Differential expression of vascular endothelial growth factors and their receptors in multiple myeloma

Teresa Kimlinger
Michael Kline
Shaji Kumar
John Lust
Thomas Witzig
S. Vincent Rajkumar

Background and Objectives. Bone marrow angiogenesis is increased in patients with multiple myeloma (MM) and correlates with disease stage.

Design and Methods. Previous studies of quantifying vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFR) in plasma cells from patients at different stages of MM found no significant difference in expression between overt MM and earlier pre-malignant stages of the disease namely, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM).

Results. In this report we used quantitative flow cytometry to study cytoplasmic VEGF (cyVEGF) expression (measured as antibody binding capacity) in plasma cells from patients with MM (n=22), MGUS/SMM (n=12), and AL-amyloidosis (AL) (n=9). CyVEGF expression was higher in MM (169,591) than in MGUS/SMM (144,858), or AL (106,011) although these differences were not statistically significant. Using an indirect VEGFR assay that measures VEGF binding, we found VEGF receptors on plasma cells from all groups of patients, with the lowest expression on plasma cells from normal individuals. We detected VEGF R1, VEGF R2, and VEGF R3 on plasma cells from all groups of patients and found receptor expression predominantly in the subset of CD45-positive plasma cells.

Interpretation and Conclusions. This study supports the concept that VEGF is involved in the pathogenesis of MM, and suggests that VEGF may differentially affect a subset of plasma cells.

Key words: multiple myeloma, plasma cell, vascular endothelial growth factor, vascular endothelial growth factor receptor, CD45.

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All authors from the Division of Hematology, Department of Internal Medicine, Mayo Clinic, Rochester, MN, 55905, USA.

Correspondence:
S. Vincent Rajkumar, MD,
Division of Hematology, Mayo Clinic
200 First St SW, Rochester,
MN, 55905, USA.
E-mail: rajks@mayo.edu

Multiple myeloma (MM) is an incurable disease characterized by a clonal proliferation of plasma cells in the bone marrow.[1] Clinical manifestations include bone lesions, anemia, hypercalcemia, and kidney failure. Monoclonal gammopathy of undetermined significance (MGUS) is a benign precursor condition of MM in which bone lesions are absent. Individuals with MGUS progress to MM at a rate of about 1 % per year but there are no clinical features or laboratory tests that can reliably predict progression. The progression to MM is marked by changes in the clonal plasma cells as well as in the bone marrow microenvironment.[2] One of the characteristic changes in the microenvironment is an increase in bone marrow angiogenesis associated with MM and a poor prognosis.[3-7] An increased or abnormal level of angiogenesis has been recognized for many years as a key feature of solid tumor diseases and has been increasingly associated with poor prognosis in hematologic diseases.[8]

Increased angiogenesis may result from a variety of cytokines and other factors. Vascular endothelial growth factor (VEGF) is the most well characterized pro-angiogenic factor and is produced by multiple cell types in MM and other malignancies.[9,10] Plasma cells in MM secrete VEGF, which acts on vascular endothelial cells.[11,12] VEGF also stimulates stromal cells to produce interleukin-6, a potent growth factor for myeloma cells, which in turn stimulates increased expression and secretion of VEGF. VEGF may function as a survival factor, increasing the expression of Bcl-2, Mcl-1, and other anti-apoptotic factors in the plasma cells and the surrounding stromal cells.[10,13,14] VEGF activity is mediated mainly through VEGF R1 (flt-1), VEGF R2 (kdr), VEGF R3 (flt-4) and neuropilin. VEGF receptors are expressed on hematologic cell lines, bone marrow stromal

cells, and primary myeloma plasma cells, making it possible that autocrine or paracrine pathways could be involved in increased angiogenesis and the progression of MM disease.[15]

In previous studies, we demonstrated that there is an increase in the degree of angiogenesis from MGUS to smoldering multiple myeloma (SMM), an intermediate condition, to newly diagnosed MM and relapsed MM. [7] To further explain these differences, efforts were made to quantify VEGF and VEGF receptor levels between plasma cells from normal controls, patients with light chain amyloidosis (AL), MM, MGUS, and those with SMM. These studies were done using immunohistochemistry on bone marrow slides as well as using isolated CD138 positive plasma cells to perform quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative western blot measurements. We found no significant differences in expression between plasma cells from the different groups of patients in these studies.[16] The findings of our original studies may have been limited by low levels of antigen expression, heterogeneity of expression within the plasma cell population, or by the low percentage of plasma cells in a given sample. To address these limitations, in this study we used sensitive flow cytometric methods to identify plasma cells by their characteristic CD38/45 staining pattern and to measure the expression of VEGF and VEGF receptors.

Design and Methods

Samples

This study was approved by the Mayo Foundation Institutional Review Board, and was conducted in accordance with United States federal regulations and the Declaration of Helsinki. All bone marrow samples used in these studies were excess material and were processed within 24 hours of collection.

Cytoplasmic VEGF staining

Cytoplasmic VEGF expression (cyVEGF) was measured on 43 whole bone marrow samples: amyloid (n=9), MGUS (n=5) SMM (n=7), and MM (n=22). For each sample, 20×10^6 cells from whole bone marrow were washed in phosphate buffered saline (PBS) and lysed in ammonium chloride buffer (ACK) before placing $1\text{-}2\times10^6$ cells in each of two tubes. Two milliliters of freshly prepared Becton Dickinson (BD) FACS™ lysing solution (#349202 BD Biosciences, San José, CA, USA) were added to each tube and incubated for 10 minutes at room temperature. The cells were pelleted and resuspended in 0.5 mL of freshly prepared FACS™ Permeabilizing Solution (#340973 BD Biosciences) for an additional 10 minutes of incubation. Two milliliters of 3% PBS bovine serum albumin (BSA) were added to each tube and the tubes were then centrifuged for 5 minutes at 300 g. CD38-allophycocyanin (APC) (#340677 BD Biosciences) and CD45-peridinin chlorophyll protein (PerCP) (#340665 BD Biosciences) were added to each tube. Mouse IgG2-PE conjugated with phycoerythrin (PC) (#555574 BD Biosciences) was added to tube one while VEGF-PE (#IC2931P, R & D Systems, Minneapolis, MN, USA) was added to tube two. The samples were incubated for 15 minutes at room temperature, in the dark. Two milliliters of PBS were added to each tube and mixed; the tubes were then returned to the dark for a 15 minute soak. The tubes were centrifuged for 5 minutes at 300 g. The pellets were resuspended in 0.5 mL of 1% paraformaldehyde and stored at 4°C until run on the BD FACSCalibur. Plasma cells were identified by their characteristic CD38/45 staining pattern. Lymphocytes and granulocytes were identified using CD45/log side scatter (SSC) patterns.[17] With each run, Quantum™ Simply Cellular Beads (#815, Bangs Laboratories, Fishers, IN, USA) were stained with the same VEGF antibody to create a standard curve for calculation of antibody binding capacity (ABC) values. Using values from the standard curve generated by the VEGF-stained set of beads, a VEGF ABC value and an isotype control ABC value (background) were calculated for the plasma cells, lymphocytes, and granulocytes from each patients' sample.[18,19] The difference between VEGF and background was calculated and a mean ABC value for each group of patients was determined. Patients with MGUS and SMM were considered a single group for this analysis.

Direct staining of VEGF receptors

Bone marrow from 67 individuals with amyloid (n=9), MGUS (n=11), SMM (n=14), MM (n=23), and normal cases (n=10) was processed for direct staining of VEGF receptors 1, 2 and 3. For each sample, 20×10^6 cells from whole bone marrow were washed in PBS and lysed in ACK buffer. The cells were incubated with mouse Ig to limit Fc binding. Four tubes were set up for each sample. In individual tubes, PE-labeled VEGF R1, R2, or R3 antibodies (# FABSP3P, R & D Systems) or isotype control (# 340761 BD Biosciences) were added along with CD38 APC (BD Biosciences) and CD45 PerCP (BD Biosciences). The tubes were incubated for a minimum of 15 minutes at room temperature, in the dark, and then the cells were washed once in PBS. The pellets were resuspended in 1% paraformaldehyde and incubated at 4°C until run on the BD FACScalibur flow cytometer. Plasma cells were identified by CD38/45 staining pattern and analyzed for percent positive receptor staining. Plasma cell subsets, lymphocytes, monocytes and granulocytes were also identified and analyzed.

VEGF binding capacity of plasma cells

FLUOROKINE™ recombinant human VEGF biotin kit (# NFVEO, R & D Systems) was used according to the manufacturer's instructions to examine the VEGF binding capabilities of plasma cells. A total of 46 bone marrow samples [amyloid (n=5), MGUS (n=9), SMM (n=7), MM (n=20) and normal cases (n=5)] were studied. One tube (tube 1) of ACK lysed whole bone marrow cells was incubated with VEGF biotin. In tube 2, VEGF biotin pre-incubated with a blocking antibody was added to the cells (specificity control), while in a third tube a non-specific biotinylated protein (negative control) was added. All cells were preincubated with mouse Ig to block Fc binding. After 1 hour at 4°C, FITC-avidin (included in the kit), CD38-APC (BD Biosciences) and CD45-PerCP (BD Biosciences) were added to each tube. Cells were incubated for an additional 30 minutes at 4°C, washed twice and run on a BD FACScalibur flow cytometer. Gating and plasma cell identification were performed as described in the previous section. The percent of signal blocked (specificity control) was calculated by comparing the FITC intensity (channel number) of the blocked VEGF peak (tube 2) to the FITC intensity of tube 1. This approach does not determine the identity of the receptor, but indicates the presence of any VEGF receptors.

Surface VEGF staining

Surface bound VEGF was directly measured on plasma cells from 63 individuals [(amyloid (n=4), MGUS (n=9), SMM (n=22), MM (n=17), and normal cases (n=7)]. Bone marrow cells (20×10^6) were washed in PBS and lysed in ACK buffer. Surface expression of VEGF (sVEGF) was determined using routine color flow cytometry with VEGF-PE (R & D Systems)/CD45 PerCP (BD Biosciences)/ CD38 APC (BD Biosciences). A separate isotype control tube was set up for each sample. The tubes were incubated for a minimum of 15 minutes at room temperature in the dark. Cells were washed once in PBS. The pellets were resuspended in 1% paraformaldehyde and incubated at 4°C until run on the BD FACScalibur flow cytometer. sVEGF expression was determined on the plasma cell population as a whole and on the individual CD45-positive and CD45-negative fractions. The median expression and the proportion of cases with plasma cells expressing >20% sVEGF were calculated. Subsets of patients' plasma cells that were not clonally restricted were removed from the analysis.

Confirmation of clonality

In two separate tubes, cytoplasmic kappa and lambda staining was done to determine clonality of the plasma cell populations. After staining for CD38 and CD45, the cells were washed and incubated for 15 minutes in Medium A (# GAS001, Caltag, Burlingame, CA, USA).

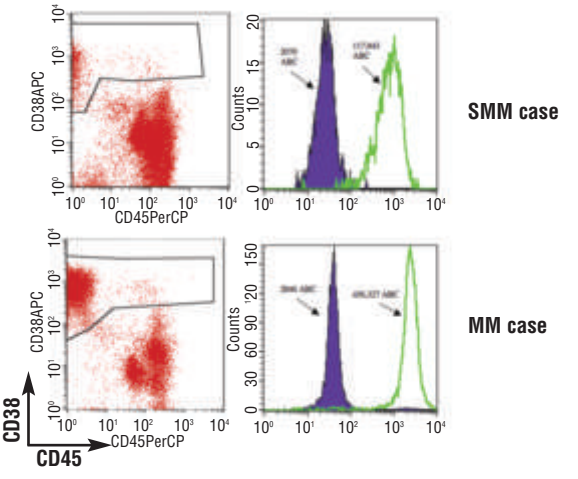

Figure 1. Example of the analysis of plasma cell cytoplasmic VEGF (cyVEGF) levels. For each patient, the entire plasma cell population was gated first using side scatter (SSC)/forward scatter (FWD) parameters to include only mononuclear cells (*not shown*) then by using CD45/CD38 characteristics. The isotype control value (filled histogram) was subtracted from the VEGF value. In this example, the antibody binding capacity (ABC) value for the case of smoldering multiple myeloma (SMM) is 117,943 while that of the multiple myeloma (MM) case is 436,327.

Two milliliters of PBS were added to each tube and spun. Either anti-κ fluorescein isothiocyanate (FITC - BD Biosciences 348063) or anti-λ FITC (BD Biosciences 348073) was added and the pellets were resuspended in Medium B (Caltag GAS002) and incubated for an additional 15 minutes. The cells were washed with 2 mL PBS. The pellets were resuspended in 1% paraformaldehyde and incubated at 4°C until run on the BD FACScalibur flow cytometer.

Results

Cytoplasmic VEGF staining

Representative data are shown in Figure 1. In contrast to previous comparisons of VEGF expression obtained using quantitative RT-PCR and western blotting, flow cytometric measurements revealed slight differences in cytoplasmic VEGF levels between the plasma cell populations of the patients with different diseases. The average cyVEGF ABC value was higher in MM (n=22 patients, mean ABC 169,591) than in MGUS/SMM (n=12, mean ABC 144,858), or AL (n=9, mean ABC 106,011) although the differences were not statistically significant (p=0.67) (Figure 2). Plasma cell cyVEGF ABC values were higher than the values determined for lymphocytes (mean, 61,945) or granulocytes (mean, 35,239).

VEGF receptor expression on patients' plasma cells and cell subsets

In the indirect assay using biotinylated VEGF, plasma

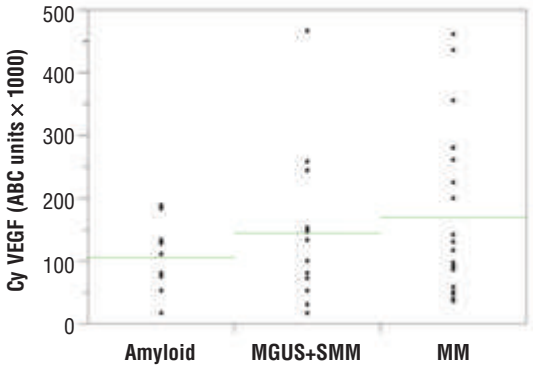

Figure 2. Summary of cyVEGF assay results. A VEGF standard curve was constructed for each run using a mixture of five beads with calibrated amounts of anti–mouse reagent stained with mouse anti VEGF -PE antibody. The PE geometric mean of each bead population was used to create a standard curve for ABC. The average ABC value was slightly higher in MM patients (n=22, mean ABC 169,591) than in MGUS/SMM patients (n=12, mean ABC 144,858), and AL patients (n=9, mean ABC 106,011). These differences were not statistically significant (*p* value=0.67).

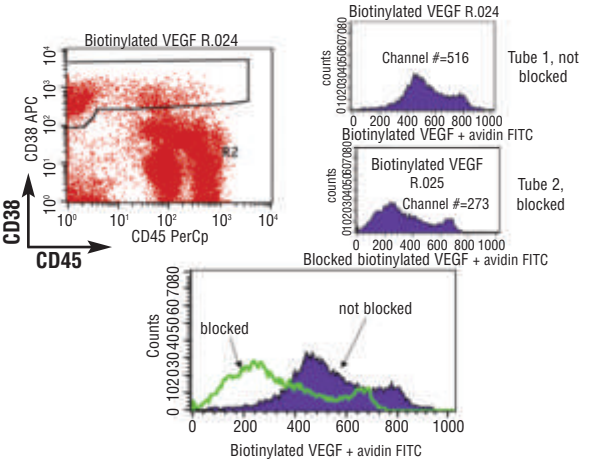

Figure 3. Indirect analysis of plasma cell VEGFR expression. In this method, VEGF receptor expression was indirectly determined by the binding of biotinylated VEGF plus FITC- avidin. In this example, a majority of the plasma cells in tube 1 (without a blocking antibody) showed positive levels of FITC binding. When an antibody blocking VEGF was added, the binding of VEGF to the cells was markedly decreased (tube 2).

Table 1. Results of staining for VEGF binding on normal and malignant bone marrow plasma cells using an indirect VEGFR staining method and flow cytometry.

All plasma cell gate	*Number of patients studied*	*Staining intensity (Mean channel number)*
Normal plasma cells	5	125
Malignant plasma cells		
Amyloidosis	5	435
MGUS	9	490
SMM	7	598
Multiple myeloma	20	537

MGUS: monoclonal gammopathy of undetermined significance; SMM: smoldering multiple myeloma.

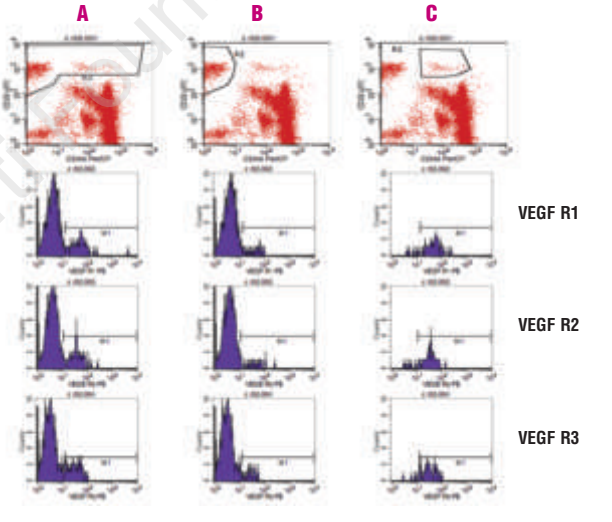

Figure 4. Direct detection of VEGF receptors on plasma cells. Using this method, the heterogeneity of the plasma cell population for VEGF R expression is demonstrated by the differences in the staining patterns of CD45-negative vs. CD45-positive plasma cells from the same individual. (A) Staining on the entire plasma cell population. The marker was set using the isotype control (*not shown*). There is a very small receptor-positive subset. (B) The CD45-negative population displays less VEGF receptor staining. (C) The CD45-positive subset is nearly all receptor-positive.

cells from all groups of patients bound VEGF (> 97% positive) at high intensity. The specificity of VEGF binding was confirmed by a significant decrease in peak FITC channel numbers in the presence of a blocking antibody (Figure 3). A similar level of VEGF binding was seen in monocytes (>93%) and a lower intensity was observed in lymphocytes and granulocytes (*data not shown*). Interestingly, plasma cells from patients with the various diseases bound significantly higher levels of VEGF (higher intensity – higher channel number) than did normal samples (Table 1).

To further investigate the specificity of VEGF binding to plasma cells we performed direct staining for VEGF R1, VEGF R2, and VEGF R3. Staining for these three receptors on plasma cells did not reveal any significant differences among the groups of patients (Table 2). Most cases had a small subset of plasma cells that expressed VEGFR; however, the frequency of cases with >20% of the plasma cells expressing VEGFR was low in all groups (Figure 4). Backgating on this positive subset revealed heterogeneity among the plasma cell subsets such that the vast majority of the VEGFR staining was within the CD45-positive subset (Table 2). Cytoplasmic kappa and lambda staining confirmed the monoclonal nature of cells expressing VEGF receptors (Figure 5). Using CD45/SSC parameters it was possible to separately gate on lymphocytes, monocytes, and granulocytes. Lymphocytes were typically negative for all three

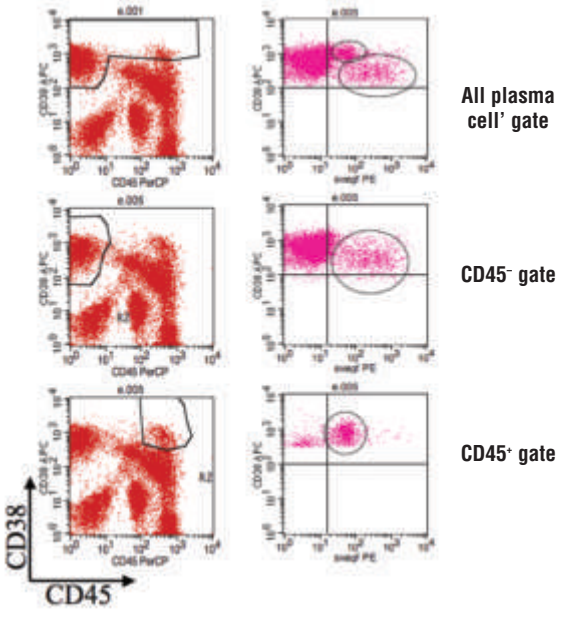

Figure 5. Measurement of VEGF present on the surface of plasma cells. The *all plasma cell gate* was mostly surface VEGF-negative with two small populations staining positive for VEGF. One population had slightly dimmer CD38 staining and a more diffuse pattern with a wide spectrum of VEGF staining. This population was contained within the CD45-negative plasma cell subset. A second population had a much more discrete pattern of VEGF staining (dimmer than the first population) and had slightly brighter CD38 staining. This population was determined to be in the CD45-positive plasma cell subset. Clonality was determined for each patient and subset. Polyclonal populations (except in the normal cases) were excluded from the analysis.

VEGF receptors; and monocytes and granulocytes showed a well-defined staining pattern for VEGF receptors (*data not shown*).

Surface VEGF staining

To extend our findings of a discrepancy in VEGFR expression between plasma cell CD45 subsets, we stained plasma cells for surface bound VEGF (sVEGF). The median percentage of plasma cells staining positive for sVEGF expression was less than 20% for all plasma cells in all groups studied (Figure 5; Table 3). Of particular interest was the distribution of very small populations of sVEGF positive plasma cells that were segregated according to their CD45 expression. These small populations may be lost in routine methods of analysis that examine median/average staining values or proportions of positive cases. When the median sVEGF was re-analyzed based on CD45 expression, the median sVEGF staining was less than 20% of cells in the CD45-negative subset in all groups studied. In contrast, the CD45-positive plasma cell subset had higher median sVEGF expression in all groups (Table 3). A high proportion of all cases (greater than 70%) in the CD45-positive subset expressed >20% sVEGF.

Table 2. VEGFR expression in normal and malignant plasma cells.

		Panel A	
	VEGF R1	VEGF R2	VEGF R3
AL (n=9)	1/9	1/9	1/9
MGUS (n=11)	1/11	1/11	1/11
SMM (n=14)	2/14	2/14	3/14
MM (n=23)	0/23	0/23	1/23
Normal (n=10)	0/10	0/10	2/10
		Panel B	
	VEGF R1	VEGF R2	VEGF R3
AL (n=6)	4/6	4/6	4/6
MGUS (n=6)	5/6	3/6	3/6
SMM (n=13)	6/13	6/13	5/13
MM (n=21)	14/21	14/21	13/21
Normal (n=6)	5/6	3/6	5/6
		Panel C	
	VEGF R1	VEGF R2	VEGF R3
AL (n=6)	0/6	0/6	0/6
MGUS (n=6)	0/6	0/6	0/6
SMM (n=12)	0/12	0/12	1/12
MM (n=21)	0/21	0/21	0/21
Normal (n=5)	0/5	0/5	1/5

Panel A: Number of cases with >20% expression of VEGFR when all monoclonal plasma cells were included in the gate; panel B: Number of cases with >20% expression of VEGFR when the analysis was restricted to the CD45⁺ plasma cell subset; panel C: Number of cases with >20% expression of VEGFR when the analysis was restricted to the CD45⁻ plasma cell subset.

Table 3. Surface VEGF staining.

All plasma cells	*N.*	*Median % of cells positive*	*Number of cases with greater than 20% expression*
Amyloid	4	18.0	1(25%)
MGUS	9	18.3	3 (33%)
SMM	22	16.8	10 (45%)
MM	17	14.6	4 (24%)
Normal	7	16.5	2 (29%)
CD45-negative PC	*N.*	*Median % of cells positive*	*Number of cases with greater than 20% expression*
Amyloid	2		1(50%)
MGUS	5	15.9	2 (40%)
SMM	19	17.7	8 (42%)
MM	15	11.7	3 (20%)
Normal	6	8.1	2 (33%)
CD45-positive PC	*N.*	*Median % of cells positive*	*Number of cases with greater than 20% expression*
Amyloid	1		1(100%)
MGUS	6	39.2	5 (83%)
SMM	15	53.0	14(93%)
MM	14	47.9	10 (71.4%)
Normal	5	42.2	4 (80%)

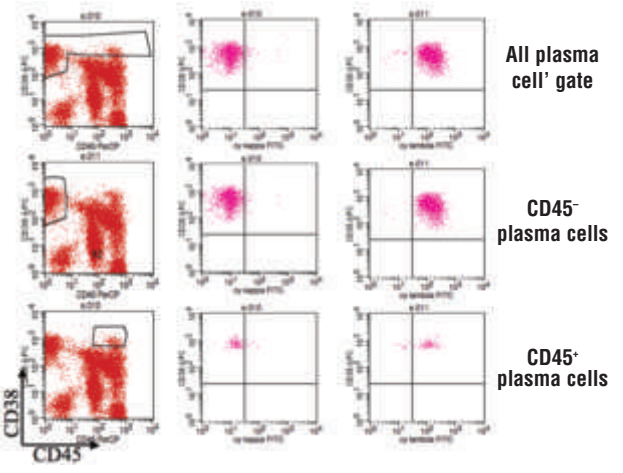

Figure 6. Verification of plasma cell clonality. Clonality was determined for each of the plasma cell and subset populations to ensure that the cells of interest were myeloma plasma cells. In this representative data set all plasma cell populations are clearly clonal for the λ light chain.

Discussion

In MM, there is evidence that increased angiogenesis in the bone marrow is an indication of aggressive disease and a poor prognosis. Multiple factors including VEGF, interleukin-6, basic fibroblastic growth factor and insulin-like growth-factor-1 appear to be involved in autocrine and paracrine relationships between tumor cells and stroma which influence angiogenesis.[11,12,20-22] In this context VEGF is particularly important and has been studied extensively. In addition to its role as a pro-angiogenic factor, VEGF acts as a regulator of cellular growth, survival, and migration. In the marrow microenvironment VEGF appears to be secreted by the tumor cells as well as by the stromal cells and the secretion by myeloma cells is stimulated by interleukin-6 which is, in turn, secreted by stromal cells in response to VEGF stimulation[11] resulting in a positive feedback loop. Adhesion of the myeloma cells to marrow stromal cells results in up-regulation of VEGF as well as interleukin-6 secretion by the tumor cells and the stromal cells.[12] In addition we have previously demonstrated the presence of the VEGF receptors flt-1 and KDR on myeloma cells from patients with different stages of disease and have also demonstrated functional activity for these receptors.[15] Other studies have shown that VEGF has multiple effects on myeloma cells including up-regulation of anti-apoptotic proteins such as Mcl-1 as well as increased migration mediated through activation of protein kinase C and proliferation mediated through the raf kinase pathway.[2,23,24] It is possible that subtle increases in plasma cell VEGF levels between patient groups, such as that observed with the MM cases, could contribute to the growth and survival of these malignant tumor cells. There is also evidence that VEGF can directly or indirectly affect the activity of osteoclasts resulting in the lytic bone lesions characteristic of MM.[25] In this study, we used quantitative flow cytometric methods to standardize quantification of VEGF expression on unique plasma cell populations within a heterogeneous mixture. This technique was highly sensitive and, like cell sorting, allowed discrimination and quantification of cytoplasmic VEGF and VEGF receptors among cell subsets within individual patients.

Our results show that plasma cell cyVEGF expression was slightly higher in MM samples than in SMM/MGUS or AL samples, this perhaps being explained by an over-expression of VEGF in a subset of patients with MM (Figure 2). These results are in agreement with the immunochemistry results of Vacca *et al.* who demonstrated that levels of VEGF-A were higher in MM cases than in MGUS cases.[21] Animal studies have demonstrated that angiogenesis is exquisitely sensitive to very small changes in VEGF concentrations, consistent with the *angiogenic switch* concept.[26,27] The overall higher levels of cyVEGF in MM cases seen in this study could suggest that increased VEGF in MM may be, at least partly, related to an increase in production of VEGF by these plasma cells and may contribute to the higher levels of angiogenesis and disease progression. However, the lack of statistically significant differences (which may be in part related to sample size issues) and the overlapping ranges of cyVEGF expression between the disease groups indicated that factors other than cyVEGF expression are important in disease progression, and support our earlier findings using immunostaining, RT-PCR and western blotting of minimal differences in expression across the disease stages.[16]

Given these results we examined the expression of VEGF receptors on plasma cells and plasma cell subsets to examine whether differences in their expression may contribute to the differences in the effect of VEGF. Previous studies have not been consistent regarding the expression of VEGF receptors on myeloma cells, likely a reflection of the sensitivity of the techniques employed.[10,11,15] Using nested RT-PCR we demonstrated the presence of VEGF R1 and VEGF R2 on most myeloma cell lines and primary patient cells.[15] Direct evaluation of VEGF receptors (VEGF R1, VEGF R2, and VEGF R3) on the surface of plasma cells revealed no differences in receptor expression between the normal controls and the groups of AL, MGUS, SMM, and MM patients. VEGF receptors were expressed at a low level in the plasma cell gate as a whole. This is in agreement with our previous studies that found low expression by nested RT-PCR techniques.[15] The indirect estimation of VEGF receptors using VEGF binding ability as a measure demonstrated higher levels of binding than were expected from the observed level of VEGF receptor expression. This is likely because of the presence of

other receptors for VEGF, such as neuropilin, which may be present on plasma cells. This aspect needs further study. The subset of plasma cells expressing VEGF receptors was identified by a close review of the flow dot plots of receptor-positive cells within the plasma cell gate. This population was restricted primarily to the CD45-positive plasma cell subset, a small fraction of the total clonal plasma cell population. This subset could easily be missed when using other techniques involving the entire plasma cell population.

The differential expression of VEGF receptors based on CD45 expression is of interest. CD45 (leukocyte common antigen) is a tyrosine phosphatase expressed on most hematopoietic cells, including B cells, and its expression decreases as plasma cell maturation progresses. CD45-positive and negative plasma cell subsets have been described, and the CD45-positive subset correlated with growth response to interleukin-6 and better survival after therapy.[28-31] Models of plasma cell development and maturation have suggested that the CD45 plasma cell subsets contain phenotypically and functionally different types of clonal plasma cells. In particular, CD45 expression on plasma cells appears to identify tumor populations with different proliferation, maturation, and apoptotic rates.[32,33] Recent work from our group has correlated higher percentages of plasma cells expressing CD45 with early disease in comparison with late stage MM.[34] CD45 expression was also directly correlated with median overall survival and inversely correlated with high levels of angiogenesis.

The existence of VEGF receptors on the CD45-positive plasma cells may allow an autocrine pathway involving the most actively cycling plasma cell population. The low numbers of plasma cells in this subset may explain the negative findings in other studies.

These findings are very similar to those reported concerning the expression of interleukin-6 receptor.[30] It is likely that the proliferative fraction of tumor cells has higher expression of receptors for various cytokines and hence is responsive to these growth factors. Together, these findings support the possibility of differential plasma cell regulation, which may have therapeutic consequences regarding the proliferative tumor cell fraction. This work highlights potentially significant differences between subsets of patients' plasma cells, which may be relevant for understanding and treating myeloma.

TK: performed the experiments and wrote the paper: MK: performed experiments; SK: design of experiments and writing of the manuscript; VR: design of experiments and writing of the manuscript; TW: design of experiments and writing of the manuscript. JL: design of experiments and writing of the manuscript. The authors declare that they have no potential conflicts of interest.

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