFGF but this increase was not found in the other 6 patients with extramedullary disease when compared with patients without extramedullary disease. VEGF and bFGF did not correlate with each other while HGF showed a weak correlation with VEGF and a stronger one with bFGF. Moreover, VEGF correlated with features of disease activity, such as C-reactive protein, and  $\beta_2$ -microglobulin, while both bFGF and HGF showed an inverse correlation with albumin level. No correlation was found between VEGF, bFGF and HGF levels and age, M protein level, osteolytic lesions, or percentage of BM plasma cells. Since angiogenic factors may be released by normal cells in response to hypoxia, we also evaluated erythropoietin (EPO) levels (which correlate with the hypoxic stimulus) both in PB and BM plasma of these patients but none of the measured angiogenic factors correlated with EPO levels.

**Interpretation and Conclusions.** Several soluble factors may play a role in the angiogenic activity described in MM but their contribution to the progression of disease may be different. The finding of higher levels of these factors in BM than in PB might

indicate that the bone marrow environment is their major source. Concentrations of angiogenic factors parallel the activity of disease and are independent of the hypoxic stimulus.

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Some recent studies have shown the importance of neovascularization in the biology of multiple myeloma (MM) and it has been demonstrated that bone marrow angiogenesis parallels progression of disease.<sup>1,2</sup> Confirmation of these biological observations came from recent clinical trials that have also shown the efficacy of an antiangiogenic treatment in patients with relapsing MM.<sup>3</sup> However, so far little is known about the mechanism that promotes angiogenesis within the complex cytokine network that regulates the biology of MM. Angiogenesis is a not yet fully elucidated multistep process in which many cells and cytokines may play a role. Some soluble factors with angiogenic activity either in normal or in neoplastic conditions have been identified,<sup>4</sup> but which of these factors is involved in MM angiogenesis remains to be determined as does their hierarchy.

The first candidate is vascular endothelial growth factor (VEGF). This factor is considered as a key mediator of embryonic and physiologic postnatal angiogenesis.<sup>5</sup> Moreover, elevated VEGF levels have been found in the sera of up to 65% of patients affected by several type of metastatic cancers, suggesting that it plays a role in neoplastic progression.<sup>6</sup> As far as concerns plasma cell dyscrasias, elevated VEGF levels have been found in patients affected by POEMS syndrome, a disease closely related to MM.<sup>7</sup> Also, it has been demonstrated that VEGF mRNA is present in plasma cells from both MM cell lines and patients.<sup>8</sup> The VEGF produced can induce secretion of IL-6 (the most important myeloma growth factor) from stromal cells and, in turn, IL-6 stimulates the secretion of VEGF, thus determining a mutual co-operation between the two factors.<sup>9</sup>

Another potent inducer of angiogenesis is basic fibroblast growth factor (bFGF). High bFGF levels have been found in serum and urine<sup>10,11</sup> of patients with several types of cancer and, it has been indicated, are an independent predictor of poor prognosis in lymphoproliferative disorders such as chronic lym-

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phocytic leukemia<sup>12</sup> and non-Hodgkin's lymphoma.<sup>13</sup> Elevated concentrations of this factor were found in plasma cell extracts from MM patients and its level correlated with activity of disease. Moreover, neutralizing anti-bFGF antibodies strongly inhibit the angiogenic activity present in plasma cells of patients with active myeloma.<sup>2</sup> That bFGF has a role in MM pathobiology is also supported by studies of its low affinity receptor, syndecan-1. This molecule is constantly present in plasma cells of patients with MM and is considered a useful marker of detecting malignant plasma cells in the blood or bone marrow.<sup>14</sup> Functional studies in MM have shown that syndecan-1 has an effect on tumor cell growth, survival, cell adhesion and may modulate myeloma bone disease.<sup>15</sup> However, the clinical significance of the bFGF bound to myeloma cells by syndecan-1 needs further elucidation.

A third possible candidate for angiogenesis in MM is hepatocyte growth factor (HGF). This is a pleiotropic cytokine capable of stimulating growth of several epithelial cells and inducing blood vessel formation.<sup>16</sup> In addition, HGF can promote formation and activation of osteoclasts.<sup>17,18</sup> HGF is normally produced by mesenchymal cells, including bone marrow stromal cells. However, HGF mRNA and its receptor (c-met) are also expressed in human myeloma cell lines<sup>19</sup> and in plasma cells from patients with multiple myeloma.<sup>20</sup> Increased levels of HGF have been found in the sera of MM patients at diagnosis and it has been found that high levels predicted poor response to therapy and survival.<sup>21</sup> There are no reports so far on HGF level in the bone marrow of MM patients.

In this study we measured levels of the three above mentioned factors in plasma obtained from both bone marrow and peripheral blood of 34 patients affected by plasma cell neoplasia. This study was conducted to provide more information on the role of these angiogenic molecules in MM pathobiology. In addition, levels in bone marrow and peripheral blood were compared in order to identify the better source of information for identification of a surrogate marker of angiogenesis in MM patients.

## Design and Methods

### Patients

Thirty-four patients with a median age of 65 years (range 24-84) were evaluated in this study. The series included 1 patient with solitary extramedullary plasmacytoma, 17 patients with a diagnosis of MM at presentation and 16 with previously treated MM. The clinical features of these patients are shown in Table 1. After informed consent peripheral venous blood and bone marrow aspirates were collected in sterile tubes using EDTA as anticoagulant and centrifuged at 1000 g for 10 minutes within 30 minutes of collection. Plasma was divided into aliquots and stored at -20°C for VEGF and HGF assays and at -70°C for bFGF determination.

The choice of plasma instead of serum is justified by the fact that it has been demonstrated, at least for VEGF, that platelets can release this factor during clot formation, leading to a false increase in the

**Table 1. Clinical features of patients.**

N° of patients	34
Median age (years)	65 (range 42-79)
Stage	
I	9
II	6
III	18
Solitary plasmacytoma	1
Median Hb (g/dL)	11 (range 6-14)
Median WBC (x10 <sup>6</sup> /L)	5.5 (range 1.9-15.3)
Median PLT (x10 <sup>6</sup> /L)	175 (range 37-380)
Ig type	
IgG	18
IgA	12
κ	1
λ	3
Extramedullary localization	7/34
Median % BM plasma cells	20 (range 0-90)
Median albumin (g/L)	3.8 (range 2.5-4.7)

serum. Plasma measurement, therefore, seems to be more reliable.<sup>22</sup>

### ELISA assay

The concentrations of the soluble factors were determined in plasma samples by enzyme-linked immunosorbent assay (ELISA) (VEGF, HGF, and bFGF Quantikine R&D System, Minneapolis, MN, USA and EPO RG Instruments GmbH, Germany) according to the manufacturers' instructions. Briefly, these assays employ the quantitative sandwich enzyme immunoassay technique with monoclonal antibodies specific for each growth factor (VEGF, HGF, bFGF and EPO) pre-coated onto a microplate. Standard controls and samples (100 µL of plasma) are pipetted into the wells in duplicate. After growth factor binding and washing, an enzyme-linked polyclonal antibody specific for each growth factor is added to each well. After thorough washing, a substrate solution is added to the wells and color develops in proportion to the amount of growth factor bound in the first step. The optical density of each well was determined by a microplate reader at 450 nm. The value for the blank was subtracted from both the standard controls and samples. A standard curve was created by plotting the logarithm of the mean absorbance of each standard versus the logarithm of the known growth factor concentration. Concentrations are reported as picograms per milliliter. Normal ranges in plasma: VEGF 0-115 pg/mL, HGF 301-960 pg/mL, bFGF 0-14.6 pg/mL, EPO 6-25 mU/mL. The minimum detectable levels are: VEGF 5 pg/mL, HGF 40 pg/mL, bFGF 3 pg/mL.

### Statistical analysis

Correlations between two parameters were evaluated by the Spearman rank correlation analysis using an INSTAT Graph Pad program. All *p* values were 2-tailed. Values ≤ 0.05 were considered as statistically significant.

**Table 2. Concentration of angiogenic factors in different groups of patients.**

Patients	VEGF		bFGF		HGF	
	BM	PB	BM	PB	BM	PB
9 MM stage I	94 (37)	43 (32)	60 (50)	16 (17)	1912 (1817)	884 (689)
24 MM stage II-III	278 (438)	125 (148)	181 (224)	42 (34)	4226 (3182)	1288 (746)
<i>p</i>	0.2	0.01	0.2	0.039	0.05	0.07
1 Sol. Plasm.	542	264	132	84	1519	775

BM = bone marrow, PB = peripheral blood. Normal values in PB = VEGF 0-115 pg/mL, bFGF 0-14 pg/mL, HGF 301-960 pg/mL. Mean values ( $\pm$  standard deviation) in pg/mL.

**Table 3. Concentrations of angiogenic factors in different groups of patients.**

Patients	VEGF		bFGF		HGF	
	BM	PB	BM	PB	BM	PB
Extramedullary disease (7 patients)	245 (394)	104 (140)	242 (172)	58 (30)	3282 (3052)	1303 (710)
No extramedullary disease (27 patients)	229 (207)	102 (82)	118 (253)	29 (40)	3363 (2515)	1090 (789)
<i>p</i>	0.28	0.5	0.29	0.14	0.5	0.5

BM = bone marrow, PB = peripheral blood. Normal values in PB = VEGF 0-115 pg/mL, bFGF 0-14 pg/mL, HGF 301-960 pg/mL. Mean values ( $\pm$  standard deviation) in pg/mL.

## Results

In all patients the concentration of each angiogenic factor was higher in bone marrow (BM) than in peripheral blood (PB). In particular, the median BM/PB ratio was 1.7, 4.2, and 2.2 for VEGF, bFGF, and HGF respectively. A good correlation was found between BM and PB concentrations of VEGF ( $r=0.58$ ,  $p=0.0003$ ) and HGF ( $r=0.70$ ,  $p<0.0001$ ), while for bFGF the correlation coefficient was less significant ( $r=0.47$ ,  $p=0.007$ ). One of the reasons for this last low correlation coefficient might be the interindividual difference in the ratio between BM and PB bFGF. In a few patients, this ratio was very high, mainly because of the low PB levels. As a consequence, the mean bFGF BM/PB ratio was much higher than the median (98 vs 4.2), while for VEGF and HGF, mean and median BM/PB ratio values were quite similar (2.6 vs 1.7 for VEGF and 2.7 vs 2.2 for HGF, respectively).

For the purposes of this study, we analyzed patients with stage I MM and patients with stage II-III MM separately. Moreover, data from a single patient with extramedullary solitary myeloma were extrapolated.

**Table 4. Concentrations of angiogenic factors in different groups of patients.**

Patients	VEGF		bFGF		HGF	
	BM	PB	BM	PB	BM	PB
Untreated (17 patients)	191 (281)	103 (178)	144 (171)	26 (28)	3081 (779)	1069 (195)
Previously treated (16 patients)	268 (477)	103 (58)	147 (229)	44 (34)	3927 (782)	1253 (191)
<i>p</i>	0.26	0.04	0.8	0.1	0.4	0.29

BM = bone marrow, PB = peripheral blood. Normal values in PB = VEGF 0-115 pg/mL, bFGF 0-14 pg/mL, HGF 301-960 pg/mL. Mean values ( $\pm$  standard deviation) in pg/mL.

Nine patients had stage I MM and in these patients we found that the concentration of each angiogenic factor, both in BM and PB, was lower than in the 24 patients with stage II-III MM. The difference in some cases was not statistically significant, probably because of the small number of patients in each group. However, for each angiogenic factor, the BM concentration was always higher than the PB one and the highest ratio was again observed for bFGF (Table 2). In addition, the mean PB level of each angiogenic factor was higher than normal in patients with stage II-III but not stage I MM. The single patient with extramedullary solitary plasmacytoma had high levels of VEGF both in BM and PB as well as high levels of bFGF especially in PB, while HGF levels were within the normal range.

However, when we evaluated all seven patients in our series who had extramedullary disease and compared them with patients without extramedullary disease, we were unable to find any significant difference for any of the factors either in BM or in PB (Table 3).

We also found that untreated patients had lower levels of each angiogenic factor than previously treated patients but in most cases the difference was not statistically significant (Table 4). However, it should be underlined that chemotherapy, especially steroids<sup>23</sup> and interferon<sup>24</sup> might have an anti-angiogenic effect. Most of our previously treated patients were receiving steroids and three patients were treated with interferon. Therefore, it is possible that treatment lowered the angiogenic factor levels, thus reducing the difference between untreated and previously treated patients. We then correlated each angiogenic factor with some well known parameters of prognosis and tumor load in MM, such as age, M protein level, Hb, WBC count, platelet count, C reactive protein (CRP) concentration,  $\beta_2$  microglobulin ( $\beta_2$ -M), serum calcium level, creatinine, alkaline phosphatase, lactate dehydrogenase (LDH), percentage of bone marrow plasma cells, and number of osteolytic lesions. For this correlation we limited the

**Table 5. Correlation coefficients (r) between angiogenic factors measured in BM and some disease features.**

	VEGF	p	bFGF	p	HGF	p
CRP	0.82	0.002	0.32	NS	0.23	NS
B2	0.65	0.01	0.33	NS	0.39	NS
% PC	0.3	NS	0.39	NS	0.47	NS
Alb	-0.09	NS	-0.83	0.0003	-0.77	0.002
LDH	0.1	NS	0.86	<0.0001	0.64	0.02
Hb	-0.3	NS	-0.23	NS	-0.47	NS
WBC	0.45	NS	0.01	NS	0.05	NS
PLT	0.69	0.007	-0.36	NS	-0.47	NS
EPO	-0.11	NS	0.17	NS	0.56	NS

analysis to the 17 untreated patients to avoid the possible confounding effects of the treatment. Since correlation coefficients were similar in BM and PB we will refer to BM values from now on. As shown in Table 5, VEGF correlated with CRP,  $\beta_2$  microglobulin, and platelet count. bFGF and HGF correlated with LDH and inversely with albumin. None of the factors showed a significant correlation with percentage of BM plasma cells.

Among the three angiogenic factors, we found that VEGF and bFGF were not correlated each other ( $r=0.07$ ) while BM (but not PB) HGF showed a weak correlation with VEGF ( $r=0.4$ ,  $p=0.01$ ) and a stronger correlation with bFGF ( $r=0.79$ ,  $p=0.0007$ ).

Since angiogenic factors may be released by normal cells in response to hypoxia, in 14 patients we also measured the EPO levels (which correlate with hypoxic stimulus) in both BM and PB plasma. None of these patients was receiving exogenous EPO. We found a good correlation between BM and PB EPO levels ( $r=0.99$ ,  $p=0.03$ ). However, none of the factors showed a significant correlation with EPO (Table 5).

## Discussion

Our study was prompted by the recent experimental evidence of increased angiogenic activity in multiple myeloma.<sup>2,3</sup> Several clinical studies on angiogenesis in neoplastic diseases have shown that the evaluation of this process can add prognostic and predictive information to the currently used staging systems.<sup>25</sup> In addition, some preclinical studies have indicated that inhibition of circulating angiogenic factors may be a good strategy to block angiogenesis. It is therefore crucial not only to identify which of these factors is important in angiogenesis in MM but also to make available a useful tool for monitoring the response to angiogenic therapy.

Beside microvessel counting by immunostaining methods, angiogenesis may be evaluated more easily by angiogenic factor concentrations. We therefore measured the concentrations of three factors that have a well-known angiogenic potential: VEGF, bFGF, and HGF. The study was carried out on plasma obtained from both peripheral blood and bone marrow of patients affected by plasma cell dyscrasias. We found that BM levels of each angiogenic factor were invariably higher in BM than in PB, thus indi-

cating the bone marrow environment as a major producer of these factors, and as a corollary, that bone marrow is a more reliable source of information for the measurement of angiogenic factor concentration. This was especially true for bFGF, whose concentration in some patients was below the limit of detection in PB but high in BM.

We have also confirmed that, in MM, angiogenesis parallels activity of disease. In fact, the concentrations of all three angiogenic factors were higher in more advanced disease. However, each factor's contribution to the pathophysiology of disease may be different. In fact, in this study, VEGF concentration correlated with features of aggressive disease, such as CRP and  $\beta_2$ -M, while bFGF and HGF levels were mainly negatively correlated with albumin (which mirrors the host status). In addition, HGF and especially bFGF, showed a strong correlation with LDH values. However, this parameter cannot be considered as an indicator of disease activity because all but one of the patients had LDH values within the normal range. On the other hand, we did not find any correlation between any of the angiogenic factors and some important parameters of tumor burden such as anemia, M component levels and osteolytic lesions, although for this last, it is possible that a more accurate evaluation of bone lesions such as bone histomorphometry or other biochemical indicators of bone remodeling could reveal a correlation between angiogenic growth factors and bone disease. Indeed, both VEGF<sup>26</sup> and HGF<sup>17,18,27</sup> have been shown to be able to induce osteoclastic activation *in vitro*. In summary, our study does not provide any evidence for a hierarchy in MM angiogenesis among the three studied factors. However, a recent study showed that HGF can increase the expression of VEGF in human keratinocytes and the VEGF receptor flk1 in human endothelial cells<sup>28</sup> leading to the hypothesis that HGF may actually be the orchestra conductor in the scenario of angiogenesis in myeloma.

All of the three studied factors have potential angiogenic activity. However, in most patients with extramedullary lesions, in whom more invasive disease is postulated, angiogenic factor concentrations were in the same ranges as in other patients. So, it is possible that in MM the angiogenic factors that we have studied affect the proliferation of disease and the homing of myeloma cells in the bone marrow<sup>29</sup> but do not have an effect on trafficking of malignant plasma cells. Indeed, many cells may play a role in the development of neovascularization in MM and the sources of VEGF, bFGF, and HGF may be different. The possibility that the angiogenic factors we studied have different sources is derived from the observation that, in our study, no correlation was found between VEGF and bFGF. In addition, although it has been demonstrated *in vitro* that neoplastic plasma cells can produce each of these factors,<sup>2,8,19</sup> no correlation was found with the percentage of BM plasma cells. Finally, the evidence that these factors can be produced by cells other than plasma cells, comes from the finding of high concentrations of VEGF and bFGF in the bone marrow of the patient with solitary extramedullary (without detectable plasma cells in BM) plasmacytoma. It is therefore prob-

able that accessory cells can produce and/or accumulate these angiogenic factors. Megakaryocytes and platelets are well-known to play a role in accumulating VEGF in other neoplastic disease<sup>30</sup> and in our study a correlation between VEGF and platelet count did exist while both bFGF and HGF showed an inverse correlation (although not significant) with platelet count.

In conclusion, our data indicate that the three angiogenic factors we studied may be important markers of different aspects of angiogenesis in MM. However, their use as indicators of progression in MM awaits larger studies and longer follow-up.

### Contributions and Acknowledgments

*FDR and GAP designed the study. FDR wrote the manuscript. GAP conducted the statistical analysis. MPA and GS performed the laboratory experiments and their analysis. SB, GG and PMF followed the patients, collected the clinical data and helped in evaluation and interpretation of the data. RG supervised the study and critically revised the manuscript.*

*The order of names takes into account the relative contribution given by all authors. RG is cited last as senior author.*

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### Disclosures

*Conflict of interest: none.*

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### Manuscript processing

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### Potential implications for clinical practice

- ◆ Evaluation of angiogenesis in MM may be introduced into clinical practice in the near future if these preliminary results are confirmed and if a prognostic role for angiogenesis is demonstrated into current clinical practice.<sup>31-33</sup>

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