Journal of BUON **16:** 98-103, 2011

ORIGINAL ARTICLE

Clinical and histopathological study of angiogenesis in multiple myeloma

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Summary

Purpose: *Angiogenesis is an essential component in the growth and progression of multiple myeloma (MM). We studied the clinical significance of angiogenesis in patients with MM estimated by precise counting of the number of vessels (i.e. microvessel density, MVD) and compared these results with the results obtained using semi-quantitative grading of angiogenesis.*

Methods: *Fifty-nine newly diagnosed cases of MM were analyzed with respect to clinical features, laboratory findings, histological features, angiogenesis parameters, and response to treatment. Bone marrow microvessels were examined using immunohistochemical staining for CD34. Bone marrow angiogenesis was estimated by two different methods. The mean number of vessels per area in each sample was characterized as the MVD. Microvessels were counted manually on light microscopy in 3 hot spots at ×400 magnification. Semi-quantitative estimation of angiogenesis was based on visual assessment of slides at ×100 magnification. Each slide was assigned as low, intermediate or high intensity of angiogenesis.*

Results: *The median MVD was 15 vessels per 3 hot spots (range 1-89). Intensity of angiogenesis was assigned as low in 24 (40.7%) patients, intermediate in 17 (28.8%) and high in 18 (30.5%). Significant correlation between intensity of angiogenesis (estimated using both methods) and histological grade, extent of bone marrow infiltration, proliferative activity of myeloma cells and poor survival was found. Semi-quantitatively assessed intensity of angiogenesis additionally correlated with clinical stage. There was a statistically highly significant correlation between MVD and semi-quantitatively estimated intensity of angiogenesis ($p < 0.001$).*

Conclusion: *Tumor-associated angiogenesis is an important prognostic feature in MM and should be routinely done on bone marrow biopsies of these patients. Simple semi-quantitative grading of angiogenesis can be recommended for daily practice, as an alternative method for complicated and time-consuming estimation of MVD.*

Key words: angiogenesis, clinical significance, immunohistochemistry, methods, multiple myeloma, prognosis

Introduction

An increased or abnormal level of angiogenesis has been recognized for many years as a key feature of solid malignancies and has been increasingly associated with the pathogenesis of hematological diseases [1]. Altered angiogenesis is one of the characteristic changes in the bone marrow myeloma microenvironment [2]. The pathophysiology of MM-induced angiogenesis is complex and involves both direct production of angiogenic cytokines by plasma cells and their induction within the microenvironment. The latter are secreted by stromal cells, endothelial cells and osteoclasts, and promote plasma cell growth, survival and migration, as well as paracrine cytokine secretion and angiogenesis in the bone marrow milieu [3,4].

There has been growing evidence that neoangiogenesis (new vessel formation) has an integral role in the pathophysiology of MM [2]. There is also evidence that angiogenesis progressively increases from monoclonal gammopathy of undetermined significance (MGUS) to advanced disease [2], and that bone marrow angiogenesis is a predictive factor for poor survival in newly diagnosed MM [5-7]. Recently, we reported that the extent of bone marrow angiogenesis estimated by precise counting of the number of vessels per area

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Received 16-05-2010; Accepted 23-09-2010

(i.e. MVD) is an indicator of biological potency of the malignant clone and a predictor of poor survival in newly diagnosed MM [8]. The purpose of this study was to establish the clinical significance of angiogenesis in patients with MM estimated by precise counting of the number of vessels (i.e. MVD) and compare these results with those obtained using semi-quantitative grading of angiogenesis.

Methods

Patients

Bone marrow biopsy specimens were obtained from 59 patients with MM diagnosed between 1997 and 2000. The diagnosis of MM was made according to the criteria of the Chronic Leukemia-Myeloma Task Force [9]. Staging of the disease was done according to Durie and Salmon classification system [10]. Bone marrow aspirates and biopsies were taken at the same time from each patient and examined with conventional light microscopy and immunohistochemically. A number of clinical variables were particularly analyzed: age, gender, clinical stage, presence and extent of bone lesions, serum immunoglobulin, serum and urine immunoelectrophoresis, albumin, C-reactive protein, $ß_2$-microglobulin, calcium, creatinine, 24-h proteinuria, hemoglobin concentration and platelet count. Classification of the histological grade of plasma cell differentiation was done according to the model proposed by Sailer et al. [11] and was defined as low, intermediate and high grade plasma cell malignancy. Classification of the histological stage (i.e. the extent of bone marrow infiltration) was done according to the model proposed by and Bartl et al. [12] as follows: stage I or low grade bone marrow infiltration [plasma cells <20% of all nucleated cells (ANC)]; stage II or intermediate infiltration (plasma cells 20-50% ANC); and stage III or high grade bone marrow infiltration (plasma cells >50% ANC). All patients were treated with conventional chemotherapy: 32 patients received vincristine, adriamycin, dexamethasone (VAD), 14 vincristine, melphalan, cyclophosphamide, prednisone (VMCP), 11 vincristine, carmustine, melphalan, cyclophosphamide, prednisone (VBMCP) and one patient received prednisolone/melphalan combination. Response to therapy was evaluated according to the criteria of Chronic Leukemia-Myeloma Task Force [9]. Overall survival was calculated from the time of diagnosis until death from any cause, and observation ended at the time of the last contact with patients last known to be alive. The observation time ranged from 27 to 63 months (median 32).

Immunohistochemical studies

MVD was assayed by means of the avidin/biotin/peroxidase complex method (LSAB 2, DAKO), using aminoethylcarbazole as chromogen. Bone marrow trephine biopsy was done from the posterior iliac crest using Jamshidi needle. The bioptic specimen measured at least 15 mm. Bone marrow specimens were then fixed in B5, embedded in paraffin and decalcified with EDTA. The 3 μm thick sections were deparaffinized in xylene and rehydrated in water. For CD34 and Ki-67 immunodetection a heat-induced epitope retrieval method was used before immunostaining. Namely, sections were placed in 0.01 mmol/L citrate buffer at PH 6.0 and heated twice in a microwave over for 5 min. For immunodetection of Ki-67 antigen DAKO prediluted antibody was used. Each sample was processed for immunohistochemical identification of microvascular endothelial cells with anti-CD34 (DAKO) antibody at a 1:50 dilution for one hour at room temperature. After development of the chromogen all slides were counterstained with hematoxylin. Control sections were immunostained under identical conditions, substituting the primary antibody with buffer solution. The specificity of the immunohistochemical assay for CD34 was also tested using three normal bone marrow biopsies. All of the bone marrow tissues were evaluated at 100× and 400× magnification (microscope Leica DMLS, eyepiece HC plan 10×, objective lenses 10× and 40×) and independently analyzed by two of the authors.

Quantitative estimation of angiogenesis

Quantitative estimation of angiogenesis or MVD was done according to Weidner et al. [13], who used this method for estimation of angiogenesis in patients with breast carcinoma. Slides were first scanned at 100× magnification to determine three areas with the maximum number of microvessels (hot spots). The hot spots were then examined at ×400 magnification. Microvessels were counted in each of these hot spots at ×400 magnification. Any red-stained endothelial cell or endothelial cell cluster that were clearly separated from adjacent microvessels were considered a single, countable microvessel and vessel lumens were not a prerequisite to define a structure as microvessel. MVD was estimated by determining the average number of vessels in each of the 3 hot spots. The results were expressed as the average number of vessels per 400× power field. Large vessels and vessels in the periosteum or bone were excluded.

Semi-quantitative estimation of angiogenesis

Semi-quantitative estimation of angiogenesis was

based on visual assessment of slides at ×100 magnification and defined according to the method of Rajkumar et al. [2]. Angiogenesis intensity of the samples was defined as low, intermediate or high.

Proliferative index

Proliferative index was defined according to Lai et al. [14] as follows: low (<10% Ki-67+ plasma cells), intermediate (10-20% Ki-67+ positive plasma cells) or high proliferative index (>20% Ki-67+ plasma cells).

Statistical analysis

Statistical analysis was performed using SPSS PC version 8 statistical processing software. Group comparison was based on chi-square distributions for categorical variables. For continuous variables Student's t-test for normally distributed variables and Mann-Whitney test for all other variables were used. Statistical dependence between two variables was assessed by Spearman rank correlation test. Survival of myeloma patients was plotted according to the Kaplan-Meier method and curves were compared by using log-rank test. All statistical tests were two-sided and a p-value<0.05 was considered as statistically significant.

Results

Clinical data

Clinical data are shown in Table 1.

Table 1. Clinical and histological characteristics of 59 myeloma patients

Characteristics	*No. of patients*	*Percent*
Age (years)		
Median (range)	63 (38-80)	
Gender		
Male/female	32/27	54/46
Stage (Durie-Salmon)		
I	4	7
II	16	27
III	39	66
M-component		
IgG	35	59
IgA	13	22
light chain	11	19
Type of light chain		
Kappa	42	71
Lambda	17	29
Calcium (mmol/L)		
>2.6	19	32
≤ 2.6	40	68
β_2-microglobulin (mg/L)		
Median (range)	7.1 (1.3-20.7)	
CRP (mg/L)		
Median (range)	16.1 (0-95)	
Histological grade		
Low	31	53
Intermediate	21	35
High	7	12
Bone marrow plasma cells (%)		
<20	6	10
20-50	32	54
>50	21	36
Response to therapy		
Yes	31	53
No	28	47

Immunohistochemical results

Median MVD in the myeloma samples was 15 vessels per 3 hot spots (range 1-89; Figure 1). In contrast, MVD of normal bone marrow samples was 2,3 and 7 vessels per 3 hot spots, respectively. The median MVD was used as a cut-off value for the classification of patients with low and high grade of angiogenesis. Namely, MVD was determined as low grade angiogenesis when its value was < 15 vessels per 3 hot spots, and high grade angiogenesis when the value was ≥ 15 vessels per 3 hot spots. Intensity of angiogenesis estimated by semi-quantitative grading was assigned as low in 24 (40.7%) patients, intermediate in 17 (28.8%) and high in 18 (30.5%). Immunoreactivity to Ki-67 antibody ranged from 1 to 36% (median 7.0). Low proliferative index was found in 39 patients, while intermediate and high proliferative index were found in 12 and 8 patients, respectively.

Correlation between MVD and semi-quantitatively estimated intensity of angiogenesis

There was highly significant association between MVD and semi-quantitatively estimated intensity of angiogenesis (Spearman rank correlation coefficient, $\rho=0.922$; $p<0.001$).

Clinicopathological parameters in relation to MVD and semi-quantitatively estimated intensity of angiogenesis

MVD was in positive correlation with proliferative activity, percent of bone marrow plasma cells, histological grade and response to initial chemotherapy. On the contrary, there was no significant correlation between MVD and age, serum albumin, hemoglobin, serum calcium, clinical stage, platelet count and bone marrow fibrosis.

Semi-quantitatively estimated intensity of angiogenesis was in positive correlation with clinical stage,

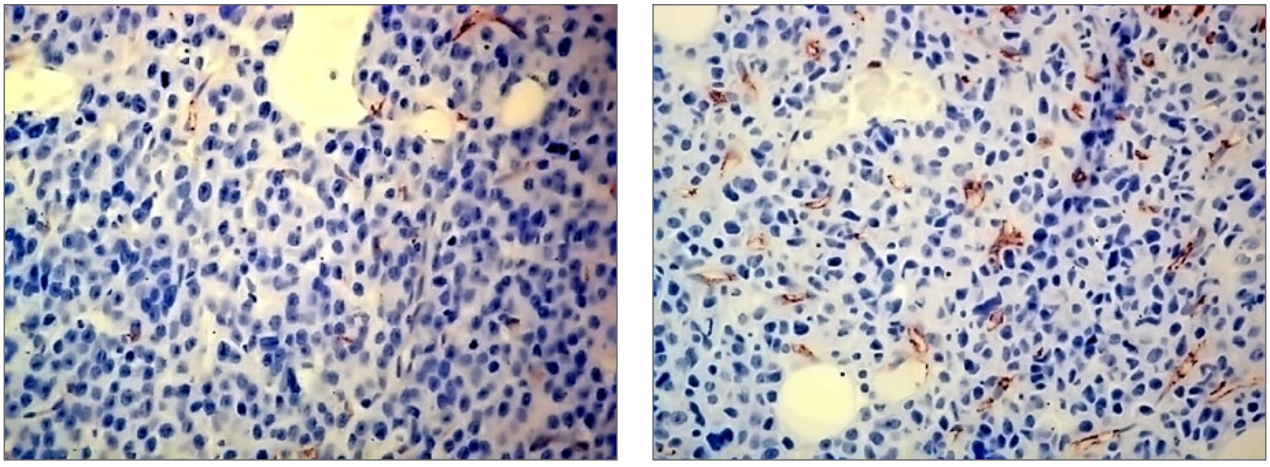

Figure 1. Bone marrow biopsy specimens with immunohistochemical staining for CD34 antigen illustrating low grade (left) and high grade of angiogenesis (right) (×400).

proliferative activity, percent of bone marrow plasma cells, histological grade and response to initial chemotherapy. There was no significant correlation between semi-quantitatively estimated intensity of angiogenesis and age, serum albumin, hemoglobin, serum calcium, platelet count and bone marrow fibrosis.

Prognostic significance

Twenty-nine patients died during follow up. The Kaplan-Meier actuarial median survival of 59 analyzed patients was 34 months. Univariate analysis showed that the following clinicopathological parameters were significantly associated with overall survival: age, hemoglobin, extent of osteolytic lesions, platelet count, serum calcium, CRP, $ß_2$-microglobulin, histological grade, bone marrow infiltration, proliferative activity of myeloma cells and response to initial chemotherapy. The median survival of patients with low MVD (<15 vessels per 3 hot spots) was 46 months, compared with 22 months for patients with high MVD (≥15 vessels per 3 hot spots) (p=0.009; Figure 2). The median survival of patients with low, intermediate and high intensity of angiogenesis was 46, 38 and 13 months, respectively. Survival analysis of myeloma patients according to intensity of angiogenesis estimated by semi-quantitative grading method showed that there was no significant difference in survival between patients with low and intermediate grade angiogenesis (p >0.05). However, there was significant difference in survival between patients with low and high grade angiogenesis (p=0.0094), and patients with intermediate and high grade angiogenesis (p=0.0001; Figure 3).

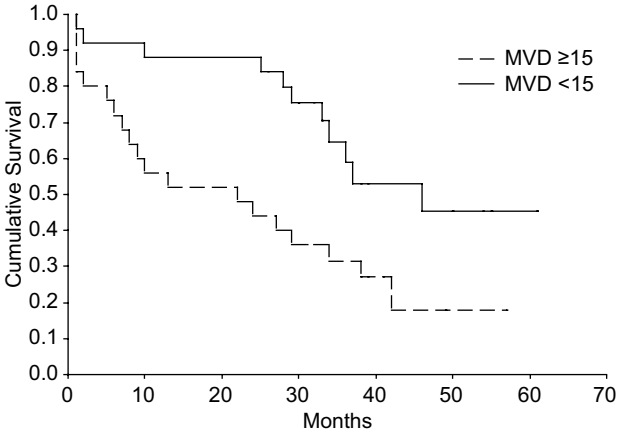

Figure 2. Survival of myeloma patients according to MVD (Kaplan-Meier). There was significant difference in survival between patients with low MVD (<15 vessels per 3 hot spots) and patients with high MVD (≥15 vessels per 3 hot spots) (p=0.009).

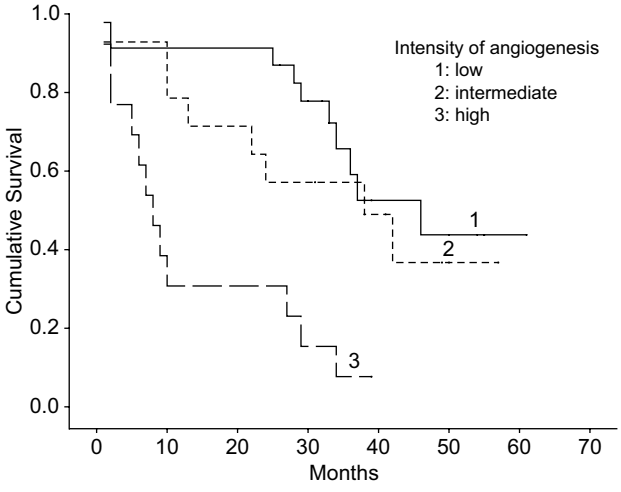

Figure 3. Survival of myeloma patients according to intensity of angiogenesis estimated by semi-quantitative grading (Kaplan-Meier). There was no significant difference in survival between patients with low and intermediate grade angiogenesis (p >0.05). There was significant difference in survival between patients with low and high grade angiogenesis (p=0.0094), and patients with intermediate and high grade angiogenesis (p=0.0001).

Discussion

Angiogenic activity of a tumor can be determined directly and indirectly. A direct measure of tumor angiogenic activity is MVD in tumor sections, analyzed by immunohistochemistry using monoclonal antibodies (mAbs) which recognize some of endothelial cell antigens (CD105, CD34, FVIII). In this study anti-CD34 was used because this antibody reacts only with endothelial cells and a few hematopoietic cells (blasts), which makes blood vessels easily recognizable [7]. The choice of mAbs for visualization of blood vessels was important in studies of angiogenesis in MM since significant differences in clinical results were found after staining with anti-CD34 and anti-CD105 antibodies [20]. Namely, Kumar and colleagues [20] showed that the anti-CD105 mAb was significantly more sensitive than the anti-CD34 mAb in visualizing blood vessels both in controls and MM samples, but on the other hand, only significant association between CD34+ MVD and survival was found, whereas there was no difference in survival between patients with low and high CD105+ MVD. In that study, the grade of angiogenesis was estimated using the hot spot method [13] for precise counting of the number of microvessels as well as simple semi-quantitative visual assessment of slides according to the method of Rajkumar et al. [2].

We established significant correlation between the grade of angiogenesis (estimated by using both methods) and the grade of bone marrow infiltration by plasma cells, plasma cell morphology and proliferative activity of MM cells. However, the semi-quantitative grading was additionally discriminative for clinical stage (i.e. advanced myeloma displayed more intensive angiogenic activity). These results are in concordance with literature data [8,15,16] and point to angiogenesis as one of the major determinants of tumor growth and disease activity. Our study confirmed the results of previous studies that MVD is an adverse prognostic factor in MM regarding overall survival [5-8,17-19] and response to initial therapy [8,19]. Survival analysis also showed that high grade of angiogenesis estimated semi-quantitatively is highly correlated with poor outcome. According to this analysis, patients with low and intermediate grade of angiogenesis have similar prognosis, and should be analyzed as an unique group. In other words, visual estimation of bone marrow slides should particularly characterize the presence of high grade of angiogenesis and underline such a finding in histopathological reports of MM patients. Such information can be very helpful for clinical decision about the use of antiangiogenesis agents in every individual patient.

We found highly significant correlation between MVD and grade of angiogenesis estimated by semi-quantitative analysis. Good correlation between angiogenesis estimated by visual grading and that determined by MVD assessment was also found in other studies [20]. These findings are very important for daily practice because simple semi-quantitative visual assessment of intensity of angiogenesis can be adequate substitution for complicated and time-consuming MVD assessment.

Conclusions

We conclude that angiogenesis is a biologically relevant and important prognostic feature in MM that can be targeted by antiangiogenesis therapy. Thus, we strongly suggest that estimation of angiogenesis should be routinely done on bone marrow biopsies of these patients, particularly using simple, quick and reliable semi-quantitative grading.

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