Angiogenic Markers in Plasma Cell Myeloma Patients Treated with Novel Agents

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Abstract. *Background: Angiogenesis plays an important role in pathogenesis and progression of plasma cell myeloma (PCM). Novel agents such as thalidomide, lenalidomide and bortezomib, have in part antiangiogenic mechanisms of action. In this study, we examined angiogenic markers in patients with PCM and correlated these markers to treatment response to novel agents. Patients and Methods: We included 93 patients newly diagnosed with PCM treated with novel agents thalidomide or lenalidomide (immunomodulatory drugs; IMiDs), bortezomib, or a combination of IMiD and bortezomib. A panel of serum angiogenic markers was assessed by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) before and in the course of the therapy. The response evaluation was performed after three cycles of therapy. The patients were divided into responders [(stringent complete remission (sCR), complete remission (CR), very good partial response (VGPR)] and non-responders [(partial response (PR) stable disease (SD), progressive disease (PD)]. Results: The CR-plus-VGPR rate was 45% in the IMiD-based group (13/29), 52% in the bortezomib-based group (16/30) and 58% in the combination group (20/34). Baseline levels of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), tumor necrosis factor-α (TNFα), and angiopoietin-2 (ANG2) correlated positively with advanced disease stage ($p<0.005$ in each case). Regarding all 93 patients, levels of VEGF, soluble VEGF receptor-2 (sVEGFR-2), basic fibroblast growth factor (bFGF), placental-derived growth factor (PGF), ANG2, HGF and neuropilin-1 (NRP1) were significantly different in responders compared to non-responders. The levels of these angiogenic factors were significantly different in the IMiD-based group and the combination group after therapy but not in the bortezomib group. Conclusion: The mode of action of IMiDs possibly leads them to have a greater antiangiogenic effect than bortezomib and thus the levels of angiogenic markers was more influenced by IMiD-based therapies in PCM. This study contributes in the understanding of the mode of action of novel agents in the treatment of PCM.*

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Plasma cell myeloma (PCM) is a disease which accounts for about 1% of all neoplasias and more than 10% of all hematological malignancies. It has a poor prognosis, with a median survival of 3-5 years despite all treatment approaches, including intensive chemotherapy followed by hematopoietic stem cell transplantation (1). The introduction of new therapeutic strategies, such as lenalidomide and bortezomib, which target malignant plasma cells to affect their interactions with the bone marrow microenvironment, has changed the management of PCM and has improved survival rates (2, 3). Bortezomib is a proteasome inhibitor known to induce apoptosis, reverse drug resistance of PCM cells, and block cytokine effects, cell adhesion, and angiogenesis in the myeloma cell microenvironment, all of which support the proliferation and migration of neoplastic plasma cells (4).

PCM was the first hematological malignancy in which an increased angiogenesis rate was detected (5). Angiogenesis, or new blood vessel formation, is fundamental to the growth and spread of tumors. New vessel formation in the bone marrow seems to play an important role in the pathogenesis of PCM (6). Increased bone marrow microvessel density in patients with PCM appears to also be an important prognostic factor (7). Malignant plasma cells can secrete various cytokines, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF), all known for their proangiogenic activity (8, 9). It was shown that PCM cells are capable of secreting VEGF in response to interleukin-6 (IL6) stimulation; in response to that VEGF stimulation, microvascular endothelial cells and bone marrow stromal cells secreted IL6, a potent growth factor for

malignant plasma cells, thus closing a paracrine loop (6). Tight control of angiogenesis is maintained by a balance of endogenous antiangiogenic and proangiogenic factors (10). VEGF plays a key rate-limiting role in promoting tumor angiogenesis and exerts its effects by binding to one of three receptor tyrosine kinases: VEGF receptor-1 (VEGFR-1; fms-like tyrosine kinase-1, FLT1), VEGFR-2 (human kinase domain region, KDR/murine fetal liver kinase-1, FLK1), and VEGFR-3 (FLT4) (11).

The aim of this study was to investigate several serum/plasma angiogenic markers VEGF, sVEGFR-2, bFGF, placental growth factor (PGF), angiopoietin-2 (ANG2), HGF, tumor necrosis factor-alpha (TNFα), IL6, and neuropilin-1 (NRP1) in patients with PCM and to correlate these markers with disease stage and response to novel agents.

Patients and Methods

Patients. The study was performed according to the regulations of the local Ethics Committee (approval number 334/10). The study population included 93 patients newly diagnosed with MM (60 men, 33 women; median age=59 years, range=30-75 years) fulfilling the International Myeloma Working Group diagnostic criteria (1). Baseline patient characteristics are shown in Table I. Patients with acute or chronic infection, inflammatory process, and liver or kidney diseases were excluded from the study. None of the patients had received any myeloma-related therapy prior to the study. For each patient, a baseline peripheral blood sample was drawn prior to therapy, and after three cycles of chemotherapy. All patients received treatment with novel drugs, using either thalidomide or lenalidomide (IMiD)-based regimens (n=29) (such as melphalan, prednisone and thalidomide; lenalidomide and dexamethasone), bortezomib-based regimens (n=30) (such as bortezomib, melphalan and prednisone; bortezomib, cyclophosphamide and dexamethasone; bortezomib and dexamethasone), or a combination of IMiD and bortezomib-based regimen (n=34) (such as bortezomib, thalidomide and dexamethasone). Response to treatment and relapse/progression events were classified according to consensus guidelines (12). The study population was divided into two groups after therapy: responders (stringent complete response, complete response, very good partial response) and non-responders (partial response, stable disease, progressive disease).

Factor analysis. Venous blood samples were collected from all patients, centrifuged at rcf 1000 × *g* for 10 min, aliquoted into separate vials and finally stored at −70˚C until assay, at the end of the study, to avoid interassay variability. Serum levels of VEGF, sVEGFR-2, bFGF, PGF, ANG2, HGF, TNFα, IL6, and NRP1 were measured with a quantitative sandwich enzyme-linked immunosorbent assay, using monoclonal antibodies against the under study molecules, from commercially available test kits according to manufacturer's instructions (Table II). For each patient, levels were measured at diagnosis and after chemotherapy, when treatment response was assessed.

Statistical analysis. Association of angiogenic markers with stage of disease was assessed by one-way analysis of variance (ANOVA).

Table I. *Baseline characteristics of patients*.

No. of patients	93
Age, median (range), years	59 (30-75)
Gender (male/female)	60/33
Immunological subtype	
IgG	54
IgA	22
IgM	2
IgD	1
Light chain	13
Nonsecretory	1
ISS	
I	10
II	50
III	33
Durie-Salmon	
I	5
II	28
III	60
Renal function: creatinine	
<2 mg/dl	83
>2 mg/dl	10
Induction regimen	
IMiD-based	29
Bortezomib-based	30
Combination (IMiD/bortezomib-based)	34

ISS: International Staging System; IMiD: immunomodulatory drug.

Alteration of angiogenic markers from pre-treatment to post-treatment were assessed among responders and non-responders using paired *t*-test or Wilcoxon test as appropriate (repeated-measure analysis of variance method was used to compare the changes in angiogenic levels pre-treatment and post-treatment in subgroups with different treatment responses). A value of $p<0.05$ was required for statistical significance.

Results

Out of the 93 patients, 29 were treated with an IMiD-based regimen, 30 with a bortezomib-based regimen and 34 patients with the combination (IMiD/bortezomib). The complete response plus very good partial response rate was 45% in the IMiD-based group (13/29), 52% in the bortezomib-based group (16/30) and 58% in the IMiD/bortezomib combination group (20/34). Baseline levels of VEGF, HGF, TNFα, and ANG2 correlated positively with advanced disease stage by the International Staging System ($p<0.005$ in each case).

We found no correlation between baseline serum levels of angiogenic factors and response to therapy. Regarding all 93 patients, levels of VEGF, bFGF, PGF, ANG2, HGF and NRP1 significantly decreased post-treatment (after three cycles of therapy) in responders, whereas in non-responders there were no significant changes (Table III). Levels of

Table II. *Details of used sandwich enzyme-linked immunosorbent assay (ELISA) kits.*

Antigen	Kit	Kit number	Intra-assay CV%	Mean MDD pg/ml
VEGF	Human VEGF Quantikine ELISA Kit	DVE00	5.1%	9
sVEGFR-2	Human VEGF R2/KDR Quantikine ELISA Kit	DVR200	3.3%	11.4
bFGF	Human FGF basic Quantikine ELISA Kit	DFB50	4.5%	3
PGF	Human PlGF Quantikine ELISA Kit	DPG00	4.1%	7
ANG2	Human Angiopoietin-2 Quantikine ELISA Kit	DANG20	6.5%	21.3
HGF	Human HGF Quantikine ELISA Kit	DHG00	5.3%	40
IL6	Human IL-6 Quantikine ELISA Kit	D6050	2.1%	0.7
TNFα	Human TNF-alpha Quantikine ELISA Kit	DTA00C	4.3%	5.5
NRP1	Human Neuropilin-1 Quantikine ELISA Kit	DNRP10	6.9%	9.33

All from R&D systems, Minneapolis, USA. ANG2: angiopoietin-2; bFGF: basic fibroblast growth factor; HGF: hepatocyte growth factor; IL6: interleukin-6; NRP1: neuropilin-1; PGF: placental-derived growth factor; TNFα: tumor necrosis factor-α; sVEGFR: soluble vascular endothelial growth factor receptor; VEGF: vascular endothelial growth factor. CV: Coefficient of variation; MDD: minimum detectable dose.

Table III. *Levels of angiogenic markers in serum from 93 patients with plasma cell myeloma, before and after three cycles of therapy with novel agents. Patient groups were divided into responders and non-responders.*

	Responders			Non-responders		
	Baseline	After 3 cycles	*p*-Value	Baseline	After 3 cycles	*p*-Value
VEGF (pg/ml)	423±123	270±75	<0.005	399±155	449±123	0.15
sVEGFR-2 (pg/ml)	2153±723	4523±871	<0.005	1923±788	2470±688	0.73
bFGF (pg/ml)	8±4	4±2	0.04	9±3	11±5	0.67
PGF (pg/ml)	423±77	301±44	<0.005	510±155	600±99	0.45
ANG2 (pg/ml)	553±158	289±99	<0.005	622±202	570±175	0.34
HGF (pg/ml)	1260±245	723±201	<0.005	1011±155	950±102	0.52
IL6 (pg/ml)	79±17	50±13	<0.005	66±14	33±12	0.03
TNFα (pg/ml)	44±12	37±9	0.07	33±14	40±12	0.08
NRP1 (ng/ml)	320±79	622±99	<0.005	379±82	423±99	0.81

Responders: Stringent complete remission, complete remission, very good partial response; non-responders: partial response, stable disease, progressive disease. ANG2: angiopoietin-2; bFGF: basic fibroblast growth factor; HGF: hepatocyte growth factor; IL6: Interleukin-6; NRP1: neuropilin-1; PGF: placental-derived growth factor; TNFα: tumor necrosis factor-α; sVEGFR: soluble vascular endothelial growth factor receptor; VEGF: vascular endothelial growth factor.

sVEGFR-2 and NRP1 significantly increased with therapy in responders compared to non-responders. IL6 levels significantly decreased in both responders and non-responders under therapy. We found no significant changes in TNFα levels after therapy.

Regarding changes in levels pre-treatment *versus* post-treatment in the different treatment groups, the changes in levels of angiogenic factors were significantly greater in responders compared to non-responders in the IMiD-based group and the combination group (Table IV) after therapy but not in the bortezomib-based group. Regarding IL6 levels changes pre-treatment *versus* post-treatment, there was a significantly greater decrease in responders compared to non-responders in all treatment groups. There was no significant changes regarding levels of TNFα.

Discussion

Tumor angiogenesis plays a key role in the pathogenesis and progression of PCM (6). Thereby, pro- and antiangiogenic growth factors and cytokines regulate the angiogenic process. Bone marrow angiogenesis, as measured by microvessel density, has been shown to be markedly elevated in myeloma compared to its premalignant state, monoclonal gammopathy of unknown significance (7). Approved agents with antiangiogenic mechanisms of action for the treatment of PCM are thalidomide, lenalidomide, and bortezomib. Earlier studies have shown that thalidomide had antiangiogenic activity in a rabbit model of corneal neovascularization that was induced as a response to bFGF (13). Thalidomide and the newer IMiDs have also been shown to significantly

Table IV. *Changes of baseline to post-treatment serum levels of angiogenic markers in responders and non-responders under novel therapies.*

	IMiD		Bortezomib		Combination	
	Responders	Non-responders	Responders	Non-responders	Responders	Non-responders
VEGF (pg/ml)	–117±72	13±25	–56±42	5.7±15	–132±56	–0.8±23
p-Value	<0.005		0.12		<0.005	
sVEGFR-2 (pg/ml)	2370±972	1020±553	1072±422	782±182	3520±1899	1175±589
p-Value	<0.005		0.27		<0.017	
bFGF (pg/ml)	–5±4	–2±1	3±2	5±3	–12±8	–6±4
p-Value	<0.005		0.08		<0.005	
PGF (pg/ml)	–100±57	24±44	77±66	25±22	–253±137	–79±56
p-Value	<0.005		0.95		<0.005	
ANG2 (pg/ml)	–270±144	–112±77	–35±17	55±39	–334±188	–157±88
p-Value	<0.014		0.19		<0.005	
HGF (pg/ml)	–353±177	– 117±55	122± 66	99±56	–123±85	–49±33
p-Value	<0.014		0.31		<0.005	
IL6 (pg/ml)	–20±17	–11±9	–99±35	–45±33	–133±76	–80±47
p-Value	<0.005		0.07		<0.005	
TNFα (pg/ml)	–10±9	–17±12	22±19	40±25	–17±14	–23±19
p-Value	0.08		0.52		0.07	
NRP1 (ng/ml)	320±156	161±77	51±37	102±81	440±278	202±125
p-Value	<0.005		0.83		<0.005	

Responders: Stringent complete remission, complete remission, very good partial response; non-responders: partial response, stable disease, progressive disease. IMiD: Immunomodulatory drug; combination: IMiD and bortezomib-based regimen. ANG2: angiopoietin-2; bFGF: basic fibroblast growth factor; HGF: hepatocyte growth factor; IL6: interleukin-6; NRP1: neuropilin-1; PGF: placental-derived growth factor; TNFα: tumor necrosis factor-α; sVEGFR: soluble vascular endothelial growth factor receptor; VEGF: vascular endothelial growth factor.

reduce the expression of angiogenic factors VEGF and IL6 in PCM, thereby reducing angiogenesis and hence contributing to clinical activity in PCM (14, 15). Lenalidomide, an analog of thalidomide, has shown greater efficacy than thalidomide in myeloma, with reduction in angiogenesis through inhibition of VEGF secretion being one of its mechanisms of action (16). In a recent study by Maffei *et al*., the antiangiogenic effect of lenalidomide was examined *in vitro* and *in vivo* in patients with chronic lymphocytic leukemia (CLL) (17). Lenalidomide influenced the cross talk between CLL cells and endothelial cells and reduced plasma levels of VEGF in patients with CLL. Patients who responded to lenalidomide showed a more pronounced decrease of VEGF and bFGF than did patients with stable or progressive disease (17). The proteosomal inhibitor bortezomib is well-established in the treatment of myeloma and in addition to its proteosomal inhibitory effects, it has been shown to have significant inhibitory effects on endothelial cell proliferation and migration, as well as in the down-regulation of VEGF and angiopoietin expression by endothelial cells (18, 19).

In our study, we found changes in angiogenic levels under therapy with novel agents, especially IMiD-based regimens, in patients with PCM. Thereby, we found a difference in responders compared to non-responders for the majority of the markers examined. The available data on angiogenic markers under therapy with novel agents in PCM are controversial. Our findings are in line with other studies which also found changes in angiogenic markers under therapy. In a study by Pour *et al*., serum levels of VEGF and HGF significantly decreased in responders compared to non-responders (20). In a study by Pappa *et al*., there was also a significant decrease of serum VEGF levels post-treatment (21). In contrast, a study by Cibeira *et al*. found no significant differences in angiogenic markers in responders compared to non-responders (22). The reason for these controversial results could be different patient cohorts. We only included patients with newly-diagnosed PCM before therapy and with normal renal function.

Biomarkers are molecular, cellular or functional parameters that are indicative of a particular genetic, epigenetic or functional status of a biological system (23, 24). Not all patients with PCM benefit from such therapies with an antiangiogenic mode of action, and some who benefit initially might develop treatment failure, as well as showing some adverse effects. Thus, the development of biomarkers for antiangiogenic therapies is urgently needed to select those patients most likely to benefit, to prevent unnecessary toxicity in patients with resistant disease and to avoid high therapy costs (25). Non-responsiveness and failure to antiangiogenic treatment can be the result of intrinsic tumor resistance or acquired resistance. Different

mechanisms can explain such resistance, including redundant angiogenic factors, with up-regulation of alternative angiogenic signals, induction of hypoxia, selection of more aggressive tumor cells, recruitment of bone marrow-derived pro-angiogenic cells and inflammatory cell invasion, modification of vascular pericyte coverage and vessel co-option (25). The evaluation of angiogenic parameters in serum/plasma samples with standard immunogenic assays is an attractive method for monitoring antiangiogenic therapies, not only because of its feasibility and its low costs. To date, the relevance of soluble biomarkers in the blood has not been fully investigated due to the fact that most candidate biomarkers were evaluated retrospectively and prospective validation is lacking.

In conclusion, the changes in several angiogenic markers in patients with response to novel agents indicate that the rate of angiogenesis is possibly reduced after successful treatment for PCM. In the groups treated with IMiD-containing therapy, we found significant changes of angiogenic markers in responders compared to non-responders, whereas in the bortezomib-based group the difference in angiogenic markers was not significant. The mode of action of IMiDs may have a greater antiangiogenic effect than bortezomib and thus the levels of angiogenic markers was more influenced by IMiD-based therapies in PCM. Baseline levels of angiogenic markers were not predictive of response to novel agents.

Conflicts of Interest

No Author has any conflict of interest to report.

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