ABSTRACT

We studied the prognostic value of angiogenesis grading and microvessel density estimation in newly diagnosed multiple myeloma. Seventy-five patients with newly diagnosed myeloma, treated on Eastern Cooperative Oncology Protocol E9486 and Intergroup study 0141 (S9321) at the Mayo Clinic, were studied. Bone marrow microvessels were examined using immunohistochemical staining for von Willebrand factor. Determination of microvessel density and angiogenesis grading was done in a blinded manner. There was a strong correlation between microvessel density and the plasma cell labeling index, rho 0.42, P < 0.001. Angiogenesis grade was also significantly associated with the plasma cell labeling index. Fifteen % of patients with low-grade angiogenesis had a high labeling index (>1 %). In contrast, 47% of patients with intermediate or high-grade angiogenesis had high labeling indices (P = 0.02). Overall survival was significantly different among those with high-, intermediate-, and low-grade angiogenesis, with median times of 2, 4, and 4.4 years, respectively (P = 0.02). Similarly, patients with microvessel density >50/x400 field had poorer survival compared with those with 50 or fewer microvessels/field, median survival 2.6 versus 5.1 years, respectively (P = 0.004). There was a strong association between angiogenesis grade and microvessel density (P < 0.001). We conclude that bone marrow angiogenesis is a predictor of poor survival in newly diagnosed myeloma. Angiogenesis is correlated with the plasma cell labeling index but not the bone marrow plasma cell percentage. A simple visual grading of angiogenesis is an efficient alternative to microvessel density estimation.

INTRODUCTION

Multiple myeloma accounts for 1% of all malignancies and >10% of malignant hematological neoplasms (1, 2). In 1999, ~13,800 new cases of myeloma will be diagnosed and >11,000 patients will die of the disease in the United States (1). The median survival in patients treated with conventional chemotherapy is about three years. Survival appears to be improved with high-dose therapy and autologous stem cell transplantation (3–6).

Angiogenesis is the formation of new blood vessels and occurs physiologically during embryonal growth, wound healing, and in the female genital system during the menstrual cycle. Angiogenesis is also important for the proliferation and metastases of most malignant neoplasms (7–9). In the absence of angiogenesis, tumors cannot grow beyond 1–2 mm in size (8). Increased angiogenesis has been found to be an adverse prognostic factor in several solid tumors (8–14). Targeting tumor angiogenesis with novel antiangiogenic agents appears to be a promising and exciting therapeutic approach and is the subject of intense investigation. Although many initial studies were done on solid tumors, angiogenesis appears important in hematological malignancies as well (15, 16). There is evidence that increased bone marrow angiogenesis occurs in myeloma and is related to disease activity (17–19). Angiogenesis in myeloma also appears to be correlated with the PCLI3 (17). PCLI is a measure of the proliferative activity of neoplastic plasma cells and is an independent predictor of poor survival in myeloma (20).

The extent of angiogenesis in the bone marrow can be assessed using an immunohistochemical stain for vWF (Factor VIII-related antigen) using standard methods (10, 14, 17, 21). A quantitative assessment is performed by determining the MVD, or the percentage of unit surface area occupied by microvessels (11, 17). Such assessments can also be done using computerized image analysis (12, 21, 22).

The purpose of this study was to assess the prognostic value of bone marrow angiogenesis in patients with newly diagnosed multiple myeloma.

PATIENTS AND METHODS

Patients and Data Collection. Seventy-four patients with newly diagnosed multiple myeloma, treated on Eastern...
Cooperative Oncology Group protocol E9486 and Intergroup study 0141 (S9321) at the Mayo Clinic, on whom bone marrow core biopsy samples were available were studied. Patients were enrolled in these trials between March 1988 and December 1997. Information on prognostic factors including β-2M, bone marrow plasma cell percentage, and PCLI were used to study correlation with bone marrow angiogenesis grade and MVD.

No patients were lost to follow-up. All patients gave written informed consent for research bone marrow and blood samples and again prior to stem cell mobilization. Approval of the protocol by the Mayo Institutional Review Board was obtained in accordance with federal regulations and the Declaration of Helsinki.

**Microvessel Staining.** Bone marrow biopsy specimens used in this study were prepared from paraffin-embedded blocks. Immunohistochemical staining for vWF was performed by a labeled streptavidin-biotin peroxidase method on the Ventana ES automated immunohistochemistry stainer (Ventana Medical Systems, Tucson, AZ) using buffers and detection reagents supplied by the manufacturer. Deparaffinized tissue sections were subjected to protease digestion on the instrument with Protease #2 for 12 min prior to immunostaining. The primary antibody (DAKO A0082; Dako, Carpinteria, CA; diluted 1:2000) was incubated with tissue sections for 24 min. The aminoethyl carbazole detection kit (Ventana Medical Systems) was used for antigen visualization; sections were counterstained with a light hematoxylin and then coverslipped with Kaiserí’s glycerol jelly (Mayo Medical Laboratories, Rochester, MN). Paraffin sections of well-vascularized tonsil were run with each batch to serve as a positive control, and a section stained with nonimmune rabbit immunoglobulin was used as a negative control for each sample tested.

**Estimation of Angiogenesis Grade and MVD.** All estimations were done in a blinded manner. Bone marrow angiogenesis was estimated by two separate methods, angiogenesis grading and MVD estimation. Initially, slides were scanned at ×100, ×200, and ×400, and based on the extent of vWF staining, each slide was assigned an angiogenesis grade: low, intermediate, or high. This grading was based solely on visual assessment. The entire stained sample was considered when assigning the angiogenesis grade.

Next, using standard methods (12, 15, 23), bone marrow MVD was estimated on all samples. For MVD estimation, each slide was first scanned at ×100 to determine three “hot spots” defined as areas with the maximum number of microvessels. The slides were then examined at ×400, using a ×10 ocular and ×40 objective lens. Microvessels were counted in each of the three hot spots at ×400. Large vessels and vessels in the periosteum or bone were excluded. Areas of staining with no discrete breaks were counted as a single vessel. The presence of a lumen was not required. MVD was estimated by determining the average number of vessels in each of the three hot spots and expressing the result as number of vessels per ×400 high power field.

**Labeling Index.** Bone marrow PCLI was assessed at diagnosis. PCLI is a reflection of the plasma cell proliferative activity and was performed using a slide-based immunofluorescence method on bone marrow samples as described elsewhere (20, 24). A PCLI of ≥1% was classified as high.

<table>
<thead>
<tr>
<th>Table 1 Patient characteristics</th>
<th>All patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td>No.</td>
</tr>
<tr>
<td>Total number of patients</td>
<td>74</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>51</td>
</tr>
<tr>
<td>Female</td>
<td>23</td>
</tr>
<tr>
<td>Protocol treatment</td>
<td></td>
</tr>
<tr>
<td>E9486</td>
<td>54</td>
</tr>
<tr>
<td>S9321</td>
<td>20</td>
</tr>
<tr>
<td>Bone marrow angiogenesis grade</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>27</td>
</tr>
<tr>
<td>Intermediate</td>
<td>30</td>
</tr>
<tr>
<td>High</td>
<td>17</td>
</tr>
<tr>
<td>Bone marrow microvessel density</td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>37</td>
</tr>
<tr>
<td>&gt;50</td>
<td>37</td>
</tr>
<tr>
<td>PCLI ≥1%</td>
<td>24</td>
</tr>
</tbody>
</table>

**Statistical Analysis.** Overall survival was calculated from the date of registration onto the clinical trials to the date of death or date last known alive. Survival analysis was done using the method described by Kaplan and Meier (25). Differences between survival curves were tested for statistical significance using the two-tailed Wilcoxon test. The Fisher exact test was used to compare differences in nominal variables; the rank-sum test or the Kruskal-Wallis test was used for continuous variables. Correlation between continuous variables was studied using the Spearman Rank correlation. Multivariate analysis was conducted using Cox’s proportional hazards model (26).

**RESULTS**

Patient characteristics are listed on Table 1. No patient had received treatment for myeloma prior to assessment of bone marrow angiogenesis and other prognostic variables. No patient was lost to follow-up.

**Bone Marrow Angiogenesis.** Immunostaining for vWF, demonstrating increased microvessels in four representative patients, is shown in Fig. 1. The median MVD for the cohort was 50 (range, 2–106). Seventeen patients (23%) had a high angiogenesis grade (Table 1). There was a statistically significant association between MVD and angiogenesis grade (P < 0.001; Table 2).

**Correlation between Measures of Angiogenesis and Known Prognostic Factors in Myeloma.** There was a significant association between PCLI and angiogenesis grade (Fig. 2). Fifteen % of patients with low-grade angiogenesis had high PCLI (>1%), whereas 47% of patients with intermediate or high-grade angiogenesis had high PCLI (P = 0.02). Similarly, when MVD was analyzed as a nominal variable, 14% of patients with a low MVD (≤50) also had a high PCLI. In contrast, 52% of patients with high MVD had a high PCLI (P < 0.001). PCLI was also significantly correlated with MVD when analyzed as continuous variables (rho 0.42; P < 0.001).

There was no relationship between β-2M and angiogenesis grade (P = 0.43) or MVD (P = 0.97). There was also no relationship between bone marrow plasma cell percentage and angiogenesis grade (P = 0.47) or MVD (P = 0.18).
Bone Marrow Angiogenesis and Response to Therapy.

There was no statistically significant relationship between response rate and measures of bone marrow angiogenesis. The response rate among patients with low-, intermediate-, and high-grade angiogenesis was 86, 70, and 65%, respectively ($P = 0.47$). The median MVD for both responders and nonresponders were similar, 72 and 74, respectively ($P = 0.85$).

Survival Analysis. Fifty of the 74 patients have died, and the median survival of the cohort is 4 years. Overall survival was significantly different among those with high-, intermediate-, and low-grade angiogenesis, with median times of 2, 4, and 4.4 years, respectively ($P = 0.02$; Fig. 3). Similarly, patients with high MVD ($>50$) had poorer survival compared with those with low MVD ($\leq 50$), median survival 2.6 years versus 5.1 years, respectively ($P = 0.004$; Fig. 4). Neither angiogenesis grade nor MVD was predictive for survival in a multivariate model that included PCLI, $\beta$-2M, and bone marrow plasma cell percentage.

DISCUSSION

Several recent studies indicate that bone marrow angiogenesis is increased in myeloma (15, 17, 22, 27, 28). There is also evidence that bone marrow angiogenesis correlates with the proliferation of neoplastic plasma cells and may be important in disease progression and activity (17).

Earlier, we reported that MVD in bone marrow samples from patients with myeloma is increased compared with control samples (15). In that study, we assessed MVD before transplantation and at the time of response in 13 patients with relapsed or refractory myeloma (7 complete and 6 partial responders) using immunostaining for vWF. Baseline MVD was significantly different between patients with myeloma and normal controls, mean (± SD) 294 (± 115)/mm$^2$ versus 93 (± 26)/mm$^2$ ($P = 0.001$). After transplantation, MVD continued to be high compared with controls, mean (± SD) 230 (± 68)/mm$^2$ ($P = 0.003$).

Table 2  Correlation between angiogenesis grading and MVD

<table>
<thead>
<tr>
<th>Angiogenesis grade</th>
<th>No. of patients</th>
<th>Median MVD</th>
<th>MVD, range</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>27</td>
<td>24</td>
<td>2–42</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>30</td>
<td>66</td>
<td>35–91</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>17</td>
<td>92</td>
<td>67–106</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Fig. 1  Bone marrow biopsy specimens from four different patients, with immunohistochemical staining for vWF, illustrating increased microvessels in newly diagnosed multiple myeloma. ×200.

Table 2

<table>
<thead>
<tr>
<th>Angiogenesis grade</th>
<th>No. of patients</th>
<th>Median MVD</th>
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<td>&lt;0.001</td>
</tr>
</tbody>
</table>
No difference was noted in complete or partial responders compared with pretransplant values. There was a tendency to increased relapse-free survival with decrease in posttransplant MVD. We concluded that increased angiogenesis occurs in myeloma marrow and suggested that increased angiogenesis persists even after complete response.

The present study was done to test the hypothesis that increased bone marrow angiogenesis may be associated with an unfavorable outcome in myeloma. We show that both angiogenesis grading and MVD estimation are adverse prognostic factors in patients with newly diagnosed myeloma. An MVD of 50 was chosen as the cutoff in survival analysis, because it represented the median MVD of the cohort. Preliminary data from Munshi et al. (28) using CD34 staining to identify microvessels provide additional support to this hypothesis. In their study of 36 patients with newly diagnosed myeloma, increased angiogenesis was an adverse prognostic factor for survival.

Bone marrow angiogenesis was not an independent predictor for survival. This may be explained by the significant correlation between PCLI and measures of angiogenesis. To show modest differences, it would require a much larger sample size to demonstrate the prognostic value of angiogenesis, independent of PCLI. We have previously made similar observations with other important prognostic factors in myeloma. For example, because of the strong association that exists between the presence of cytogenetic abnormalities and plasmablastic morphology, we were unable to demonstrate the prognostic significance of cytogenetic abnormalities independent of the plasmablastic phenotype (29). The same finding relationship has been described between PCLI and the presence of cytogenetic abnormalities as well (30). It is important to note that the correlation between MVD and the PCLI is not perfect (rho 0.46); therefore, MVD cannot be used as a surrogate for the PCLI. A much larger sample size is needed to test the hypothesis that angiogenesis is independent of other known prognostic factors in myeloma, and the present study did not have the power to detect modest differences in survival. We are in the process of initiating a large study to address this question.

Our finding that patients with increased bone marrow angiogenesis have poor survival in myeloma gains additional relevance in view of the various antiangiogenic approaches that are being explored today. Therapy targeted against angiogenesis represents a novel, exciting, and possibly less toxic way to treat malignant disease (31–33). Singhal et al. (34) have used thalidomide as an antiangiogenic agent and have shown promising activity in a group of heavily pretreated patients with myeloma. Most patients in their study had failed stem cell transplantation, and the overall response rate (>50% reduction in M protein level) was 20–25%. Preliminary data from our institution confirms a 20–25% response rate with thalidomide in relapsed myeloma (35). We are presently studying bone marrow samples from patients treated with thalidomide to assess the effect of this agent on bone marrow angiogenesis and other angiogenic cytokines. Future trials will test other antiangiogenic agents such as 2-methoxy estradiol or antibodies against angiogenic proteins such as VEGF.

The mechanism behind the increased angiogenesis in myeloma is not fully understood. There are data that myeloma cells express the potent angiogenic cytokines, VEGF and basic fibroblast growth factor (36–38). Preliminary data using reverse transcription-PCR techniques indicate that VEGF isoforms,
VEGF121 and VEGF165, are expressed by myeloma cells both in studies of bone marrow samples from patients with myeloma and on various myeloma cell lines (37, 39). Stimulation of human microvascular endothelial cells and bone marrow stromal cells with VEGF induces a significant increase in interleukin 6 secretion in a dose-dependent manner (39). Furthermore, stimulation of cells from the 8226 myeloma cell line with interleukin 6 leads to an increase in VEGF secretion. Additional studies to identify the mechanisms leading to increased angiogenesis in myeloma are ongoing.

Our study indicates that angiogenesis grading is highly correlated to MVD estimation. It also appears to be equally effective as a prognostic factor. Angiogenesis grading is much less time consuming and more practical for clinical use.

It is still not clear whether the increased angiogenesis seen in myeloma is important in the pathogenesis of the disease or merely an epiphenomenon related to cytokine overexpression by the neoplastic plasma cells. The correlation of increased bone marrow angiogenesis to the PCLI and the prognostic value of angiogenesis shown in this study support the hypothesis that angiogenesis may play a role in the pathogenesis and progression of myeloma. The response observed with thalidomide therapy further strengthens this hypothesis. However, further studies are needed to ascertain the role of angiogenesis in myeloma and other hematological malignancies. If angiogenesis is determined to be an important factor, it would offer additional new targets for therapy.

In summary, we show that increased angiogenesis grade and MVD predict poor survival in patients with newly diagnosed myeloma. The extent of bone marrow angiogenesis is correlated with the PCLI. Angiogenesis grading is highly correlated with MVD estimation and appears to be a more practical alternative.

REFERENCES


Clinical Cancer Research

Prognostic Value of Bone Marrow Angiogenesis in Multiple Myeloma

S. Vincent Rajkumar, Traci Leong, Patrick C. Roche, et al.


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