Bone Marrow Angiogenesis in 400 Patients with Monoclonal Gammopathy of Undetermined Significance, Multiple Myeloma, and Primary Amyloidosis

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ABSTRACT

Purpose: To determine whether bone marrow (BM) angiogenesis progressively increases along the spectrum of plasma cell disorders ranging from monoclonal gammopathy of undetermined significance (MGUS) to advanced myeloma.

Experimental Design: Four hundred patients with the following disorders were studied: MGUS (76 patients); smoldering (indolent; early-stage) multiple myeloma (SMM; 112 patients); newly diagnosed, active multiple myeloma (MM; 99 patients); relapsed (advanced) multiple myeloma (RMM; 26 patients); and primary amyloidosis (AL; 87 patients). Forty-two normal control BM samples were studied for comparison. BM angiogenesis was studied in a blinded manner by immunohistochemical staining for CD34 to identify microvessels.

Results: The median (range) microvessel density (MVD) per ×400 high power field was 1.3 (0–11) in the controls, 1.7 (0–10) in AL, 3 (0–23) in MGUS, 4 (1–30) in SMM, 11 (1–48) in newly diagnosed MM, and 20 (6–47) in RMM; P < 0.001. MVD was significantly higher in MGUS, SMM, newly diagnosed MM, and RMM compared with controls and AL; P < 0.001. MVD was not significantly different between controls and AL. By grading, high-grade angiogenesis was present in 0% of controls and AL, 1% of MGUS, 3% of SMM, 29% of newly diagnosed MM, and 42% of RMM; P < 0.001. MVD correlated with the BM plasma cell labeling index (r = 0.46, P < 0.001) and BM plasma cell percentage (r = 0.5, P < 0.001). Survival was 28 months in SMM and newly diagnosed MM with high-grade angiogenesis, compared with 53 months for those with low- and intermediate-grade angiogenesis; P = 0.02.

Conclusions: BM angiogenesis progressively increases along the spectrum of plasma cell disorders, from the more benign MGUS stage to advanced myeloma, indicating that angiogenesis may be related to disease progression.

INTRODUCTION

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 (1, 2). It is also important for the proliferation and metastases of most malignant neoplasms. In the absence of angiogenesis, tumors cannot grow beyond 1–2 mm in size. Increased angiogenesis is an adverse prognostic factor in several tumors (3, 4). Although many initial studies have been performed on solid tumors, recent evidence indicates that angiogenesis is increased and is important in hematological malignancies as well (5–7).

MM is part of a spectrum of plasma cell disorders, which includes MGUS and SMM/Stage 1 myeloma. Patients with MGUS and SMM are at risk for progression to MM, but do not require therapy (8, 9). Recent evidence indicates that BM angiogenesis is markedly increased in myeloma and has prognostic value in the disease (10, 11). Just as in solid tumors, angiogenesis may play a role in the transformation from the premalignant stage (MGUS) to active myeloma.

The purpose of this study was to estimate the extent of BM angiogenesis in the spectrum of plasma cell disorders including MGUS, SMM, newly diagnosed MM, and RMM. Normal controls and patients with primary AL, a plasma cell proliferative disorder defined primarily by monoclonal light-chain fragment deposition rather than tumor bulk, were studied for comparison. We hypothesized a progressive increase in BM angiogenesis from normal BMs through the various stages of plasma cell disorders.
PATIENTS AND METHODS

Patients and Data Collection. BM samples from 400 patients with plasma cell disorders seen at the Mayo Clinic (Rochester, MN) were studied: MGUS (76 patients); SMM (112 patients); newly diagnosed MM (99 patients); RMM (26 patients); and AL (87 patients). MGUS, SMM, and newly diagnosed MM were defined according to criteria we published previously (12). We also studied 42 normal control BM samples for comparison. Information on prognostic factors, including β-2M, BM plasma cell percentage, and PCLI, was used to study the correlation with BM angiogenesis grade and MVD. No patient was lost to follow-up. Approval of the study by the Mayo Institutional Review Board was obtained in accordance with federal regulations and the Declaration of Helsinki.

CD34 Immunostaining. The extent of BM angiogenesis was assessed by standard immunohistochemical methods to identify BM microvessels (3, 12, 13). Briefly, CD34 immunostaining was performed with a labeled streptavidin-biotin peroxidase method, as described previously (12), on a Ventana ES automated immunohistochemistry stainer (Ventana Medical Systems, Tucson, AZ) using buffers and detection reagents supplied by the manufacturers. Deparaffinized tissues were pretreated with EDTA (pH 8.0) in a steamer for 30 min followed by incubation with the tissue sections for 32 min. The aminoethyl carbazole detection kit (Ventana Medical Systems) was used for antigen visualization; sections are counterstained with a light hematoxylin and then cover-slipped with Kaiser’s glycerol jelly (Mayo Medical Laboratories, Rochester, MN). Paraffin sections of well-vascularized tonsill were run with each batch as a positive control, and a section stained with nonimmune rabbit immunoglobulin was used as a negative control.

Angiogenesis Grading and MVD Estimation. All estimations were performed in a blinded manner. Methods used in our laboratory have been published previously and are similar to standard methods used by other investigators (11, 12). We have shown that these methods have a high degree of reproducibility with low interobserver variability (11). BM trephine core biopsies were used. Thin sections of the core biopsy sample were stained for CD34. For simple grading, slides were scanned at ×100, ×200, and ×400 magnification, and based on the extent of microvessel staining, each slide was assigned an angiogenesis grade as described previously (11): low, intermediate, or high. Briefly, BM trephine biopsy specimens stained for CD34 were classified into low-, intermediate-, and high-grade angiogenesis based on visual evaluation of the entire stained field under ×200 magnification. The assessment of low-, intermediate-, and high-grade angiogenesis was based primarily on visual impression of the number of CD34 positive microvessels seen in the entire biopsy section. This visual impression is arbitrary but has been found to correlate to a very high degree with manual counting of MVDs (11). For MVD estimation, each slide was first scanned at ×100 magnification to determine three “hot spots” defined as areas with the maximum number of microvessels. The hot spots were then examined at ×400 magnification using a ×10 ocular and ×40 objective lens. Microvessels were counted in each of the three hot spots at ×400 magnification. 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 of the 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. BM PCLI was assessed at diagnosis. PCLI was a reflection of the plasma cell proliferative activity and was performed with a slide-based immunofluorescence method on BM samples as described elsewhere (14, 15). A PCLI of ≥1% was classified as high.

Statistical Analysis. Overall survival was defined as the interval from diagnosis to either death or last contact. An event was defined as a death from any cause, unless otherwise indicated. Kaplan-Meier methodology was used to estimate survival distributions (16). The two-tailed Wilcoxon test was used to assess whether survival from diagnosis differed among categories. Associations between MVD and various clinical, histological, and laboratory variables obtained at the time of the BM study were also studied. Fisher’s exact test was used to assess the significance of the difference between the distributions of categorical data. The Wilcoxon rank-sum test or the Kruskal-Wallis test was used to assess whether continuous variables differed significantly among categories. Multivariate analysis was conducted with Cox’s proportional hazards model (17). All data were analyzed by with SAS software (SAS, Inc., Cary, NC).

RESULTS

BMs from 400 patients with plasma cell disorders seen at the Mayo Clinic were studied: MGUS (76 patients), SMM (112 patients), newly diagnosed MM (99 patients), RMM (26 patients), and AL (87 patients).

BM Angiogenesis. Angiogenesis was significantly increased in newly diagnosed MM (Fig. 1). The median (range) MVD per ×400 high power field was 1.3 (0–11) in the controls, 1.7 (0–10) in AL, 3 (0–23) in MGUS, 4 (1–30) SMM, 11 (1–48) in newly diagnosed MM, and 20 (6–47) in RMM; P < 0.001 (Fig. 2). MVD was significantly higher in SMM, newly diagnosed MM, and RMM compared with controls, MGUS, and AL; P < 0.001. MVD was also significantly higher in MGUS compared with controls (P < 0.001). There was no significant difference in MVD between controls and AL. Patients with RMM had significantly higher MVD compared with newly diagnosed, untreated MM (P = 0.02).

Results were similar if angiogenesis grading was substituted for MVD. By grading, high-grade angiogenesis was present in 0% of controls and AL, 1% of MGUS, 3% of SMM, 29% of newly diagnosed MM, and 42% of RMM; P < 0.001 (Fig. 3).

Correlation of BM Angiogenesis with Marrow Involvement, PCLI, and Other Prognostic Factors. There was a significant relationship between the BM PCLI and angiogenesis grade in SMM/newly diagnosed MM (Figs. 4 and 5). Similarly, MVD correlated positively with the BM PCLI (P = 0.46, P < 0.001). MVD was also correlated to BM plasma cell % (P = 0.5, P < 0.001; Fig. 6). There was no correlation between MVD and age, serum monoclonal protein level, β-2M, or serum creatinine.
BM Angiogenesis and Survival. For survival analysis, patients were followed until death, and median follow-up among those still alive was 68 months. BM MVD was a prognostic factor for survival in the group of patients with SMM and newly diagnosed MM; $P = 0.02$. Survival was 28 months in SMM/newly diagnosed MM with high-grade angiogenesis, compared with 53 months for those with low-grade angiogenesis; $P = 0.02$ (Fig. 7). Other prognostic factors for survival were PCLI ($P < 0.001$), BM plasma cell percentage ($P = 0.003$), and $\beta$-2M ($P = 0.01$). On multivariate analysis, despite being highly correlated to the PCLI, MVD appeared to have a trend toward independent prognostic value ($P = 0.05$).

Increased angiogenesis was not a significant predictor of progression to myeloma in MGUS or SMM.

DISCUSSION

Folkman (1, 2) has suggested that premalignant cancer cells (such as in situ carcinomas or dormant metastatic deposits) acquire the ability to induce microvessel formation at some point by altering the balance between pro- and antiangiogenic cytokines (“angiogenic switch”). The result of this putative switch is the mutually favorable relationship that forms between cancer cells and supporting stromal/endothelial cells. Thus tumor cells release proteins that promote the growth of supporting cells and new vessel formation. In return, the newly formed microvessels provide the growing tumor with oxygen and other nutrients critical for cancer cell growth, as well as release of important cytokines that promote cancer cell proliferation.
Our hypothesis is that myeloma evolves from preexisting MGUS, and it is possible that the angiogenic switch may be responsible, at least in part, for the progression to newly diagnosed MM (12). In this study, we show that the degree of angiogenesis progressively increases along the various stages of myeloma progression. As expected, angiogenesis was not significantly increased in AL compared with normal controls. There is a slight increase in BM angiogenesis in a small subset of patients with MGUS compared with controls, but no specific clinical or laboratory marker identified these patients. The number of patients with MGUS who had increased angiogenesis was too small to determine whether there was a greater likelihood of progression to newly diagnosed MM. Angiogenesis is significantly increased in SMM compared with MGUS and in newly diagnosed MM compared with SMM. In relapsed newly diagnosed MM, there is a further increase in angiogenesis.

Although this study does not provide a causal relationship between angiogenesis and disease progression, we believe that the induction of angiogenesis is an important and significant first step. However, because some patients with newly diagnosed MM have low-grade angiogenesis and, conversely, some with SMM have high-grade angiogenesis, other mechanisms of disease progression are likely involved as well. These may include cytogenetic changes that promote myeloma cell proliferation, resistance to apoptosis, and invasion. We believe that progression from MGUS to newly diagnosed MM likely involves critical events in both the clonal plasma cell and the microenvironment that changes the balance between pro- and antiangiogenic cytokines in favor of increased BM angiogenesis (angiogenic switch). This occurs infrequently and accounts for the very low rate of progression from MGUS to myeloma. When it occurs, angiogenesis triggers increased proliferation of plasma cells and transformation to myeloma (18). We are not able to prove this finding in this study because of the low rate of transformation of MGUS to myeloma. A large cohort of MGUS was included in this study, but a much larger sample size (>1000) will be needed to show a difference in angiogenesis between patients who progressed and those who did not. Although the Mayo Clinic has one of the largest databases of MGUS in the world, we are limited in the number of samples available to study because BM biopsies are not performed routinely in MGUS. Furthermore, although the risk of progression from MGUS to myeloma is 1% per year, many patients are elderly and die of competing causes of death. In fact, the true progression to myeloma is <10% at 15 years of follow-up (19). We have recently identified high-risk groups of MGUS with increased risk of progression (19), and we are planning prospective studies to identify factors involved in progression.

In a small study, Vacca et al. (10) showed that BM angiogenesis is increased in newly diagnosed MM compared with MGUS. The present study confirms these observations in a much larger cohort and includes normal controls and AL as comparison groups to make the findings more conclusive. Recently, Vacca et al. (20) demonstrated the angiogenic ability of myeloma cells in an in vitro CAM angiogenesis assay. In this model, 76% of purified myeloma samples from patients were angiogenic versus 20% of MGUS samples. A linear correlation was present between the angiogenic activity seen in the CAM angiogenesis assay and marrow vascularization estimated by
analysis of microvessels. These observations suggest that the increase in MVD in newly diagnosed MM is a marker for the angiogenic stimulus ongoing in the BM.

The present study has several important findings. It demonstrates for the first time a progressive increase in angiogenesis across a wide spectrum of plasma cell disorders. Previous studies by us and from the University of Bari (10, 21) had smaller sample sizes and did not include the entire spectrum of plasma cell disorders. This study thus provides additional basis and rationale for initiating and investing resources to future studies of angiogenesis and plasma cell disorders. This is also the first report to study SMM. Unlike MGUS, where the risk of progression to myeloma is 1% per year, patients with SMM have a risk of progression of 25% per year. However, SMM is a distinct entity, separate from newly diagnosed, symptomatic MM because therapy is not needed and patients may go for several years without progression. This report also demonstrates no increase in angiogenesis in AL compared with controls. This is an expected finding because AL occurs because of the unique structure of the light chain fragment involved (amyloidogenic light chain), rather than uncontrolled proliferation of neoplastic plasma cells. We also show for the first time that MVD is slightly, but to a statistically significant extent, increased in MGUS compared with a large cohort of normal controls. Finally, the study shows that RMM, a difficult entity to treat and one in which thalidomide, an antiangiogenic agent is active, has significantly more angiogenesis than newly diagnosed, active MM.

Increased angiogenesis in this study was a marker of poor survival in patients with SMM/newly diagnosed MM. This confirms our earlier observation in a smaller cohort. In that study of 75 newly diagnosed MM patients, overall survival was significantly longer in patients with low-grade angiogenesis (53 months) compared with patients with high-grade (24 months) or intermediate-grade angiogenesis (48 months); \( P = 0.018 \) (11). Angiogenesis was not a predictor of progression to MM in either SMM or MGUS. As discussed previously, this is likely related to the low event rate of newly diagnosed MM in these groups. Munshi and Wilson (22) and Sezer et al. (23) have also demonstrated recently the prognostic value of angiogenesis in myeloma. Although angiogenesis is prognostic for survival, we have shown that there is no correlation between a change in MVD and remission status (7, 24). In contrast, Sezer et al. (25) found a significant decrease in angiogenesis with successful therapy, and the reason for the discrepancy in findings is not clear. There is also no correlation between angiogenesis and the...
deletion of chromosome 13 (26). A positive correlation is present between angiogenesis and BM plasma cell involvement, but we were unable to find a correlation with β-2M as noted in an earlier report (27).

Increased plasma cell proliferation measured by the PCLI in myeloma occurs with increased angiogenesis (10, 11). This was seen in the present study, as well as in earlier studies by us and by other groups (10, 11). This suggests that increased angiogenesis may be promoting plasma cell proliferation and supports a causal relationship between angiogenesis and disease progression in myeloma. An opposing point of view is that increased angiogenesis is merely an epiphenomenon related to increased cytokine expression by the proliferating myeloma cells. The challenge in hematologic malignancies is to prove a cause and effect relationship between increased angiogenesis and cancer progression. These studies are needed to establish that increased angiogenesis in hematologic malignancies is not merely an epiphenomenon but a critical part of the disease pathogenesis. We believe two types of studies are required to prove the pathogenetic role of angiogenesis in myeloma. The first type is a serial follow-up study of MGUS patients over time to demonstrate that the increased angiogenesis occurs right before progression. We are planning such a study, but it requires significant amount of time to complete because only 1% of patients with MGUS progress to myeloma each year (19). The second type of study is one that shows the response of myeloma to antiangiogenic therapy. Although thalidomide has antiangiogenic properties and is effective in myeloma, it has several other potential mechanisms of action. A drug that has antiangiogenesis as its sole mechanism, such as endostatin or angiostatin, must show efficacy in myeloma. Preliminary data from Fuji et al. (28) show that endostatin does induce regression of myeloma in a mouse model of myeloma, supporting a pathogenic role for angiogenesis.

Although angiogenesis is increased in myeloma, the underlying mechanism of this phenomenon is unclear. Several cytokines are involved in promoting tumor angiogenesis and two of the most important are VEGF and bFGF (29–31). There is now data that show an increased level of expression of VEGF occurs in the marrow in patients with myeloma and may be the cause of the increased angiogenesis seen in this disease (32–34). VEGF levels appear to decrease after effective therapy of myeloma. In studies with MGUS and myeloma, we have found that VEGF is overexpressed by myeloma cells based on immunostaining, ELISA, and reverse-transcription PCR (35). Because VEGF is a major angiogenic cytokine, this provides the basis for the increased angiogenesis seen in myeloma. We have also similarly found overexpression of bFGF, as well as the receptors for bFGF (33). VEGF receptors also seem to be overexpressed by myeloma cells. VEGF and bFGF likely mediate the increased angiogenesis seen in this study.

In addition to its important role in the initiation of angiogenesis, VEGF may play a more direct role in myeloma by its effects on myeloma cell migration and proliferation (34, 36). A paracrine role for VEGF has also been proposed. Stimulation of myeloma cell lines with interleukin-6 leads to an increase in VEGF secretion. Similarly, stimulation of human umbilical vein endothelial cells and BM stromal cells with VEGF induces a significant increase in interleukin-6 secretion in a dose-dependent manner.

bFGF may also play a role in myeloma angiogenesis. Sezer et al. (37) have found an increased level of serum bFGF in myeloma, which decrease with effective chemotherapy. Vaccaro et al. (20) have shown that antibodies to bFGF cause a significant inhibition (>50%) of the angiogenesis induced by myeloma cells in the CAM angiogenesis assay. High bFGF levels correlate with poor survival, but also with response to thalidomide therapy (38). Besides VEGF and bFGF, vaccaro et al. (20, 39) have also found increased expression of aquaporin 1 and matrix metalloproteinase-2 that correlates with the increased angiogenesis seen in myeloma.

These observations provide a rationale for testing antiangiogenic therapy in newly diagnosed MM. On the basis of its antiangiogenic properties in animal models and in in vitro systems, and its availability for clinical trials, thalidomide was tested and found effective in RMM (40, 41). We are currently evaluating 2-methoxyestradiol, a metabolite of estrogen that has antiangiogenic properties, as a potential therapeutic agent in myeloma. Novel analogues of thalidomide are also being developed and tested in an attempt to minimize the adverse effects while increasing clinical efficacy (42, 43). One such analogue (CC5013) is now undergoing Phase I testing at the Dana-Farber Cancer Institute and the University of Arkansas. Phase II trials are planned to start next year. Future studies will examine the precise role of angiogenesis in the pathogenesis of newly diagnosed MM and the use of other novel antiangiogenic agents in therapeutic trials.

Finally, this study confirms previous observations by us that a simple visual method of angiogenesis grading can adequately substitute for cumbersome MVD estimations (11).

REFERENCES
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