The evaluation of angiogenesis and matrix metalloproteinase-2 secretion in bone marrow of multiple myeloma patients before and after the treatment

Bolkun L†, Lemancewicz D1,2*, Sobolewski K3, Mantur M4, Semeniuk J1, Kulczynska A1, Kloczko J1, Dzieciol J2

1 Department of Hematology, Medical University of Bialystok, Bialystok, Poland
2 Department of Human Anatomy, Medical University of Bialystok, Bialystok, Poland
3 Department of Medical Biochemistry, Medical University of Bialystok, Bialystok, Poland
4 Department of Clinical Laboratory Diagnostics, Medical University of Bialystok, Bialystok, Poland
5 Department of Pediatrics, Pediatric Gastroenterology and Allergology, Medical University of Bialystok, Bialystok, Poland

† These authors contributed equally to this work

ABSTRACT

Purpose: Angiogenesis appears to be a prominent feature of many hematological disorders, particularly in multiple myeloma (MM). Progression in MM also involves secretion of the metalloproteinases (MMPs). In this study, the expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and its receptor, in bone marrow trephine biopsy (TB) of thirty six MM patients before and after the treatment or during progression was examined. The MMP-2 secretion was assessed from the same patients.

Material/Methods: Immunohistochemical staining of bone marrow specimens for angiogenic factors and microvessel density (MVD) and bone marrow aspirates for Western blot analysis of MMP-2 expression was performed.

Results: In active, untreated MM patients, we found statistically significant differences in the expression of angiogenic factors according to the patients after the anti-angiogenic treatment. We found statistical differences of the expression of angiogenic factors between the group of patients with a response after the treatment and the patients who had progression during the treatment. The data showed statistically significant decreased MVD after the treatment. The results showed statistically significant differences between initial secretion of MMP-2 in active, untreated MM patients and patients with a response after the treatment and patients with progression during the treatment.

Conclusions: We showed that not only decreased expression of angiogenic cytokines is present after the anti-angiogenic treatment but also activity of MMP-2 in MM patients who responded to the treatment. Combination therapy with the inhibition of the activity of MMPs could represent an interesting therapeutical approach in MM.

Key words: angiogenesis, MMP-2, multiple myeloma, trephine biopsy

INTRODUCTION

Multiple myeloma (MM) is a very devastating malignancy characterized by slow proliferation of plasma cells infiltration of bones with a high capacity to destroy bone matrix. Angiogenesis is a required step in the progression of tumor growth, invasion, and metastasis [1]. The resulting effect is a painful lesion, where bone is eroded and filled with myeloma cells that suppress and replace the normal marrow components [2]. Increasing levels of tumor angiogenesis have been associated with poor prognosis for a variety of hematologic malignancies and solid tumors [3]. Malignant plasma cells can secrete various cytokines, including vascular endothelial growth factor (VEGF), basic fibroblast...
growth factor (bFGF). Those cytokines have been invoked as responsible for driving the process of neovascularization in solid tumors and hematologic malignancies [4-8]. Progression also involves secretion of the extracellular matrix-degrading enzymes such as metalloproteinase-2 (MMP-2 or 72-kD type IV collagenase) and MMP-9 (92-kD type IV collagenase) by tumor cells [9]. Metalloproteinases (MMPs) are genetically distinct but structurally related proteinases consisting of five subgroups: collagenases, gelatinases, stromelysins, membrane-type MMPs, and other MMPs [10]. MMPs are Zn2+ and Ca2+ requiring enzymes capable of degrading almost all extracellular matrix and basement membrane components in normal tissue remodeling, and especially in tissue destructive diseases, including odontogenic tumors and cysts [11,12]. Matrix MMPs play a critical role in MM patients bone remodeling and tumor invasion. In human solid tumors such as colon, breast, lung carcinomas, and melanoma, angiogenesis and MMP-2/MMP-9 overexpression occur simultaneously during invasion and metastasis, but are downregulated or even absent in hyperplastic or normal tissue and in situ tumors [13-15]. The precise roles of MMPs in bone resorption are still unknown. However, MMPs involved in bone resorption may be provided by either the osteoblasts (MMP-2, MMP-13 and MMP-3), or the osteoclasts (MMP-9) [12-14].

In this study, the expression of VEGF, bFGF and its receptor, in bone marrow trephine biopsy (TB) was examined in MM active, untreated patients, non active MM patients after the anti-angiogenic treatment (at least partial response-PR and complete response-CR) and patients with progression during the treatment and patients with stable disease. The MMP-2 secretion by bone marrow plasma cells was assessed from the same patients.

**PATIENTS AND METHODS**

Thirty six patients with a new diagnosed MM were studied. The median age of patients at the time the samples were obtained was 60 and the range was 42-71. Twenty four of them were male and twelve were female. Complete blood count, erythrocyte sedimentation rate, total protein/albumin, monoclonal protein level (M protein), albumin concentration, β2 microglobulin (β2m), serum calcium level (Ca), lactate dehydrogenase (LDH) protein, immune electrophoresis, renal function tests and skeletal surveys were performed in all cases. Bone marrow aspiration and TB samples were obtained. Bone marrow aspirate was used for Western blot studies. Paraffin-embedded TB samples were used for immunohistochemical stains. All patients at the time of diagnosis were divided into three groups based on International Staging System (ISS): nine patients were at I stage, fifteen patients were at II stage, and twelve were at III stage (Tab. 1). Patients’ initial treatment of MM depended on their age and comorbidities. For all studied patients the induction regimens were thalidomide, dexamethasone and cyclophosphamide (CTD) prior to maintenance therapy or autologous stem-cell transplantation. The evaluation was done after six cycles of chemotherapy with the same methods as diagnosis or at the moment where the progression was suspected. Informed written consent was obtained from all participants and the Ethics Committee of the Medical University of Białystok approved the study (approval number R-I-003/173/2006). In the control group (age- and sex- matched), bone marrow samples were obtained from twenty four patients undergoing bone marrow biopsy for clinical indications, who were eventually classified as having normal bone marrow.

**Immunohistochemistry for VEGF, bFGF and its receptor**

Immunohistochemical examinations were conducted with the help of specific antibodies. Primary antibodies were used by dilution according to the manufacturer’s instructions: a dilution of 1:10 for VEGF (DAKO Cytomation), a dilution of 1:50 for bFGF (Santa Cruz Biotechnology, Santa Cruz, CA), and a dilution of 1:10 for fibroblast growth factor receptor, FGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA). All sections required microwave heating for receptor retrieval. A detecting kit was LSAB+KIT (DAKO, Cytomation). DAB (3,3′-diaminobenzidine) was used as a chromogen. If the reaction was positive brown-colored antigen-antibody complexes appeared in the position of a protein receptor. Intensity of the positive reaction was defined according to a scale described by Kumar et al. [5]: -, indicates no staining; +, weak staining (0%-30% plasma cells positive); ++, weak to moderate staining (31%-60% plasma cells positive); and ++++, strong staining (more than 60% plasma cells positive).

![Table 1. Clinical features of active, untreated multiple myeloma patients.](image-url)
Using a separate section from each biopsy sample stained with plasma cell clone VS38c (DAKO) an estimate of plasma cells percentage was made. (Fig. 1).

**Measurement And Grading of Bone Marrow Angiogenesis**

Microvessel density (MVD) was assessed in the TB samples using previously described methods used by other investigators [16,17]. All estimations were performed in a blinded manner. For simple grading, slides were scanned at x100, x200, and x400 magnification, and based on the extent of microvessel staining, each slide was assigned an angiogenesis grade as described previously: low, intermediate, or high [16]. Briefly, bone marrow TB specimens stained for CD34 were classified into low-, intermediate-, and high-grade angiogenesis based on visual evaluation of the entire stained field under x200 magnification (Fig. 2). 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. The average number of vessels which stained with CD34 was first scanned at x100 magnification to determine three hot spots defined as areas with the maximum number of microvessels. The hot spots were then examined at high-powered fields (x400 magnification) using a x10 ocular and x40 objective lens. 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.

**Western Blot Analysis**

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) was performed according to the method described by Laemmli [18]. Samples of anti-coagulated bone marrow aspiration supernatants containing 20 μg of protein were subjected to electrophoresis (Fig. 3A). After SDS/PAGE, gels were allowed to equilibrate in 25 mM Tris, 0.2 M L-glycine, 20% (v/V) methanol for 5 min, and the proteins were transferred to nitrocellulose membranes with 0.2-µm pore diameter (catalogue No. N8017; Sigma Aldrich, Steinheim, Germany) at 100 mA for 1 h using a Sigma Semi-Dry Blotter. Nitrocellulose membranes were then incubated with one of the following primary monoclonal antibodies: against MMP2 (catalogue No. MAB9021; R&D Systems) or against MMP9 (catalogue No. MAB9091; R&D Systems) at 1: 1,000 dilutions in 5% dried defatted milk in TBS-T [20 mM Tris-HCl buffer, pH 7.4; 150 mM NaCl; 0.05% (v/v) Tween-20] for 1 h. To evaluate MMP expression, species-specific secondary antibodies were added at 1: 5,000 dilutions. Incubation was continued for 30 min with slow shaking. After that, nitrocellulose membranes were washed with TBS-T (5 times for 5 min) and treated with Sigma-Fast BCIP/NBT reagent (catalogue No. B1911; Sigma Aldrich). The levels of MMP-2 expression in bone marrow plasma cells were determined by scanning densitometry (Fig. 3B).
MMP were assessed by scoring the intensity of the bands by a computerized image analysis (APPLE, Cupertino, CA).

**Statistical Analysis.**
Statistical comparison between MM group and control group was made using the non parametric Mann–Whitney test. Mean values of the measured parameters were expressed as mean ± SD. One way analysis of variance (ANOVA) with the Kruskal–Wallis was used for the analysis of differences between groups. The Spearman’s rank correlation test was used to examine correlations between various parameters. A p value <0.05 was considered to be significant.

**RESULTS**

Tables 2 and 3 show the mean ± SD values for each of the variables measured in the group of MM patients before and after the treatment. For the purposes of this study, we analyzed patients with stage I, stage II and stage III according to the ISS of MM separately. Nine patients had stage I and in these patients we found that the expression of each angiogenic factor was lower than in the patients with stage II and stage III (Tab. 2). In analysis of group of active, untreated MM patients, we found statistically significant differences in the expression of angiogenic factors according to the group of patients after the anti-angiogenic treatment (Tab. 3). Additionally we found statistical differences of the expression of angiogenic factors between the group of patients with a response (PR + CR), non-active patients and the group of patients who had progression during the treatment or who did not respond to the treatment (Tab. 3). We also found that treated patients who had stable disease after the treatment had lower expression of each angiogenic factor than untreated, active MM patients but in the most of cases the difference was not statistically significant (Tab. 3). We then correlated each angiogenic factor with some well known parameters of prognosis and tumor load in MM such as, hemoglobin (HGB), M protein level, albumin concentration, β2m, serum Ca level, LDH, IgG level and percentage of bone marrow plasma cells in TB. As shown in Tab. 4, VEGF and bFGF and FGFR2 significantly correlated with serum Ca level (p<0.0003 and p<0.02 and

| Table 2. Expression of angiogenic cytokines by immunohistochemistry in active, untreated multiple myeloma patients according to the ISS. |
|-----------------|-----------------|-----------------|
| Cytokine/cytokine receptor | No. of patients | No of patients according to ISS |
| | Active, untreated patients, n=36 | Patients at I ISS \( n=9 \) | Patients at II ISS \( n=15 \) | Patients at III ISS \( n=12 \) |
| VEGF | 49.41±16.52 | 46.33±14.2 | 48.60±18.86 | 52.76±19.24 |
| bFGF | 46.08±14.40 | 41.66±24.26 | 42.60±15.32 | 50.75±18.08 |
| FGFR2 | 35.33±10.05 | 35.00±14.00 | 35.8±10.62 | 36.87±13.65 |
| MVD | 20.16±6.76 | 17.6±8.93 | 21.20±7.46 | 20.75±8.31 |
| Level of expression | +++/++ | +/++ | +++/++ | +++/+++ |
| MMP-2 (x10³) | 20.08±4.16 | 12.12±5.77 | 18.72±4.81 | 26.11±7.42 |

Values are presented as mean ±SD, ISS - International Staging System, VEGF - vascular endothelial growth factor, bFGF - basic fibroblast growth factor, FGFR2 - fibroblast growth factor receptor 2, MVD - microvessel density, MMP-2 - matrix metalloproteinase-2, * p=0.01.

| Table 3. Expression of angiogenic cytokines by immunohistochemistry in active, untreated multiple myeloma patients (MM) and in MM patients after the treatment. |
|-----------------|-----------------|-----------------|
| Cytokine/cytokine receptor | No. of patients | Patients after treatment |
| | Active, untreated patients \( n=36 \) | After treatment \( n=36 \) | with PR+CR \( n=20 \) | with stable disease \( n=10 \) | with progression \( n=6 \) |
| VEGF | 49.41±16.52 | 29.5±9.94 | 24.62±7.26 | 32.32±12.81 | 39.25±6.99 |
| bFGF | 46.08±14.40 | 35.00±11.56 | 33.62±13.64 | 36.75±6.53 | 37.75±7.13 |
| FGFR2 | 35.33±10.05 | 29.41±6.27 | 22.7±10.94 | 33.53±5.22 | 41.25±4.64 |
| MVD | 20.16±6.76 | 13.50±2.27 | 10.00±2.13 | 18.24±5.44 | 22.18±3.21 |
| Level of expression | ++/++ | +/++ | +/- | +/+ | +/++ |
| MMP-2 (x10³) | 20.08±4.16 | 15.34±3.62 | 4.41±2.53 | 17.22±3.70 | 28.32±8.75 |

Values are presented as mean ±SD, VEGF - vascular endothelial growth factor, bFGF - basic fibroblast growth factor, FGFR2 - fibroblast growth factor receptor 2, MVD - microvessel density (number of microvessels per x 400 field), MMP-2 - matrix metalloproteinase-2, * p=0.007 between patients with response and progression, ** p=0.05 between patients with response and progression, ***p=0.01 between patients with response and progression. #p=0.0004 between active, untreated patients and after the treatment, ##p=0.02 between active, untreated patients and after treatment.
The evaluation of angiogenesis and MMP-2 in MM patients

Concentration of MVD was significantly higher in active, untreated MM patients (the median range MVD per x400 high power field was 20.16±6.76) than in the controls 2.2±0.9 (Tab. 2). The results showed increased concentration of MVD in MM patients, but not statistically according to the ISS (Tab. 2). The data showed statistically significant decreased in MVD after the treatment (p=0.02). Additionally, the analysis showed the important differences in MVD between patients in CR+PR, non active patients and patients with stable disease and progression (p=0.001) (Tab. 3). Finally we found no statistically significant correlation between MMP-2 and the parameters like MVD and the angiogenic factors (Tab. 4).

**DISCUSSION**

Angiogenesis is a vital process connected with tumor growth, progression and metastasis [19]. Numerous studies have demonstrated that angiogenesis in tumors is the result of an imbalance between positive and negative regulators of angiogenesis [1,20]. The concentrations of these angiogenic regulators in the circulation have been used to predict tumor progression, metastasis and prognosis [21–24]. Angiogenesis appears to be a prominent feature of many hematological disorders, particularly in MM. Recent studies have reported elevated serum levels of angiogenic factors like VEGF, bFGF and their receptors and increased angiogenesis, measured by MVD of the bone marrow, in patients with hematological malignancies [25-30]. In a few published study, a higher bone marrow MVD was reported in rapidly progressive MM compared to that in non-progressive or stage I disease [31]. They also suggested that angiogenesis was directly related to myeloma cells proliferation and proposed a relationship between plasma cells growth, disease grade and bone marrow angiogenesis [32]. In our study we confirmed, that the expression of angiogenic factor was significantly higher in active, untreated MM patients. Additionally patients with stage I MM had lower expression of each angiogenic factor than in the patients with stage II and III according to ISS of MM. The difference in some cases was not statistically significant, probably because of the small number of patients in each group. VEGF activity is mediated primarily through

| **Table 4. Correlation coefficient (r) between angiogenic factors and disease features in multiple myeloma patients.** |
|----------------|----------------|----------------|----------------|----------------|
| VEGF | p | bFGF | p | FGFR2 | p | MVD | p |
| HGB [g/dl] | 0.26 | NS | 0.14 | NS | 0.22 | NS | 0.16 | 0.65 |
| Serum M Protein [g/dl] | -0.33 | NS | -0.30 | NS | -0.26 | NS | -0.29 | 0.41 |
| Serum Albumin [g/dl] | -0.009 | NS | -0.15 | NS | -0.14 | NS | -0.07 | 0.82 |
| Ca2+ [mmol/l] | 0.92 | 0.0003 | 0.69 | 0.02 | 0.75 | 0.01 | 0.86 | 0.01 |
| IgG [mg/dl] | -0.32 | NS | -0.34 | NS | -0.21 | NS | -0.21 | 0.55 |
| β2m [mg/dl] | -0.28 | NS | -0.42 | NS | -0.31 | NS | -0.21 | 0.55 |
| LDH [IU/l] | 0.17 | NS | 0.12 | NS | 0.05 | NS | 0.33 | 0.34 |
| (%o) plasma cells in TB | 0.77 | 0.0003 | 0.62 | 0.03 | 0.64 | 0.02 | 0.77 | 0.03 |
| MVD | 0.93 | <0.001 | 0.79 | 0.001 | 0.88 | 0.001 | - | - |
| MMP-2 | 0.29 | NS | 0.56 | NS | 0.49 | NS | 0.67 | NS |

NS: not significant, HGB - hemoglobin, M - monoclonal, Ca - calcium, IgG - immunoglobulin G, β2m - beta-2-microglobulin, LDH - lactate dehydrogenase, TB - trephine biopsy, MVD - microvessel density, MMP-2 - matrix metalloproteinase-2, VEGF - vascular endothelial growth factor, bFGF - basic fibroblast growth factor, FGFR2 - fibroblast growth factor receptor 2.
two receptors, VEGFR-1 and VEGFR-2 [33, 34]. Similar findings have been described in the context of bFGF. Bone marrow stromal cells express FGF receptors and on stimulation by bFGF secreted by myeloma cells, they release interleukin-6 (IL-6), which in turn stimulates the plasma cells [35]. Furthermore, the IL-6 serum level was found to be significantly higher in patients with advanced disease [36]. These findings are consistent with our results that demonstrated increased angiogenic factors expression in more advanced stage of MM disease and significant correlation between concentration of VEGF and bFGF and its receptor with the percentage of plasma cells in TB.

In a group of active, untreated MM patients, we found statistically significant differences in the expression of angiogenic factors compared to the group of patients after the anti-angiogenic regimen chemotherapy. Furthermore, we found statistical differences of the expression of factors between the group of patients with a response (PR + CR) and the group of patients who were receiving steroids and all of them were treated with thalidomide. Therefore, it is possible that treatment lowered the angiogenic factor levels, thus reducing the difference between untreated and previously treated patients without response.

Recently, a variety of factors have been identified that may regulate the process of angiogenesis. One of the described factors are MMPs. MMPs are family endopeptidases with proteolytic activity for a large range of components of the extracellular matrix. These enzymes have been implicated in the physiologic turnover of extracellular matrix and in bone remodeling in wound healing, angiogenesis, bone resorption [39]. Evidence that MMPs play a functional role in pathological processes is well documented. MMP-2 apart from degradation of denatured collagen, fibronectin, and elastin, play a special role in tumor invasion and metastasis [13-15]. It has been reported that a significant increase in MMPs activity occurs in the bone marrow environment of patients with MM [10]. The expression of MMP is induced by interleukin 17 (IL-17) that is implicated in the angiogenesis of MM [40]. Serum higher levels of IL17 are significantly elevated in MM patients compared with healthy subjects and are associated with advanced stage of MM [41]. Myeloma cells constitutively secrete MMP-9 and are able to induce the activation of latent MMP-2 (pro-MMP-2) secreted by the bone marrow environment via MMP-7 (matrilysin), which is also constitutively produced by myeloma cells [10]. Endothelial cells that enter into solid tumors are known to utilize MMPs to invade, so it is likely that MMPs are involved in the infiltration of myeloma-affected marrows by neoangiogenic vessels [12, 42]. Our data confirmed the observation that MMP-2 was significantly higher in MM patients than in control group and in non-active patient, but was at the same level in a group of patient, who had the progression or did not respond to the treatment. According to the ISS, we found significant differences of secretion of MMP-2 between the stage I of the disease to other stages: II and III according to ISS of MM.

Increased concentration of IL-6 in serum is found in patients with more advanced MM and with the higher level of plasma cell [36]. Kusano et al. [43] described IL-6 as cytokine which can upregulate the production of MMP-2. Additionally, our results showed statistically significant differences between initial secretion of MMP-2 and response achieved after the treatment: CR+PR vs SD vs PD. These results could be supported by the recent paper of Terpos et al. [44], in which inhibitor of MMP-2 (tissue inhibitor of metalloproteinases-TIMP-1) correlated with poor prognosis of MM.

At the present time, data showing that activation of MMP-2 by human myeloma cells are the major interest for the management patients with MM, with the purpose being to limit bone resorption and tumor spreading. In fact, some inhibitors of MMPs are now available and two different types of pathologies have already been the target for the use of these MMP inhibitors: metastatic cancer and disease associated with articular cartilage and bone destruction. Several recent studies report the efficiency of inhibitors in animal models of arthritis leading to a dramatic suppression of cartilage and bone destruction [45]. In a cancer, MMP inhibitors have been shown to inhibit angiogenesis. Inhibition of MMPs could slow myeloma growth by reducing angiogenesis. An MMP inhibitor (Batimastat) has been shown to be effective in a variety of murine tumor metastatic models and is now used in human cancer clinical trials [46]. We could expect that this therapeutic approach can target two major deleterious effects of MM: bone destruction and tumor spreading.

CONCLUSIONS

We showed that not only decreased expression of angiogenic cytokines is present after the anti-angiogenic treatment but also secretion of MMP-2 in MM patients who responded to the treatment. Combination therapy with the inhibition of the activity of MMP could represent an interesting therapeutic approach in MM.

REFERENCES

2. Bataille R, Chappard D, Basle MF. Quantifiable excess of bone resorption in monoclonal gammopathy is an


