Patients with multiple myeloma treated with thalidomide: evaluation of clinical parameters, cytokines, angiogenic markers, mast cells and marrow CD57+ cytotoxic T cells as predictors of outcome

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ABSTRACT

Background and Objectives

In vitro studies suggest that thalidomide has an immunoregulatory role and alters the marrow microenvironment. We assessed laboratory and clinical parameters in patients with myeloma treated with thalidomide as potential prognostic markers and looked for changes with therapy.

Design and Methods

Seventy-five patients with relapsed/refractory myeloma received thalidomide in a phase II trial. Serial samples of platelet-poor plasma and bone marrow were tested for angiogenic cytokines including vascular endothelial growth factor (VEGF), marrow microvessel-density (MVD), mast cells and CD57+ cell expression. The effects of these parameters on response rate (RR), progression-free survival (PFS) and overall survival (OS) were analyzed.

Results

Elevated baseline VEGF predicted for a superior RR (p=0.018) and PFS. Elevated CD57+ cells also predicted superior PFS (p=0.012). MVD did not predict for RR, PFS or OS, but MVD and VEGF fell significantly in responders. Multivariate analysis identified that inferior OS was associated with age >65 years (p=0.017), raised lactate dehydrogenase (p=0.001), raised hepatocyte growth factor levels (p=0.012) and low pre-treatment CD57+ cells (p<0.001).

Interpretation and Conclusions

Our findings support the suggestion that thalidomide has anti-angiogenic and immunomodulatory effects in myeloma. The preferred method for assessing angiogenesis is plasma VEGF levels and the assessment of CD57+ cells for patients with myeloma receiving novel immunomodulatory drugs should be further investigated.

Key words: myeloma, thalidomide, angiogenesis, CD57, prognosis, VEGF.

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In vitro studies indicate that thalidomide alters the marrow microenvironment and also has an immunoregulatory role. This drug was initially used to treat multiple myeloma because of a possible anti-angiogenic effect.[1] However, the mechanism of action of thalidomide in treating myeloma appears to be multifactorial and pre-clinical studies demonstrate immunomodulatory effects; modulation of cytokine levels including tissue necrosis factor-α and interleukin-6 (IL-6) within the bone marrow microenvironment, decrease in the stability of cyclo-oxygenase-2 mRNA; direct induction of apoptosis of malignant plasma cells; modulation of the expression of cell surface adhesion molecules; and indeed an anti-angiogenic effect through reduction in the levels of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).[2,3] Nevertheless, it remains unclear which of these effects are most important in terms of predicting responses and affecting progression-free and overall survival.

The immune deficit seen in myeloma patients is complex. In addition to the impaired humoral response associated with low serum levels of polyclonal immunoglobulins, T-cell and innate immunity are also affected[4] and immunosuppressive regulatory T cells are increased.[5] It has been described that the levels of $CD4^+$cells decrease with disease progression[6] while higher initial levels of $CD4^+$ cells and $CD19^+$ cells have been reported to correlate with improved overall survival in patients treated with cytotoxic agents.[7] Patients with myeloma have also been reported to have increased numbers and state of activation of natural killer (NK) cells, which may play a role in controlling myeloma progression.[8] Invariate NKT cells may also influence plasma cell growth, and there is a recent report of progression from non-progressive to progressive myeloma being associated with a marked deficiency of ligand-dependent interferon-γ production by NKT cells.[9] Recently, a number of investigators have recognized the role of $CD8^+$, $CD57^+$ cytotoxic T cells in the immunomodulation of myeloma.[4,8,10] Bone marrow angiogenesis has also been shown to be a prognostic factor in multiple myeloma and pre-clinical work suggests that individual cytokines are important in the biology of myeloma, including effects on angiogenesis.[11] VEGF can stimulate angiogenesis and growth of myeloma in both paracrine and autocrine fashions.[12] IL-6 is known to interact reciprocally with VEGF in a paracrine loop, with IL-6 production stimulating increased VEGF secretion from myeloma and stromal cells.[13] bFGF is a member of the fibroblast growth factor family produced by diverse cell types and is also thought to play a role in initiating or sustaining angiogenesis.[14] Hepatocyte growth factor (HGF) is a cytokine with strong proliferative and anti-apoptotic effects which interacts with malignant plasma cells via its tyrosine kinase receptor, Met.[15] Levels of both HGF and IL-6 have been reported to correlate with more advanced stage and aggressive myeloma, as well as affecting bone marrow angiogenesis by various autocrine and paracrine effects.[15,16] The aim of this study was a prospective assessment of various laboratory correlates involved in angiogenesis and other aspects of myeloma biology as predictors of response and long-term outcome in patients with relapsed or refractory disease treated with thalidomide. We tested bone marrow and plasma specimens from patients being treated with thalidomide in a previously reported prospective clinical trial.[17] Bone marrow biopsies were immunohistochemically stained for CD57 as a measure of cytotoxic T-cell and/or NK-cell numbers. As potential surrogate markers of an anti-angiogenic effect of thalidomide we measured microvessel-density (MVD) within the bone marrow, and measured plasma levels of VEGF, IL-6, HGF and bFGF. Bone marrow mast cells were quantified, as mast cell numbers had previously been reported to correlate with MVD through release of angiogenic secretory granules.[18] We then systematically assessed the laboratory parameters along with standard clinical parameters in order to examine their effects on response rate, progression-free survival (PFS) and overall survival (OS) and to determine how they changed during thalidomide treatment.

Design and Methods

Seventy-five patients with relapsed or refractory myeloma were enrolled in a previously published prospective multi-center phase II trial.[17] In brief, patients commenced treatment with thalidomide (Thalomid™, Celgene Corp, NJ, USA) at a dose of 200 mg per day orally, with planned dose escalation up to a total dose of 800 mg/day or an individual maximum tolerated dose (iMTD). Thalidomide was continued until progressive disease or patient intolerance. Combination therapy with corticosteroids was not permitted. The Ethics Committee of each participating institution gave approval for the study.

Platelet-poor plasma plus a bone marrow aspirate and biopsy were collected prior to trial entry and then 3-monthly during thalidomide treatment. Portions of the bone marrow and plasma were cryopreserved after collection from the patient. Enzyme-linked immunosorbent assays (ELISA) were performed on the plasma for VEGF, bFGF, IL-6 and HGF according to the instructions of the manufacturer of the ELISA kits (R+D systems, Minneapolis, USA). Each bone marrow biopsy was cut at 3 microns and mounted onto Superfrost Plus slides for immunohistochemical investigations of CD34, CD57, von Willebrand's factor (VWF) and mast cell tryptase (MCT). Sections were dried overnight at 37°C then baked at 56°C in a dry heat fan oven for 30 minutes. Each bone marrow biopsy was stained with antibodies to CD34 (clone QBEND 10, Immunotech, Marseille, France), VWF (clone polyclonal, Dako, Glostrup, Denmark), MCT (clone AA1, Dako) and CD57 (clone NK-1, Novocastra Laboratories, Newcastle on Tyne, UK).

Sections were dewaxed in xylene and rehydrated through a graded ethanol series. Antigen retrieval was per-

formed if required. All steps after antigen retrieval were performed on a Dako Autostainer and all incubations were at room temperature. Endogenous peroxidase was blocked in 3% hydrogen peroxide for 10 minutes and then the primary antibody applied for 30 minutes. Sites of antibody-antigen binding were labeled by incubating slides with a biotinylated secondary antibody for 10 minutes followed by streptavidin for 10 minutes (LSAB 2 kit, K0675 Dako). Sites of antibody binding were then visualized by immersing slides in chromogen (AEC+, K 3469, Dako) for 10 minutes. Slides were removed from the Autostainer and counterstained in Harris's hematoxylin. Slides were then aqueous mounted and cover-slipped. Positive controls were included with every run to ensure reproducible staining.

MVD was assessed in the marrow biopsy, using previously described methods, as the average number of vessels which stained with CD34 or VWF, counted in three high-powered fields (×400) chosen as *hot spots* for microvessels.[11] Hot spots were identified within areas of cellular marrow which were infiltrated by malignant plasma cells, with vessels within immediate paratrabecular regions excluded. The microvessels were counted by two independent observers, who were blinded to the clinical data set, at ×400, using a ×10 ocular and ×40 objective lens. Numbers of CD57+ and MCT-positive cells were also counted as the average number of positive-staining cells in three high-powered fields infiltrated by malignant plasma cells.

Flow cytometry was performed on the cryopreserved marrow aspirates from patients with a high number of CD57+ cells in their marrow biopsy in order to define the nature of the CD57+ population (n=16). Four-color flow cytometry was performed using a Becton Dickinson FACScalibur (Becton Dickinson, San José, CA, USA). T-cell subsets and NK cells were identified among the thawed cells using various monoclonal antibody combinations. Acquisition and analysis gating was based on CD45 versus side scatter. The antibodies used included CD45 (Becton Dickinson Clone 2D1 –APC conjugated), CD3 (Becton Dickinson Clone SK7 –FITC and PerCP conjugated), CD16, 56 (Becton Dickinson Clones B73.1 and MY31 –PE conjugated), CD57 (Becton Dickinson Clone KNK-1 –FITC and PE conjugated), CD8 (Becton Dickinson Clone SK1 – PE conjugated), TCR Vα24 (Immunotech Clone C15 – FITC conjugated, Marseille, France), and TCRVβ11 (Immunotech Clone C21 – PE conjugated). Appropriate isotype controls (IgG1 FITC and IgG1 PE) were run for each patient and 7AAD incorporated into the panel to confirm sample viability. The sensitivity of the fluorescent and light scatter detectors was monitored using Becton Dickinson Calibrite beads according to manufacturer's recommendations. Data analysis was performed using Cytomics™ RXP v1 analysis software.

Statistical methods

Descriptive statistics were used to summarize baseline values for the eight laboratory variables. The Kaplan-Meier product limit method was used to estimate PFS and OS. Assessment of baseline laboratory variables with respect to OS and PFS was made using the Mantel-Cox logrank test or Cox proportional hazards regression. Multivariate analysis was performed to identify independent prognostic factors for OS and PFS, using Cox proportional hazards regression and the stepwise backward procedure, incorporating the baseline clinical variables: age, β2-microglobulin (β2M), lactate dehydrogenase (LDH), hemoglobin, serum creatinine, serum calcium, percentage plasma cells in marrow biopsy, compete or partial response to last prior chemotherapy, and chromosome 13 deletion. The removal and entry levels of significance were 0.05 and 0.01, respectively. Patients with unknown values of any of the variables were excluded from the multivariate analysis.

The effect of baseline levels of laboratory variables on response was examined using the two-sided Fisher's exact test or logistic regression, as appropriate. Spearman's rank correlation coefficient was computed to examine the relationships between laboratory and clinical variables. Wilcoxon's rank sum test was used to compare baseline levels of laboratory variables between those patients who achieved stable disease or response and those who progressed. Wilcoxon's matched pairs test was used to compare levels of laboratory variables at baseline and at the time of best response to treatment.

For the purpose of examining OS, PFS and response, the laboratory variables VEGF, HGF, IL-6 and CD57 were treated as continuous variables and transformed using natural logarithms (log); each of the other laboratory variables was dichotomized into two groups (=0 and >0). Unless stated otherwise all tests were two-sided and a p-value less than 0.05 was considered statistically significant. Statistical analyses were performed using S*plus 2000 (MathSoft Inc, Seattle, WA, USA) and StatXact v 6.0 (Cytel Software Corporation, Cambridge, MA, USA) software.

Results

As previously reported, the overall response rate to the clinical trial treatment was 28% with a further 55% of patients achieving stable disease.[17] The dose of thalidomide did not affect the likelihood of response, PFS or OS. The estimated median PFS was 5.5 months (95%CI: 3.6 - 6.8 months) and the median OS was 14.6 months (95%CI: 9.7 - >26.3 months). Descriptive statistics on baseline levels of the eight laboratory variables are presented in Table 1 and correlations between these variables as well as with the clinical variables are presented in Table 2. Not surprisingly, a strong positive correlation was found between MVD as assessed by CD34, and VWF (R =0.82; p<0.0001). As very similar results were obtained using CD34 and VWF for all the reported analyses, further results reported for MVD are those determined by CD34 only. Of note there was no correlation between the level of MVD and the plasma levels of VEGF and bFGF. There was a moder-

Table 1. Descriptive statistics for baseline levels of eight laboratory variables.

	Number of results	Mean	Standard Deviation	Median	Range
Plasma level (pg/mL)					
VEGF	71	77.0	101.5	45	0–562
FGF	72	4.3	13.9	0	0–135
HGF	70	2312	2883	1284	315–13541
IL-6	71	12.3	20.6	4	0-107
Bone marrow biopsy (average count per HPF)					
CD34	66	24.0	19.7	20	0–89
VWF	63	20.2	12.5	17	3–66
MCT	68	2.4	3.2	1	0–17
CD57	65	4.3	4.8	3	0-31

VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor; HGF: hepatocyte growth factor; IL-6: interleukin-6; VWF: von Willebrand's factor, MCT: mast cell tryptase: HPF: high-powered field (x400).

Table 2. Correlations between laboratory and clinical variables. Results with p<0.05 are bolded.

Spearman R	VEGF	bFGF	IL-6	HGF	CD34	VWF	MCT	CD57
VEGF	–	–	–	–	–	–	–	–
bFGF	0.196	–	–	–	–	–	–	–
IL-6	0.205	-0.062	–	–	–	–	–	–
HGF	0.118	-0.137	**0.463**	–	–	–	–	–
CD34	0.017	0.049	0.080	0.121	–	–	–	–
VWF	0.015	0.011	-0.003	0.022	**0.819**	–	–	–
MCT	0.009	-0.142	**-0.297**	**-0.267**	-0.140	-0.025	–	–
CD57	0.128	0.022	-0.085	-0.008	0.180	0.225	0.079	–

Spearman R	VEGF	bFGF	IL-6	HGF	CD34	VWF	MCT	CD57
Age	0.123	-0.002	0.104	**0.388**	-0.076	-0.030	**-0.274**	-0.077
B2M	-0.064	-0.040	**0.324**	**0.414**	**0.310**	0.236	**-0.435**	-0.064
LDH	0.182	0.003	0.206	0.036	-0.049	-0.047	-0.159	0.137
Hemoglobin	0.221	0.151	-0.159	**-0.296**	**-0.504**	**-0.397**	**0.304**	0.005
Creatinine	-0.108	0.019	**0.347**	**0.314**	0.147	0.126	**-0.360**	-0.101
% plasma cells IN BM	-0.237	-0.197	0.033	0.052	**0.255**	0.231	**-0.418**	-0.048

VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor; IL-6: interleukin-6; HGF: hepatocyte growth factor; VWF: Von Willebrand's factor; MCT: mast cell tryptase; β2M: β2-microglobulin; LDH: lactate dehydrogenase.

ate correlation between the markers of active myeloma, IL-6 and HGF (R=0.46; $p<0.0001$). Only a weak to moderate correlation was seen between the level of MVD as assessed by CD34/VWF and the percentage involvement of the marrow by malignant plasma cells (R=0.255; $p=0.04$), with some patients having high levels of MVD without having a large marrow infiltrate. Of interest there was a moderate negative correlation between the number of mast cells as measured by MCT and the reported percentage involvement on the bone marrow biopsy by malignant plasma cells (R= -0.42; p=0.0004), with very few mast cells being present in areas of cellular marrow in baseline samples that were packed with malignant plasma cells (Figure 1).

Bone marrow CD57⁺ cell analysis

The median number of marrow biopsy CD57+ cells at baseline was 3 per high-power field (range: 0-31). Further flow cytometry analysis of marrow aspirates demonstrated that of the lymphoid gate 71% were T cells and 15% NK cells ($CD3^-/CD16^+/56^+$). CD57 was expressed on both $CD8^+$ T cells and NK cells. Invariate NKT cells, as defined by V,11 and V·24 expression, did not express CD57. Importantly, back-gating analysis revealed that >80% of $CD57^+$ cells were cytotoxic T cells ($CD3^+/CD8^+/CD57^+$). Overall, $CD3^+/CD8^+/CD57^+$ cells accounted for up to a quarter of the marrow T-cell population.

Prognostic factors for response to thalidomide

As previously reported, the only clinical variable predictive of response to thalidomide was age, with a response rate for those 65 years or younger being 38% compared to 17% for those >65 years ($p=0.043$).[17] The likelihood of response was not affected by the iMTD of thalidomide received. Baseline plasma VEGF was the sole laboratory variable predictive of response, independent of any clinical factor; with a significant relationship between the probability of responding to treatment and the log-transformed baseline plasma VEGF level ($p=0.018$). No responses were seen in patients with a zero level of baseline VEGF. Adding age group (0≤65 vs. 1≥65) into the model incorporating log-transformed VEGF is also significant ($p=0.031$). The fitted relationship for this model is shown in figure 2, with the probability of responding to treatment estimated as: $e^{(A+B\text{-}logVEGF)}/(1+ e^{(A+B\text{-}logVEGF)})$, where A=-2.03 for age≤≤65 and A=-3.24 for age >65, and B=0.45.

Patients achieving stable disease or a response had greater baseline MVD than those who progressed, with a median baseline CD34 count of 22 (range: 0-89) vs. 12 (range: 1-20), respectively; $p=0.01$. Similarly, such patients tended to have higher baseline levels of angiogenic factors in the plasma than those who progressed; for example median VEGF=48.7 pg/mL (range: 0-562) for stable disease or response vs. 24.7 pg/mL (range: 0-130) for those with progressive disease ($p=0.078$). Higher numbers of $CD57^+$ cells in the baseline marrow was not a significant predictor for response to thalidomide (log-transformed; $p=0.127$).

Changes in laboratory parameters during thalidomide treatment

MVD fell significantly in responding patients from a median baseline count of 21 (range: 7-71) to 10 (range: 4-20) at best response ($p=0.004$); and rose in patients who progressed during the study from 10 at baseline or best response (range: 1-130) to 20 at first progression (range: 1-113) ($p=0.027$) (Figure 1 and online supplementary appendix). However, a fall in MVD was not observed in all

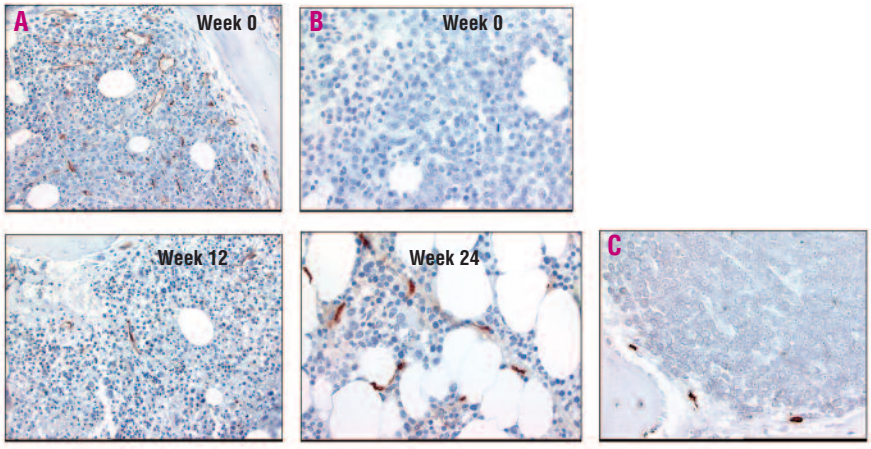

Figure 1. A. CD34 staining of bone marrow biopsy (×400 magnification) at study entry (top left) and week 12 (bottom left) in a patient demonstrating a partial response to thalidomide. **B.** Example of increasing mast cell numbers in a patient responding to thalidomide at baseline (top middle) and after 24 weeks of treatment (bottom middle). **C.** In patients with active myeloma, mast cells were most commonly observed only in areas of non-cellular marrow such as within paratrabecular regions (bottom right). Magnification ×400.

responders, with six of the 19 responders who had serial measures performed showing no change or some increase in their MVD with response. In the majority of responders in whom the MVD did fall, this did not occur in a strict linear pattern. The MVD tended to fall initially as patients responded but then often reached a plateau or even increased despite ongoing evidence of response of the patient's myeloma. VEGF levels also fell in responding patients from a median of 65.8 pg/mL (range: 9.2-562.4) to 43.3 pg/mL (range: 0–208.1) at best response (p=0.024). There was no significant change in numbers of CD57+ cells over time. Levels of bone marrow mast cells, as measured by MCT, increased significantly in responding patients from a median baseline level of 1 cell/HPF (range 0-8) to a median level at best response of 8 cells/HPF (range 1-21). The only mast cells seen at baseline in marrow specimens with a significant plasmacytosis tended to be seen within non-cellular areas such as the paratrabecular region (Figure 1). There was no significant change in any of the other laboratory parameters over time (online supplementary appendix). In addition, changes in the laboratory parameters did not significantly vary with the dose of thalidomide received.

Prognostic laboratory factors for PS and OS

Bone marrow MVD at baseline did not predict for PFS or OS. Univariate analysis revealed that only reduced CD57+ cell numbers in the baseline marrow predicted for inferior PFS (p=0.036), with baseline plasma assays including VEGF (p=0.068), HGF (p=0.079) and bFGF (p=0.076) all approaching significance. The risk of progression was estimated to be 2.1 times higher in patients with a zero baseline level of VEGF (p=0.027).

On univariate analysis inferior OS was associated with: raised baseline plasma levels of IL-6 (p=0.014), raised baseline plasma levels of HGF (p=0.005) and fewer baseline marrow CD57+ cells (p=0.003). Multivariate analysis for PFS, including relevant clinical parameters, demonstrated that independent significant factors predicting inferior PFS were a raised serum LDH, a low level of baseline plasma VEGF and fewer baseline marrow CD57+ cells. Multivariate analysis for OS, which incorporated clinical variables, demonstrated age > 65 years, raised serum LDH, raised baseline plasma levels of HGF and fewer baseline CD57+ cells as independent significant predictors of inferior OS (Figure 3). Importantly, the adverse impact of CD57+ cells on both PFS and OS was a continuous variable. For illustrative purposes, however, we dichotomized this variable into those patients with detectable CD57+ cells at baseline (n=55) and those with no detectable CD57+ cells (n=10), and compared them in a univariate analysis of overall survival (Figure 3).

Discussion

The major strength of this study is that samples for biological surrogate marker analysis were collected in a uniform manner in the context of a prospective clinical trial of thalidomide therapy for myeloma. To date, no other study in this patient group has comprehensively analyzed and correlated the predictive value of laboratory variables with standard clinical parameters. In this report we have confirmed our previously published data on the importance of age and elevated baseline LDH on OS - even when adding a variety of laboratory parameters into the analysis.[19] In addition to these clinical parameters we demonstrate that elevated baseline plasma VEGF is a positive predictor of thalidomide response and PFS and that raised plasma HGF and reduced baseline bone marrow CD57+ cells are negative predictors for PFS and OS.

In healthy individuals CD57 is expressed on up to 15% of peripheral blood lymphocytes including a subset of CD8+ (cytotoxic) T cells.[20] CD8+/57+ cells have been reported to be variably increased in patients with cytomegalovirus and human immunodeficiency virus infection, autoimmune conditions and following autologous bone marrow transplantation, in whom they may have an anti-lymphocyte proliferative capacity.[4,21,22] Increased numbers of CD8+/CD57+ cells are higher in the blood of untreated myeloma patients compared to that

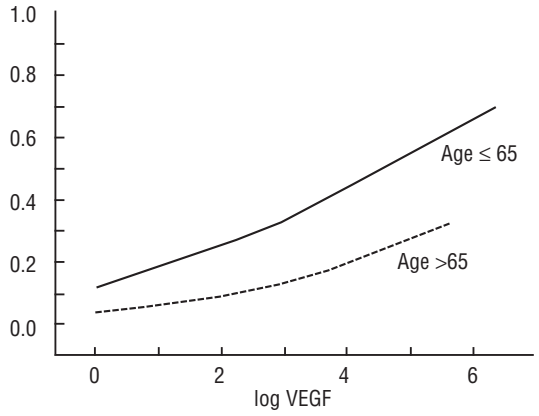

Figure 2. The fitted relationship between the probability of responding to thalidomide treatment and log VEGF for patients aged ≤65 and patients aged >65.

Factor	*Level*	*Coefficient*	*Relative hazard rate*	*95% CI*	*p value*
		Progression-free survival			
LDH	> ULN vs. ≤ ULN	1.363	3.91	1.80–8.46	0.001
Plasma VEGF (log transformed)	Continuous variable	-0.236	0.79	0.66–0.94	0.009
CD57 (log transformed)	Continuous variable	-0.495	0.61	0.42–0.90	0.012
		Overall survival			
Age	>65 vs. 65	0.997	2.71	1.19–6.16	0.017
LDH	> ULN vs. ≤ ULN	1.490	4.44	1.81–10.85	0.001
CD57 (log transformed)	Continuous variable	-0.914	0.40	0.25–0.65	<0.001
HGF (log transformed)	Continuous variable	0.630	1.88	1.15–3.08	0.012

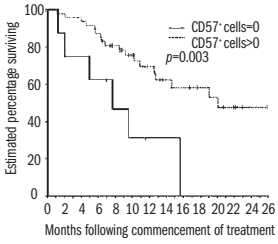

Figure 3. Independent laboratory and clinical prognostic factors from multivariate analysis of PFS and OS using the Cox proportional hazards model (*top*) and Kaplan-Meier curves of overall survival for patients with zero vs. >0 CD57 positive cells in baseline marrow. Patients with censored times are represented by tick marks (*below*).

of healthy donors has been described. The same group also identified a long-lived population of $CD8^+/CD57^+/CD28^-/perforin^+$ T-cell clones in the peripheral blood of patients with myeloma, which despite being more commonly found in patients with progressive and advanced-stage disease, was associated with superior survival.[10] Collectively our data, along with those of others, suggest that $CD8^+/CD57^+$ T cells have an immunomodulatory and potentially anti-proliferative effect in myeloma. However, the actual inter-relationship between the observed increased marrow $CD8^+/CD57^+$ T cells, disease control and thalidomide therapy remains one of conjecture. We observed no relationship between $CD8^+/CD57^+$ T-cell numbers and the likelihood of response to thalidomide, suggesting that the efficacy of thalidomide was not directly linked to augmentation of any putative $CD8^+/CD57^+$ mediated myeloma control. Similarly, we saw no change in absolute numbers of $CD57^+$ cells over time in patients responding to thalidomide, suggesting that thalidomide does not induce an expansion of $CD8^+/CD57^+$ cells. It remains possible, however, that the small number of responding patients in whom this effect was assessed and the short time to disease progression may have masked any alterations in $CD8^+/CD57^+$ number induced by thalidomide therapy. Conversely, we observed a striking effect of high numbers of baseline marrow $CD8^+/CD57^+$ cells on both PFS and OS. Again, this may reflect enhanced or maintained immunosurveillance prior to initiation of thalidomide and therefore a pre-selection for ongoing sensitivity to treatment in a subgroup of myeloma patients rather than a marker of thalidomide sensitivity *per se*. Therefore, the positive impact of $CD57^+$ cells may not be restricted to patients treated with thalidomide - this warrants further investigation using conventional cytotoxics and other biological agents including the thalidomide-derivative, lenalidomide.

In addition to its potential immunomodulatory effects, our results do support the premise that the extent of angiogenesis is a predictor of response to thalidomide. Firstly, as also described in a smaller study,[23] there was a significant relationship between the probability of responding to thalidomide treatment and the baseline level of plasma VEGF, with lower levels predicting an inferior probability of response. Secondly, although MVD itself did not predict for response, patients who had at least disease stabilization had a higher baseline MVD. Thirdly, MVD and VEGF tended to decline in responding patients, confirming observations by some, but not all, investigators.[24–26] In support of our observation, Du *et al.* and Hatjuharissi *et al.* both reported a significant decline in MVD in patients with myeloma who responded to thalidomide and Kumar et al demonstrated a similar degree of MVD reduction to that seen in our study.[24–26]

As an assessment of angiogenesis, we believe that the measurement of peripheral blood VEGF levels is both easier to perform and potentially more attractive than measuring MVD. We demonstrate that patients with a low baseline level of plasma VEGF had both a lower response rate to thalidomide treatment and an inferior PFS. However, the pitfalls of assessing angiogenesis in a tumor by MVD have been described elsewhere and we and others have demonstrated that MVD does not necessarily correlate with the degree of plasmacytosis in the marrow.[25,27] MVD in a tumor that is regressing under anti-angiogenic treatment is related to the ratio between tumor cell death and endothelial cell apoptosis.

Therefore the timing of measurement of MVD will affect the results seen, as microvessels may decline initially and then decrease more slowly or even increase as tumor cells drop out. This was the pattern shown by our serial measurements of MVD and also highlights the fact that the level of MVD, although related to the degree of plasmacytosis in the marrow does not vary in a linear fashion. In our study we found only a weak to moderate, although significant, correlation between the level of MVD and the degree of plasmacytosis in the marrow. This suggests that although changes in MVD are related to changes in the level of tumor burden, there are other factors at play – with others studies and ours showing no change or even an increase in MVD in some responders. Interestingly, Hatjiharissi *et al*., reported a significant correlation between bone marrow infiltration by plasma cells and MVD before thalidomide-dexamethasone treatment but not after response to treatment.[27] Given these considerations, direct MVD measurement may not be the optimal indicator of angiogenesis inhibition.

However, considerable variation also exists in the reported changes of the cytokines VEGF, bFGF and HGF following treatment for myeloma and sample preparation is critical.[24,25,28-31] One study correlating responses to chemotherapy with VEGF levels demonstrated results different from ours - with lower VEGF levels associated with higher complete or partial responses to chemotherapy and longer survival times.[32] These diametrically opposed results may reflect the impact of the VEGF level on the type of treatment received, with thalidomide therapy having a greater ability to affect the prognosis of patients with myeloma that is driven by VEGF, either through active angiogenesis or dependence on the marrow microenvironment. However, clearly the actions and interactions of these cytokines are complex and the heterogeneity of the results obtained in myeloma patients suggests that they are only a crude indicator of angiogenesis. In support of this is the finding, in our study, of a lack of correlation between the levels of the individual cytokines such as bFGF and VEGF, or between the levels of the angiogenic cytokines and MVD. Others have also found a similar lack of correlation between angiogenic cytokines and MVD in myeloma and solid tumors.[16,27,29] In our study, high VEGF levels enhanced the likelihood of both response and PFS, but did not affect OS. This supports the concept that other factors have a predominate effect on survival such as plasma cell proliferative capacity and, potentially, immunosurveillance. The relationships of these cytokines could be further investigated by assessing gene expression using polymerase chain reaction (PCR)-based techniques.

Angiogenesis has also been recently linked to mast cell physiology.[33] We, like others, have found low or absent mast cell marrow infiltration within most of the specimens taken at baseline,[34,35] with mast cell numbers then increasing significantly as patients responded to thalidomide. This finding may be related predominantly to changes in the level of tumor burden with a moderate negative correlation seen between the number of mast cells as measured by MCT and the reported percentage involvement on the bone marrow biopsy by malignant plasma cells. However, studies in solid tumors have also shown a variable relationship between mast cell infiltrates and MVD.[36] Our findings are in contrast to the results of Ribatti *et al*. who reported a significant positive correlation between mast cell numbers and MVD in patients with various lymphoproliferative disorders including plasma cell dyscrasias.[18] We believe that considerable methodological variation exists in enumerating mast cells in marrow biopsies and that this underlies the range of reported findings. Unlike Ribatti's group we counted mast cell numbers only within areas of cellular marrow and excluded paratrabecular regions. Furthermore, recent research suggests a difference in the pattern of infiltration between mast cell subtypes, with a more prominent correlation between MVD and numbers of tryptase$^+$/chymase$^+$ mast cells (MC_{TC}) than tryptase$^+$/chymase$^-$ mast cells (MC_T).[37] Further study is required to better define the roles of mast cell sub-populations in angiogenesis and other aspects of myeloma biology.

In summary, we propose that two phenomena, angiogenesis and immunomodulation, underpin the sensitivity of myeloma to non-cytotoxic therapies such as thalidomide. Our findings should encourage further investigation of angiogenesis utilizing newly developed techniques, which may give a more accurate reflection of the level of angiogenesis within a tumor.[38-40] The superior PFS and OS in patients with elevated levels of CD57$^+$ cells in the marrow is supported by previously published studies examining these cells in the blood and warrants further investigation to define the nature of these cells and their interactions with malignant plasma cells.

Authors' Contributions

LM: substantial contribution to conception and design of the study, acquisition, analysis and interpretation of data; DH: substantial contribution to conception and design of the study, acquisition, analysis and interpretation of data; PG: substantial contribution to acquisition, analysis and interpretation of data; MT: substantial contribution to acquisition, analysis and interpretation of data; YH: substantial contribution to acquisition, analysis and interpretation of data; MS: substantial contribution to acquisition, analysis and interpretation of data; VB: substantial contribution to acquisition, analysis and interpretation of data; DR: substantial contribution to acquisition, analysis and interpretation of data; PS: substantial contribution to conception and design, acquisition, analysis and interpretation of data; ADM: substantial contribution to conception and design of the study, acquisition of data and analysis and interpretation of data; JBZ: substantial contribution to conception and design of the study, acquisition, analysis and interpretation of data; HMP: substantial contribution to conception and design of the study, acquisition, analysis and interpretation of data.

Conflict of Interest

One of the authors (JBZ) is employed by a company (Celgene) whose product was studied in the present work. The other authors reported no potential conflicts of interest.

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