Normalization of the serum angiopoietin-1 to angiopoietin-2 ratio reflects response in refractory/resistant multiple myeloma patients treated with bortezomib

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ABSTRACT

Neoangiogenesis is involved in the pathophysiology of multiple myeloma and angiopoietins possibly contribute to myeloma-induced neovascularization. Bortezomib's antineoplastic potential includes an anti-angiogenic effect. We determined serum levels of angiopoietin-1 and angiopoietin-2 with ELISA pre- and post-bortezomib administration in 35 patients with relapsed/refractory multiple myeloma. Pre-bortezomib, serum angiopoietin-1 levels did not differ in patients and in healthy individuals, while serum angiopoietin-2 levels were elevated. Corresponding serum angiopoietin-1/angiopoietin-2 ratio was reduced in patients compared with controls. After treatment, serum angiopoietin-1 levels increased, while serum angiopoietin-2 levels decreased, therefore the angiopoietin-1/angiopoietin-2 ratio increased and normalized. This increase was significant in patients who responded to treatment. In conclusion, angiopoietin-1/angiopoietin-2 ratio normalization reflected response to bortezomib.

Key words: serum angiopoietins, serum Ang-1/Ang-2 ratio, myeloma, bortezomib.

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Introduction

Bone marrow (BM) neo-vascularization contributes to the biology of several hematologic malignancies.[1,2] The mechanisms of MM-induced neo-angiogenesis includes direct production of angiogenic cytokines by plasma cells, and the induction of their secretion by microenvironmental cells.[3,4] Cytokines such as basic fibroblast growth factor and vascular endothelial growth factor were shown to play a leading role in new vessel formation. However, other known and unknown factors are also involved and are under investigation. Angiopoietins and their receptor Tie2 are pro-angiogenic mediators that are important in developmental and postnatal vasculature homeostasis, as well as in tumor angiogenesis.[5-7] They do not seem to participate in the initial phase of vascular development, but rather play a critical role in angiogenic outgrowth, vessel remodeling, and maturation. Growth and stabilization of the vascular wall are regulated by angiopoietin-1 (ang-1) binding to Tie2 receptor. By contrast, angiopoietin-2 (ang-2) antagonizes Tie2 binding and induces vessel destabilization, which leads to the angiogenic sprouting.[8,9] The angiopoietin system was shown to be involved in the mechanisms of MM-induced angiogenesis, and angiopoietins are possibly expressed and secreted by myeloma cells although reports are contradictory.[6,7]

Bortezomib is a proteasome inhibitor producing high response rates in relapsed/resistant MM patients.[10] It mainly inactivates some key proteins implicated in cell growth and

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function, thus modulating cell cycle regulation, cytokine and growth factor secretion, immunoreceptors and adhesion molecules, apoptosis, and possibly angiogenesis. The effect of bortezomib on circulating angiopoietins levels has not yet been investigated.

This study determined the serum values of ang-1 and ang-2 in relapsed/refractory MM patients receiving treatment with bortezomib and explored possible correlations of their levels with clinical characteristics and response to treatment.

Design and Methods

Patients

In a series of 75 consecutive relapsed/refractory MM patients, uniformly treated with bortezomib according to indications,[10] 35 had available serum samples pre-bortezomib and after 4 cycles. Dexamethasone was added to 15 patients who did not respond optimally after 2 cycles of therapy. Objective response (OR) was defined as any response better or equal to partial remission according to the EBMT criteria. Pre-bortezomib patient baseline characteristics are summarized in Table 1. Median time from diagnosis to bortezomib-treatment was 30 months (range 4.7-143). The OR rate after 4 bortezomib cycles in the 35 patients evaluated (32 relapsed and 3 refractory) was 71% (CR 8.5%, PR 62.5%). Thirty-four sex and age matched healthy individuals (HI) formed the control group.

Methods

Serum ang-1 and ang-2 were determined in frozen sera collected at baseline and then on day 21 of the 4th bortezomib cycle. Measurements were made with a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, USA) according to the manufacturers' instructions. The use of sera samples for cytokine determination in patients receiving bortezomib was approved by the local Ethical Committee.

Statistical analysis

Differences between patients' serum ang-1 and ang-2 levels and their ratio versus HI were assessed by the Student's t-test (paired or unpaired, as appropriate). Differences between groups of patients according to their baseline characteristics [gender, Durie-Salmon (DS)] and ISS stage both coded as 3 *vs.* 1 and 2, creatinine ≥2 mg/dL, elevated LDH, C-reactive protein levels ≥4 mg/dL, and previous treatment lines ≥2) were assessed using the Mann-Whitney test. Correlations between serum cytokine levels and quantitative patients' characteristics (age, percentage of BM infiltration, and platelet counts) were assessed using the Spearman's ρ co-efficient. *p*-values <0.05 were considered to be of statistical significance.

Results and Discussion

Serum angiopoietins levels pre-bortezomib administration

The mean, median and range of ang-1 and ang-2 levels (pg/mL), as well as their ratio in HI and patients pre- and post-bortezomib administration, are summarized in Table 2. There was no statistically significant difference in baseline serum ang-1 levels between patients and HI (mean difference (MD) 4146; 95% confidence intervals (CI) -1497 to 9789; p=0.15). Serum ang-1 levels were lower in patients with DS stage 3 *vs.* 1 and 2 (p=0.01) and in patients with elevated LDH (p=0.03). Furthermore, ang-1 levels negatively correlated with the percentage of BM infiltration (Srho=-0.363; p=0.04) but positively with platelet counts (Srho=0.55; p=0.001). Baseline ang-2 levels were significantly higher in patients compared with HI (MD 2690; 95% CI 1431 to

Table 1. Patient's baseline characteristics prior to bortezomib administration (baseline).

		N.	*%*
Male gender		24	69
Age, median [range]		68 [43-82]	
Paraprotein type	IgG	24	69
	IgA	8	23
	BJ	3	8
Durie and Salmon Stage	I	3	8
	II	20	57
	III	12	35
ISS stage	1	10	29
	2	7	20
	3	18	51
Treatment lines	≥2	17	49
Platelet counts	<100×10^9/L	2	6
Creatinine	≥2 mg/dL	4	12
CRP	≥4 mg/L	11	33
LDH elevated		5	14
Bone marrow infiltration (%), median [range]		70 [10-90]	

Table 2. Serum values of ang-1 and ang-2 (pg/mL) and their ratio in HI and in patients pre- and post-bortezomib administration.

	Mean	*Median*	*Range*
Ang-1 HI	20785	20833	120-45940
Ang-1 pre-B	24930	24954	2518-56176
Ang-1 post-B	30327	30644	2083-59754
Ang-2 HI	1361	1202	189-3313
Ang-2 pre-B	4051	3189	452-21163
Ang-2 post-B	2657	1895	332-10742
Ang-1/Ang-2 HI	26.3	16.8	0.06-175.5
Ang-1/Ang-2 pre-B	12.0	6.8	0.4-61.4
Ang-1/Ang-2 post-B	21.2	12.4	0.2-113.9

3949; $p<0.001$). Ang-2 levels were higher in patients with ISS 3 or DS stage 3 compared with ISS 1 and 2 ($p=0.045$) or DS 1 and 2 ($p=0.049$). Furthermore, ang-2 levels positively correlated with the percentage of BM infiltration (Srho=0.336; $p=0.05$) but negatively with platelet counts (Srho=-0.474; $p=0.004$). Baseline ang-1/ang-2 ratio was reduced in patients compared with HI (MD -14.26; 95% CI -26.20 to -2.32; $p=0.02$). Ang-1/ang-2 ratio was lower in patients with ISS 3 or DS stage 3 *vs.* ISS 1 and 2 ($p=0.04$) or DS 1 and 2 ($p=0.003$), as well as in those with elevated LDH ($p=0.02$). In addition, the ang-1/ang-2 ratio correlated negatively with the percentage of BM infiltration ($S\rho=-0.437$; $p=0.01$) but positively with platelet counts ($S\rho=0.641$; $p<0.001$).

Serum angiopoietins' fluctuations post-bortezomib treatment

Bortezomib administration resulted in significantly increased serum ang-1 levels compared with HI (MD 9542; 95% CI 3624 to 15461; $p=0.002$). The increase in ang-1 values post bortezomib was significant compared with baseline (MD 5397; 95% CI 1160 to 9633; $p=0.014$; Figure 1). Serum ang-2 levels decreased but were, however still higher than HI (MD 1295; 95% CI 524 to 2066; $p=0.002$). The decrease in ang-2 values post-bortezomib was significant compared with baseline (MD -1394; 95% CI -2549 to -240; $p=0.019$; Figure 1). Patients who achieved an OR showed a greater increase in ang-1 and reduction in ang-2 compared with those who did not (*data not shown*).

The ratio of ang-1/ang-2 increased post-bortezomib administration compared with baseline (MD 9.18; 95% CI 1.23 to 17.14; $p=0.025$) and normalized, no longer differing from HI (MD -5.08; 95% CI -18.80 to 8.64; $p=0.46$; Figure 1). Significant correlations were no longer found between serum ang-1, ang-2 or their ratio with platelet counts. There are few reports on the serum levels of ang-1 and ang-2 in MM or other plasma cell dyscrasias. Circulating soluble angiopoietin-2 was recently found to be increased in MM patients,[11,12] while another study reported the serum angiopoietin-1/angiopoietin-2 ratio to be reduced in Waldenström's macroglobulinemia patients and related to disease activity.[13]

In the present study, soluble serum ang-2 levels were high in relapsed/refractory MM patients compared with HI but serum ang-1 was not. Serum ang-2 values correlated with advanced DS and ISS staging, in agreement with a previous study.[11] Given that ang-1 and ang-2 are competitive ligands of Tie2 receptor, we evaluated the serum ang-1/ang-2 ratio and found that it was significantly reduced compared with HI. This suggests that the angiopoietin balance is disrupted in favor of ang-2 in relapsed/refractory MM and thus the ang-1/ang-2 ratio is decreased, possibly leading to vessel destabilization and to angiogenic sprouting. Our results may be explained by the observation that ang-1 gene expression was found significantly up-regulated in MGUS but not in overt MM endothelial cells.[14] The reversal of the normal angiopoietin balance in favour of ang-2 has been observed in other active hematologic malignancies. It has recently been reported that ang-2 expression was significantly higher in the bone marrow of patients with AML while on the contrary, ang-1 expression did not differ from HI.[15] Increased serum ang-2 and reduced ang-1/ang-2 ratio was also reported in patients with untreated or relapsed Waldenström's macroglobulinemia, while in patients with IgM MGUS the ang-1/ang-2 ratio was comparable to HI.[13] In addition, high expression of ang-2 in malignant tissue and low or undetectable expression of ang-1 have been correlated with poor survival, high metastasis rate, and increased MVD in solid tumors.[16,17] These and our studies implicate the

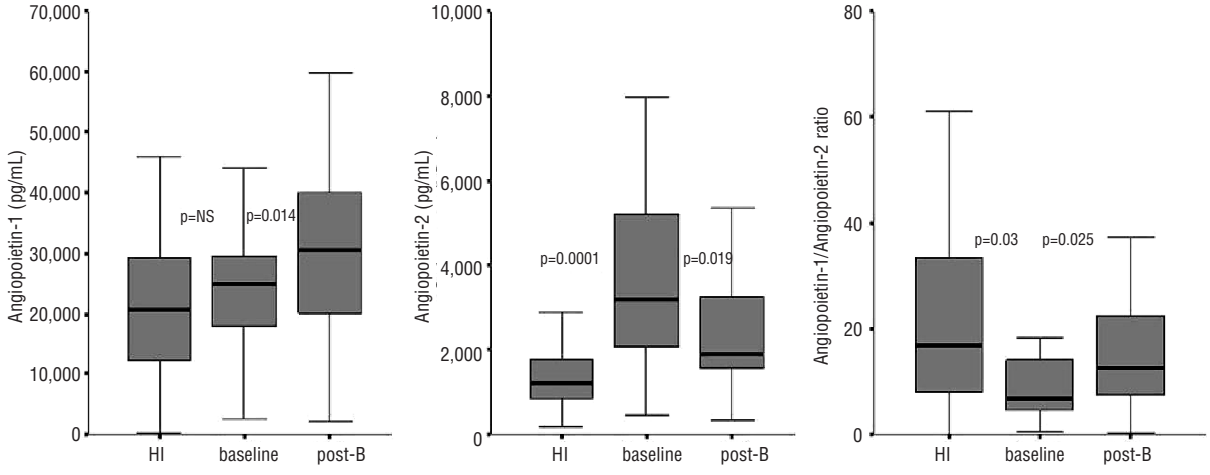

Figure 1. Serum Ang-1 and Ang-2 levels (pg/mL) and Ang-1/Ang-2 ratio pre-, post-bortezomib and in HI. The bold horizontal line represents the median value, rectangles represent the 25th and 75th percentile, while the thin vertical lines represent the 10th and 90th percentile of patients.

Tie2-angiopoietin system as a potent target for anti-angiogenic cancer therapy. In fact, there are already data in mice showing that blocking ang-2 activity by antibodies can lead to tumor growth control and a reduction in endothelial cell proliferation.[18]

We also found a significant correlation of baseline serum ang-1 levels with patient platelet counts. This may be partly due to ang-1 release from platelets upon serum sample clotting, given its consistent presence in platelets.[19] Platelet count restoration post-bortezomib may also account to some extent for the increase in ang-1 serum levels. However, the absence of a significant correlation between ang-1 levels and platelet count post-bortezomib suggests that a possible biological effect of bortezomib on the angiopoietin system further contributes to this phenomenon.

Bortezomib has been shown to induce a dose-dependent down-regulation of the expression of VEGF, IL-6, and ang-2 genes, in *ex vivo* experiments with endothelial cells from myeloma patients; the expression of ang-1 was reduced only when the highest dose of bortezomib was added.[20] In our study, bortezomib administration resulted in the normalization of the serum ang-1/ang-2 ratio. This suggests that part of bortezomibs' anti-myeloma effect may be accomplished via anti-angiogenetic action through the Tie2-angiopoietin system. However, it is unknown whether the ang-1/ang-2 normalization is related to response to bortezomib treatment or to a response to any active treatment modality.

In conclusion, patients with relapsed/refractory MM presented a low ang-1/ang-2 ratio, while response to bortezomib administration was accompanied by normalization of this ratio.

Authorship and Disclosures

KA, ET, TPV and M-C K contributed to the design, conduction and analysis of the study and wrote the paper; KA, ET, GAP, M-C K, SS, DC, MKA, EMD, TPV, CK, KT, AP, PP, and MAD performed the research, managed patients and collected data; M-C K and GAP supervised the study and reviewed the manuscript.

The authors reported no potential conflicts of interest.

References

1. Rajkumar SV, Leong T, Roche PC, Fonseca R, Dispenzieri A, Lacy MQ, et al. Prognostic value of bone marrow angiogenesis in multiple myeloma. Clin Cancer Res 2000;6:3111-6.
2. Sezer O, Niemöller K, Eucker J, Jakob C, Kaufmann O, Zavrski I, et al. Bone marrow microvessel density is a prognostic factor for survival in patients with multiple myeloma. Ann Hematol 2000;79:574-77.
3. Vacca A, Ribatti D, Roccaro AM, Frigeri A, Dammacco F. Bone marrow angiogenesis in patients with active multiple myeloma. Semin Oncol 28;543-50.
4. Molina JR, Rajkumar SV. Bone marrow angiogenesis in multiple myeloma: closing in on the loop. Haematologica 2003;88:122-24.
5. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, et al. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 1999;284:1994-8.
6. Uneda S, Matsuno F, Sonoki T, Tniguchi I, Kawano F, Hata H. Expression of vascular endothelial factor and angiopoietin-2 in myeloma cells. Haematologica 2003;88: 113-5.
7. Giulani N, Colla S, Lazzaretti M, Sala R, Roti G, Mancini C, et al. Proangiogenic properties of human myeloma cells: production of angiopoietin-1 and its potential relationship to myeloma-induced angiogenesis. Blood 2003;102:638-45.
8. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, et al. Isolation of angiopoietin-1, a ligant for the TIE2 receptor, by secretion-trap expression cloning. Cell 1996;87:1161-9.
9. Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG. The Tie-2 ligant angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. J of Cell Science 2005;118:771-80.
10. Pangalis GA, Kyrtsonis MC, Vassilakopoulos TP, Dimopoulou MN, Siakantaris MP, Emmanouilides C, et al. Immunotherapeutic and immunoregulatory drugs in hematologic malignancies. Curr Top Med Chem 2006;6:1657-86.
11. Quartarone E, Alonci A, Allegra A, Bellomo G, Calabro L, D' Angelo A, et al. Differential levels of soluble angiopoietin-2 and Tie-2 in patients with haematological malignancies. Eur J Haematol 2006;77:480-5.
12. Alexandrakis MG, Passam FH, Sfiridaki A, Kyriakou DS, Petreli E, Roussou P. Elevated serum angiogenin in multiple myeloma. Hematol J 2003;4:454-5.
13. Anagnostopoulos A, Eleftherakis-Papaiakovou V, Zervas K, Kastritis E, Tsionos K, Bamias A, et al. Serum concentrations of angiogenic cytokines in Waldenstrom's macroglobulinaemia: the ratio of angiopoietin-1 to angiopoietin-2 and angiogenin correlate with disease severity. Br J Haematol 2007; 23:560-8.
14. Vacca A, Scavelli C, Montefusco V, Di Pietro G, Neri A, Mattioli M, et al. Thalidomide downregulates angiogenic genes in bone marrow endothelial cells of patients with active multiple myeloma. J Clin Oncol 2005;23:5334-46.
15. Schliemann C, Bieker R, Padro T, Kessler T, Hintelmann H, Buchner T, et al. Expression of angiopoietins and their receptor Tie2 in the bone marrow of patients with acute myeloid leukemia. Haematologica 2006;91:1203-11.
16. Sfiligoi C, de Luca A, Cascone I, Sorbello V, Fuso L, Ponzone R, et al. Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. Int J Cancer 2003;103:466-74.
17. Etoh T, Inoue H, Tanaka S, Barnard GF, Kitano S, Mori M. Angiopoietin-2 is related to tumour angiogenesis in gastric carcinoma: possible in vivo regulation via induction of proteases. Cancer Res 2001;61:2145-53.
18. Oliner J, Min H, Leal J, Yu D, Rao S, You E, et al. Suppression of angiogenesis and tumour growth by selective inhibition of angiopoietin-2. Cancer Cell 2004;6:507-16.
19. Li JJ, Huang YQ, Basch R, Karpatkin S. Thrombin induces the release of angiopoietin-1 from platelets. Thromb Haemost 2001;85:204-6.
20. Roccaro AM, Hideshima T, Raje N, Kumar S, Ishitsuka K, Yasui H, et al. Bortezomib mediates antiangiogenesis in multiple myeloma via direct and indirect effects on endothelial cells. Cancer Res 2006;66:184-91.