Clinical Significance of Microvessel Density in Multiple Myeloma Patients

To investigate the role of angiogenesis in multiple myeloma (MM), bone marrow biopsy from 75 adults with newly diagnosed, untreated MM were evaluated. Microvessels were scored in at least 3 areas ($\times$200 fields) of the highest microvessel density in representative sections of each bone marrow specimen using immunohistochemistry for CD34. Prognostic variables were also evaluated for the overall survival. Microvessel counts were significantly higher in patients with MM (n=69.42±9.67), compared with control (n=26.81±2.85). Microvessel density had a weak correlation with percentage of bone marrow plasma cells. By univariate analysis, age, β2-microglobulin, serum albumin, serum creatinine, serum calcium, hemoglobin, platelet count, and bone marrow plasma cell percentage were correlated with survival. By multivariate analysis, age, serum albumin, serum creatinine, hemoglobin, platelet count and bone marrow plasma cell percentage were correlated with overall survival, whereas microvessel density was not. In summary, microvessel density in bone marrow of MM is significantly increased compared to control, but was not correlated with overall survival. Further studies regarding angiogeneic molecules are needed to determine the functional role of angiogenesis in MM.

Key Words : Microvessel Density; Multiple Myeloma; Survival

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INTRODUCTION

Most malignant neoplasms are considered to be dependent on angiogenesis for sustained proliferation. Angiogenesis is critical for the proliferation and metastasis of tumor and is proven to have a prognostic value in several malignancies (1-5). This new vessel formation is mediated by angiogenic molecules released by tumor cells themselves and by accessory host cells such as macrophage, mast cells, and lymphocytes. Also the newly formed endothelial cells of the tumor can stimulate tumor growth in a paracrine fashion (7, 8). It has been demonstrated that the vascular density of a tumor directly correlates with metastasis and poor outcome in patients with solid tumors (9-14). However, few data are available regarding angiogenesis in hematologic malignancies. Several investigators reported that the degree of angiogenesis correlated with the stage of B-cell non-Hodgkin's lymphoma (15) and an increased microvessel density has been demonstrated in the bone marrow of children with acute lymphoblastic leukemia (16) and acute myeloid leukemia (17).

Multiple myeloma (MM) is a human B-cell neoplasm characterized by the clonal expansion of malignant plasma cells in the bone marrow. Recently, it has been reported that bone marrow angiogenesis is correlated with the plasma cell labeling index and is increased in active myeloma (18). To investigate the prognostic significance of microvessel density in MM, we analyzed microvessel density in 75 bone marrow of MM by immunohistochemical method and verified any correlation between microvessel density and survival along with other clincopathological findings.

MATERIALS AND METHODS

Tissue samples and patients characteristics

Between January 1991 and December 1999, 79 patients were diagnosed as MM on the basis of bone marrow findings. Among them, four patients were excluded from the analysis due to the following reasons: two patients were finally diagnosed as monoclonal gammopathy of undetermined significance (MGUS) and the other two patients were excluded because of unavailable clinical data. A total of 75 patients who fulfilled the South West

Oncology Group (SWOG) diagnostic criteria (19) for MM were enrolled for this study. The plasma cell percentage was counted from each bone marrow aspirate slide. Seventy of the 75 patients received systemic chemotherapy with melphalan and prednisone, M2 protocol, or VAD (vincristine, adriamycin, and dexamethasone). High-dose chemotherapy with peripheral stem cell transplantation was done for six patients. Informations concerning the date of initial diagnosis, other clinical characteristics and death were obtained by a retrospective study. Subjects were followed until any of the followings: the date of death, the last date they were known to be alive, or the end of the follow-up. Observations were censored either at the date of last follow-up or at the last date of the follow-up period if death had not occurred. The median follow-up period was 11.8 months.

Immunohistochemical staining

Each sample was processed for immunohistochemical identification of microvascular endothelial cells with anti-CD34 antibodies (anti-HpCA-1, Immunotech, Cedex, France). We observed a highly specific and intense labeling of endothelial cells by applying anti-CD34 antibodies. No background staining was seen. Bone marrow specimens were fixed in paraformaldehyde, embedded in paraffin, and decalcified with EDTA. The 4 μm thick sections were deparaffinized in xylene and rehydrated in water. Endogenous peroxidase activity was eliminated by preincubation in 3% hydrogen peroxidase in methanol for 30 min followed by three washes in phosphate-buffered saline (PBS). All slides were preincubated with 10% normal goat serum for 20 min. The pre-diluted anti-CD34 antibody was used and applied for 60 min at room temperature. Each of the biotinylated secondary antibodies was added for 30 min followed by the streptavidin-biotin peroxidase reagent (DAKO, Carpinteria, CA, U.S.A.) for additional 30 min. After washing with PBS, the antibody was detected by means of an ABC method and developed with 3,3'-diaminobenzidine (DAKO). The slides were counter-stained with hematoxylin.

Estimation of microvessel density

The degree of angiogenesis was determined by the microvessel density in defined areas of bone marrow sections according to the method of Weidner et al. (5) and an international consensus report (20). All slides were coded and evaluated by an experienced pathologist without knowledge of patient's identity or clinical status. Each microvessel counting was performed twice. Each slide was first scanned at ×100 magnification to determine three "hot spots" defined as areas with the maxi-

mum number of microvessels. The slides were then examined at ×200 magnification. Microvessels were counted within the area defined in each of the three hot spots. Large vessels and vessels located in either periosteum or bone were excluded. Areas of staining with no discrete breaks were counted as a single vessel. Microvessel density was estimated by adding the number of vessels in each of the three hot spots and then expressed as the mean number of vessels. Nine normal bone marrows from healthy donors were used as a control group.

Statistical analysis

Differences in microvessel density between MM and control groups were analyzed by the Mann-Whitney U test for independent groups. Survival curves were calculated using the Kaplan-Meier method and compared with other prognostic variable using log-rank test. Correlation between variables was assessed by the Pearson's coefficient (r). Univariate analysis and multivariate stepwise Cox's regression analyses were performed to identify prognostic factors for survival. All statistical analyses were two-sided at a significance level of $p=0.05$, and performed using SPSS 10® statistical software.

RESULTS

Patients characteristics

The 35 male patients and 40 female patients were analyzed (Table 1). The median age was 62 yr old (range: 36-80). The most common type of multiple myeloma was IgG (50.6%), followed by IgA (22.7%), light chain (21.3%), IgD (4.0%), and one case of non-secretory type. Most of the patients had stage III (86.6%) disease and stage I and II disease were noted in 6.7% and 6.7% of the patients, respectively. The overall survival for all patients was 11.8 months (range: 0.2-86.0 months). The median plasma cell percentage in bone marrow of the patients was 48% (range: 9-100%).

Bone marrow microvessel density and its clinical significance

Microvessel counts using CD34 antibody were significantly higher in patients with MM (n=69.42±9.67) compared to control subjects (n=26.81±2.85). The representative data are shown in Fig. 1. Although the distribution of microvessel density had a weak correlation with the percentage of plasma cells in bone marrow of MM (r=0.198, $p=0.097$), there was no correlation between microvessel density and the stage of MM.

Table 1. Characteristics of 75 multiple myeloma patients

| Total No. (n)	75	
Sex (n)		
Male	35	46.7%
Female	40	53.3%
Age (yr)		
Median	62	
Range	36-80	
M-component (n)		
IgG	38	50.6%
IgA	17	22.7%
IgD	3	4.0%
Light chain	16	21.3%
Non-secretory	1	1.3%
Stage (Durie-Salmon) (n)		
Stage I	5	6.7%
Stage II	5	6.7%
Stage III (A/B)	65 (47/18)	86.6%
Microvessel density (n=71)		
Median	49	
Range	8-633	
Plasma cell percentage (%)		
Median	48	
Range	9-100	
Overall survival (months)		
Median	11.8	
Range	0.2-86.0	

By univariate analysis, patients' age, β2-microglobulin, serum albumin, serum creatinine, serum calcium, hemoglobin, platelet count, and bone marrow plasma cell percentage were correlated with overall survival (Table 2). Among those variables, the platelet count, serum calcium, and serum creatinine were the most significant factors for the survival. For the patients with platelets lower than 100,000/μL at diagnosis, the median survival was only 4.27 months, compared with 20.47 months for the patients with higher number of platelet count. Although the number of patients with higher serum calcium level above 12 mg/dL was only five, the median survival was 2.57 months for this group, compared with 20.47 months for patients with lower serum calcium level. Serum creatinine level was the most important factor influencing the overall survival. The median survival for the patients with creatinine greater than 2.0 mg/dL at diagnosis was only 3.33 months, whereas 29.37 months for the lower creatinine group. Moreover, among the 19 patients with higher creatinine level, 16 (84.2%) patients died during follow-up, whereas 28 deaths from 56 (50%) were observed in lower creatinine level.

Since the plasma cell percentage and microvessel density are continuous variables, Cox's regression hazard model was used for the analysis. By multivariate analysis, serum creatinine, plasma cell percentage, patients' age, serum albumin, platelet count, and hemoglobin level were correlated with overall survival, whereas the microvessel density was not (Table 3, Fig. 2).

Bone marrow microvessel density in patients underwent stem cell transplantation

Six out of 75 patients underwent high-dose chemotherapy with autologous stem cell transplantation. We
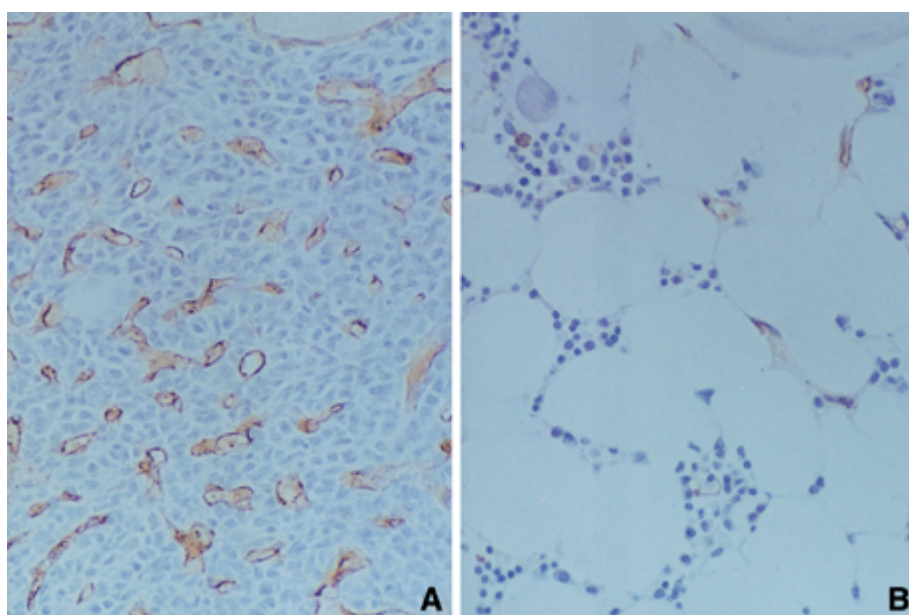
Fig. 1. Immunohistochemical staining of bone marrow sections from patient with multiple myeloma at presentation (A) and control (B) using antibodies against CD34. Note numerous microvessels in (A), compared with some rare endothelial cell clusters in (B) (×400).

Table 2. Features of 75 multiple myeloma patients and their overall survival

Features	Patient (n)	Median survival (months)	*p* value
Age (yr)			
<65	51	19.7	0.0225
≥65	24	5.67	
Sex			
Male	35	15.37	0.2548
Female	40	20.47	
Stage			
I/II	10	25.87	0.1899
IIIA	47	25.20	
IIIB	18	3.33	
Hemoglobin (g/dL)			
<8.5	50	12.47	0.0153
≥8.5	25	36.63	
Platelet count (/μL)			
<100,000	11	4.27	0.0076
≥100,000	64	20.47	
Serum albumin (g/dL)			
<3.0	25	12.10	0.0288
≥3.0	49	29.37	
Serum calcium (mg/dL)			
<12.0	68	20.47	0.0025
≥12.0	5	2.57	
Serum creatinine (mg/dL)			
<2.0	56	29.37	0.0000
≥2.0	19	3.33	
Serum β2-microglobulin (μg/mL)			
<4.0	17	25.87	0.0163
≥4.0	34	11.60	
Missing	24	29.37	
Plasma cell percentage (%)			
<30	20	39.90	0.029
≥30	55	14.90	

Table 3. Multivariate analysis of factors that influence the overall survival by Cox's regression proportional hazards model

Factors	Reference group	B	SE	*p* value	OR*	(95% confidence interval)
Serum creatinine	<2.0 (mg/dL)	1.443	0.441	0.001	4.234	(1.784-10.051)
Plasma cell%	<30.0 (%)	0.995	0.444	0.025	2.705	(1.134-6.453)
Age	<65 (yr)	0.715	0.346	0.039	2.043	(1.036-4.029)
Serum albumin	≥3.0 (g/dL)	0.643	0.364	0.077	1.902	(0.933-3.880)
Platelet count	≥100,000 (/μL)	0.584	0.441	0.185	1.794	(0.756-4.254)
Hemoglobin	≥8.5 (g/dL)	0.134	0.424	0.753	1.143	(0.497-2.626)
Microvessel density		-0.096	0.221	0.665	0.909	(0.590-1.401)

*OR: odds ratio

estimated bone marrow microvessel density before stem cell transplantation and at the time of response in 5 patients with MM (4 in complete response, 1 in partial response). Although the mean microvessel density was reduced to 33.72 after achievement of response, compared to 59.06 at diagnosis, the difference was not statistically significant (Table 4).

DISCUSSION

Angiogenesis is an important step in the progression of tumor growth, invasion, and metastasis (1, 2). In human solid tumors such as colon, breast, lung cancer, and melanoma, angiogenesis occurs simultaneously during invasion and metastasis (3-5, 9-14). In contrast, little is
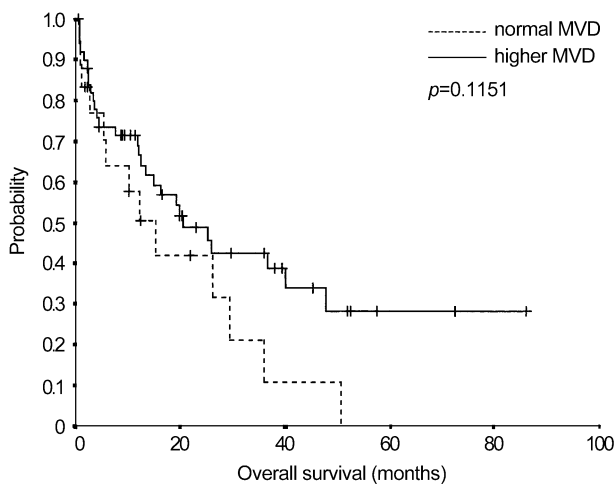
Fig. 2. Overall survival for multiple myeloma patients according to the microvessel density of bone marrow. Normal group (n= 19) was defined arbitrarily as less than 30 microvessel density and higher group (n=52) was defined as more than 30 microvessel density in bone marrow.

known regarding angiogenesis in hematologic malignancies (15-17). In this study, we observed a significant increase of bone marrow angiogenesis evaluated as microvessel density in patients with MM compared with control subjects. This finding suggests that bone marrow angiogenesis may play an important role in the neoplastic process. However, not all patients with MM had increased microvascular density and this variability may have prognostic implications. We also found that the percentage of plasma cells in bone marrow correlated with the number of microvessel density.

Vacca et al. (18) reported fivefold increase in the microvessel density in the bone marrow of patients with active MM as compared with nonactive MM and MGUS. He defined nonactive MM in posttreatment complete objective response and the off-treatment plateau phase. The progression of plasma cell tumors is accompanied by an increase of bone marrow neovascularization.

Although the microvessel density was increased in MM and correlated with plasma cell percentage of bone marrow, we could not find any correlation between microvessel density and survival. However, Vacca et al. re-

ported that bone marrow neoangiogenesis paralleled disease progression and predicted poor outcome. These controversies should be resolved by further studies with large number of patients and different study designs.

Recently, Dankbar et al. (21) reported that vascular endothelial growth factors (VEGF) are expressed and secreted by myeloma cells and that VEGF stimulate the expression of IL-6 by microvascular endothelial cells and bone marrow stromal cells. Conversely, IL-6 stimulated the expression of VEGF by myeloma cells, suggesting a paracrine role of VEGF in tumor-stroma interactions in MM. Studies combining the VEGF expression and microvessel density might be able to delineate the role of angiogenesis in MM.

Since relapse is usually inevitable in MM even after autologous stem cell transplantation, we also studied bone marrow microvessel density in patients who underwent autologous stem cell transplantation to determine whether there was persistently increased angiogenesis at the time of complete or partial response. We found that there was no significant decrease in microvessel density following stem cell transplantation, even in the setting of complete response in most cases except one. We could not observe any tendency of longer time to relapse in patients achieving a decrease in microvessel density by over 50%. This finding suggests that the persistence of anigogenesis may be explained by continued stimulus on microvessels by minimal residual disease not detectable by conventional methods and may involve potent cytokines, such as VEGF or other angiogeneic factors (22).

In conclusion, the microvessel density is increased in bone marrow of MM compared to control, but the microvessel density in MM was not correlated with overall survival. Further studies regarding other angiogeneic molecules (23, 24) are needed to determine the functional role of angiogenesis in MM.

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Table 4. Microvessel density prior to and after autologous stem cell transplantation

Patients	Microvessel density prior to stem cell transplantation	Microvessel density after stem cell transplantation	Clinical response	Time to relapse
M/57	67.7	47.0	Complete response	9 months
F/54	28.3	28.3	Partial response	8 months
F/68	27.3	19.3	Complete response	7+ months
F/57	105.7	29.3	Complete response	11+ months
F/62	66.3	44.7	Complete response	24+ months

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