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Circulating angiogenic cytokines in multiple myeloma and related disorders

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ABSTRACT. We investigated the serum concentrations of selected angiogenic cytokines including: vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor beta 1 (TGF-β1) and basic fibroblast growth factor (bFGF) in 162 patients with multiple myeloma (MM), 5 patients with Waldenström's macroglobulinaemia (WM), and 31 healthy controls. Among the MM patients there were 2 cases of primary plasma cell leukemia (PCL) and one case of extramedullary plasmacytoma. The levels of measured cytokines were correlated with the phase and stage of the disease as well as the most important clinical and laboratory parameters associated with disease activity (haemoglobin, creatinine, albumins, calcium, M-component, CRP, β2m, LDH and bone involvement). We have found correlations between serum levels of angiogenic cytokines and some parameters depicting the disease activity and advancement. The serum level of VEGF in MM patients (median 244.5 pg/mL) correlated with serum concentrations of beta-2-microglobulin (β2m) greater than 2.5 mg/L ($p = 0.0005$) and abnormal values of lactate dehydrogenase (> 425 U/L, median – 329.0 pg/mL and < 210 U/L, median – 426.6 pg/mL, $p = 0.004$ and $p = 0.04$ respectively). MM patients in stage III had higher serum levels of HGF (median – 1 411.3 pg/mL) than those in stage I (median – 1 219 pg/mL) ($p = 0.01$) according to Durie and Salmon staging, and those in phase I (at diagnosis) (median 1 555.6 pg/mL) and phase III (in progression) (median 1 309.7 pg/mL) had higher levels than those in phase II (plateau phase) (median 1 047.9 pg/mL) ($p = 0.002$ and $p = 0.02$ respectively). Significantly elevated values of HGF were found in MM patients with anaemia (median – 1 962.0 pg/mL) and hypercalcaemia (median – 2 085.6 pg/mL) ($p = 0.00001$ and 0.04 respectively). TGF-β1 (median – 33.9 ng/mL) correlated positively with high β2m values (> 2.5 mg/L) ($p = 0.04$) and was significantly higher in phase I (median – 40.1 ng/mL) than in phase II (median – 30.9 ng/mL) ($p = 0.03$) of the disease. The concentration of bFGF was significantly higher in stage III of MM (median – 3.1 pg/mL) than in stage I (median – 1.2 pg/mL) ($p = 0.04$). We found that the survival probability was statistically higher for newly diagnosed MM patients with a concentration of VEGF lower than the median value for this cytokine. The concentrations of the cytokines analyzed in patients with Waldenström's macroglobulinaemia (WM), primary plasma cell leukaemia (PCL) and non-secretory (NS) myeloma were not distinguishable from those found in MM patients. We also studied the relationship between the levels of cytokines analyzed and found positive correlations between bFGF and TGF-β1 ($\rho = 0.183$, $p < 0.02$), as well as VEGF and TGF-β1 ($\rho = 0.537$, $p < 0.001$) and VEGF and bFGF ($\rho = 0.197$, $p < 0.02$). In conclusion, our data indicate a strong relationship between angiogenic cytokine serum levels and clinical course as well as selected laboratory parameters of patients with MM.

Keywords: multiple myeloma, Waldenström's macroglobulinaemia, VEGF, bFGF, HGF, TGF-β1, angiogenic cytokines, serum levels, clinical significance

INTRODUCTION

Multiple myeloma (MM) is a malignant disease resulting from the accumulation of monoclonal plasma cells in the bone marrow and proceeding to bone destruction, anaemia and renal failure. A variety of cytokines plays major roles in the growth, differentiation and survival of MM cells [1-3]. There is growing evidence that angiogenesis is increased in MM, especially in active disease [4]. Angiogenic cytokines such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor beta 1 (TGF-β1) and basic fibroblast growth factor (bFGF), which are secreted by malignant and non-malignant cells regulate this process.

VEGF is a multifunctional cytokine stimulating the neovascularization of the tumor. VEGF protein was found in malignant cells from 75% of MM patients studied [5]. The secretion of VEGF was also detected in MM cell lines [6]. The cytokine is probably involved in the progression of MM to plasma cell leukaemia (PCL) [7]. VEGF acts by binding to one of three receptors (VEGFR-1, -2 and -3). VEGFR expression is not observed in the malignant plasma cells, but is markedly elevated in the normal marrow myeloid and monocytic cells surrounding the tumor [8-10]. VEGF serum concentration serves as a predictor of poor prognosis [11]. Recently, Podar *et al.* recognized that VEGF triggers tumor cell proliferation *via* a protein kinase C – independent Raf-1-MEK-extracellular signal-regulated protein kinase pathway [12]. Novel therapies for MM, including antiangiogenic drugs such as thalidomide (Thal) or derivative immunomodulatory drugs, inhibit VEGF secretion. VEGF inhibitors block the adhesion of MM cells to bone marrow stromal cells (BMSCs) and in this way could disrupt malignant proliferation [6]. These observations may determine a new approach to the treatment of MM [13-15].

HGF is a cytoprotective cytokine which causes regeneration of parenchyma after tissue damage in several organs [16]. The receptor for this ligand is c-Met, which was first recognized as an oncogene product responsible for the malignant transformation of an osteosarcoma cell line [17, 18]. MM cells produce HGF and express the receptor c-Met. HGF is overproduced in MM and its level may serve as a prognostic factor [19]. MM patients with elevated HGF levels have an unfavorable prognosis in terms of survival and response to treatment [20]. Derksen *et al.* demonstrated that syndecan-1, a characteristic of plasma cells is a functional co-receptor for HGF, and promotes HGF/c-Met signaling in MM cells [21]. HGF is also one of the osteoclast activating factors [22, 23].

TGF-β1 is a cytokine found in large amounts in α-granules of platelets [24]. It has an inhibitory effect on normal B-cell proliferation and Ig secretion. TGF-β1 is mainly produced by BMSCs, but is also secreted by malignant plasma cells and can regulate interleukin-6 (IL-6) secretion [13, 25]. According to Cook *et al.,* TGF-β1 produced by MM cells plays a significant role in suppressing host T cells and immune responses [26, 27]. Overproduction of TGF-β1 in MM patients was reported by Kroning *et al.* [28].

Basic FGF belongs to the family of fibroblast growth factors which exert multiple functions on various cells types affecting cell proliferation, motility, survival and differentiation, by interacting with tyrosine kinase receptors (FGFRs) and cell-surface heparan sulfate proteoglycans [29]. Expression of bFGF correlates with clinical characteristics of MM and its high level also indicates poor prognosis [4]. However, the level of bFGF may serve as a predictor for good response to the treatment of MM with Thal [30]. Patients responsive to Thal may have significantly higher concentrations of bFGF than non-responsive patients but this observation is not consistent even between the same authors [31, 32]. The FGF receptor 3 (FGFR3) is now recognized as a potential oncogene. Ectopic expression of FGFR3 originates from the translocation t(4;14) occurring in 10-25% of MM patients. FGFR3 is located on der4 and achieves an activated form which prevents apoptosis and contributes to myeloma cell proliferation [33-37].

The present study was focused on the measurement of VEGF, HGF, TGF-β1 and bFGF serum concentration in MM patients using an ELISA, and their correlation with some clinical and laboratory parameters.

PATIENTS AND METHODS

Patients

The study group consisted of 162 patients with MM, 5 patients with Waldenström's macroglobulinaemia (WM) and 31 healthy controls comparable for median age and gender distribution to the patients population. There were two cases of primary PCL and one case of extramedullary plasmacytoma localized in cervical lymph nodes among the MM patients. The patients′ characteristics are shown in Table 1.

There were 83 women and 84 men with a mean age of 66 years (range 32-86) in the patient group. The control group consisted of 18 women and 13 men with a mean age of 64 years (range 47-82). IgG myeloma was diagnosed in 108 patients, IgA in 23, IgD in one case and light chain disease (LCD) in 29 patients. Five patients (all males with a mean age of 76 years, range: 51-88)) were diagnosed with WM. Eighty seven patients were studied at diagnosis (phase I), 38 during the plateau (phase II) and 42 in relapsed disease (phase III). The plateau phase was regarded as following 3 months of stable disease and achieving complete (disappearance or decrease of M – component > 90%) or partial (M – component decrease > 50%) remission after chemotherapy. In 40 patients we performed serial examinations of cytokines in different phases of disease (in phase I & II – 18 patients, I & III – 18 and II & III – 4). The patients were staged clinically according to the Durie and Salmon system [38]. Thirty four patients were diagnosed in clinical stage I (IA – 34, IB – 0), 37 – II (IIA – 30, IIB – 7) and 96 – and III (IIIA – 71, IIIB – 25). Renal failure (substage B) was diagnosed when the level of creatinine was > 2 mg/mL

Table 1
Clinical and laboratory characteristics of MM patients

Clinical/laboratory features		Number of patients (%)
Total		162(100)
Sex(male/female)		(79/83)
Age (years) median (range)		66.3 67 (32-86)
Phase	I	82 (51)
	II	38 (23)
	III	42 (26)
Stage	I	34 (21)
	II	35 (22)
	III	93 (57)
M-Protein	IgG	108 (67)
	IgA	23 (14)
	light chain	29 (18)
	IgD	1 (0.5)
	extramedullary	1 (0.5)
Serum creatinine > 2mg/dL		32 (20)
Haemoglobin < 10g/dL		76 (47)
Albumin < 30g/L		38 (23)
Calcium	< 9 mg/dL	42 (26)
	> 11 mg/dL	20 (12)
LDH	> 425 U/L	59 (36)
	< 210 U/L	25 (15)
CRP > 5mg/L		78 (48)
β2 microglobulin > 2.5 mg/L		106 (65)
Bone disease		106 (65)

and was observed in 32 patients. Advanced bone disease was defined as when multiple lytic lesions were observed on X-ray examination. Patients were treated with different protocols adjusted for age and clinical status. Two of the MM patients received high dose chemotherapy.

Laboratory examinations

All laboratory tests were performed on the day of blood sampling for the cytokine assay. Each patient's blood sample was examined for the following parameters: complete haemogram, serum protein electrophoretogram, urea nitrogen, creatinine, calcium, lactate dehydrogenase (LDH), C-reactive protein (CRP), β2-microglobulin (β2m). Serum β2m levels were measured by means of an RIA kit. Correction for renal function was not calculated. Levels of immunoglobulins, albumin and CRP were measured by nephelometry. Normal values for CRP were considered as below 5.0 mg/L. Bone marrow examination was performed in all cases as well as an X-ray survey of flat bones i.e. in 150 patients.

Blood sampling

Venous blood samples were collected on the day of physical examination and laboratory tests. They were collected in pyrogen-free tubes, allowed to clot for 1 hour at 4^oC and centrifuged at 2 000g for 10 minutes. The sera obtained were aliquoted into separate vials and stored at – 80^oC until assayed for cytokines. Serum samples from each patient were randomly coded and the measurement of cytokines was carried out without the knowledge of the patient's clinical status or laboratory data.

Cytokine assays

The sera obtained were assayed for the concentration of VEGF, HGF, TGF-β1 and bFGF. The commercially available enzyme linked assay (ELISA) kits – Quantikine, R & D Systems Inc., USA, were used following the manufacturer's instructions. The adsorbance was read at 492 nanometers. The appropriate recombinant human cytokine was used to generate the standard curve for each assay. The concentration of cytokines in the samples was determined by the interpolation from the standard curve. This procedure has been described precisely in our previous works [39, 40]. The sensitivity limit for VEGF was 5.0 pg/mL, HGF – 40 pg/mL, TGF β1 – 7 pg/mL and bFGF – 3 pg/mL.

Statistical analysis

Statistical analysis was performed according to the procedures of the Statistics for Windows package on the IBM-compatible computer. The significance of cytokine levels in the different subsets of patients was assessed by the Mann-Whitney U, Kruskall-Wallis, Cochran-Cox and Student′s *t* tests where appropriate. P values obtained from pair-wise comparisons were corrected using the Bonferroni method. Zero values indicating undetectable levels of cytokines were included in all analyses. Correlations of analyzed cytokines with laboratory parameters and between cytokines were calculated with Spearman′s rank sum correlation coefficient. All cited are two sided and designated as statistically significant at the level < 0.05. The overall survival was estimated using the Kaplan-Meier method from onset of the disease. All endpoints were updated on 31st of August 2002. The survival period was analyzed in two groups of patients for each cytokine – below and above the median concentration value, and compared using F-Cox test.

RESULTS

The serum concentrations of VEGF, HGF, TGF-β1 and bFGF in all patients and healthy controls are shown in Table 2. Serum levels of VEGF, HGF and bFGF were detectable in all patients and all controls. Mean concentration of VEGF in the MM group was 333.1 pg/mL (range: 22.6 – 2065.3). The mean concentration of this cytokine in controls was 329.0 (range: 31.2 – 1114.2) ($p > 0.05$). The mean concentration of VEGF was highest in phase I (mean 393.5 pg/mL) and in stage IIIB of MM (mean 397.4 pg/mL), but there was no statistically significant difference between the level of this cytokine and the phase or stage of the disease (Table 2 and 3). The mean concentration of VEGF in MM patients with elevated β2m (> 2.5 mg/L) was 391.3 pg/mL, and was significantly higher than in those with lower levels of β2m – 217.1 pg/mL ($p = 0.0005$). Similar results were obtained for the correlation of VEGF and LDH. In patients with increased concentrations of LDH (> 425 U/L), the mean level for VEGF was significantly higher than in patients with normal LDH values (419.6 *versus* 234.3 pg/mL) ($p = 0.004$). Moreover, in patients with low levels of LDH (< 210 U/L) the mean VEGF concentration was also higher than in patients with normal LDH concentrations (426.6 versus 234.3 pg/mL) ($p = 0.04$). The level of this cytokine was also higher in MM patients with advanced bone disease (mean: 387.7 pg/mL in stages 2-3) than in stages 0-1 (mean: 209.7) ($p = 0.003$). VEGF concentration did not correlate with other laboratory parameters analyzed (Table 4).

The VEGF concentration in patients with WM was similar to that observed in MM patients (mean: 322.6 pg/mL, range: 32.4 – 886.7 pg/mL). In two patients with primary PCL, the VEGF concentrations were 78.1 and 174.1 pg/mL, and in non-secretory (NS) myeloma – 374.4 pg/mL.

The mean HGF level in MM patients was 1859.4 pg/mL (range: 471.7 – 4673.8). It was significantly higher than in healthy persons (mean: 1063.3 ± 483.5, range 433.1 – 2474.1) ($p = 0.0001$). The highest values of this cytokine were detected in phase I of the disease (mean: 2163.9 pg/mL). The concentration of HGF was significantly higher in phase I than in phase II (mean: 1320.0 pg/mL) ($p = 0.002$). At progression (phase III), the HGF concentration rose again and was significantly higher than in phase II (mean: 1063.3 pg/mL) ($p = 0.02$). Similar correlations were found when comparing HGF levels with stage of MM. The highest concentrations were detected in stages IIIB (mean: 2519.8 pg/mL) and IIB (mean: 2412.5 pg/mL) of the disease. There was a statistically significant difference between the level of HGF in stages IA (mean: 1538.8 – pg/mL) and IIIB ($p = 0.013$). Moreover, the HGF level was significantly higher in patients with anaemia, renal failure and hypercalcaemia (Table 4). No correlation was found between HGF and other clinical and laboratory parameters analyzed. In patients with WM, the mean level for HGF was 1169.1 (range 567.4 – 2035.3 pg/mL). However, high concentra-

Table 2
Serum levels of VEGF, HGF, TGF-β1 and bFGF in patients with different phases of MM (phase I – diagnosis, phase II – plateau, phase III – progression) and in control group.

Cytokine/receptor	All MM patients n = 162 (a)	MM phase I n = 82 (b)	MM phase II n = 38 (c)	MM phase III n = 42 (d)	Normal control group n = 17 (e)	Statistically significant comparison
VEGF						
Mean ± SD	333.1 ± 318.4	393.5 ± 373.1	261.6 ± 192.2	280.1 ± 273.4	329.0 ± 250.2	
Median	244.5	295.6	219.4	203.0	274.8	
Range	22.6 – 2 065.3	37.8 – 2 065.3	23.6 – 971.9	22.6 – 1 500.0	31.2 – 1 114.2	
HGF						b & c - p = 0.002026
Mean ± SD	1 859.4 ± 1 296.5	2 163.9 ± 1 450.5	1 320.0 ± 896.0	1 753.0 ± 1 119.4	1 063.3 ± 483.5	b & e – p = 0.000121
Median	1 319.5	1 555.6	1 047.9	1 309.7	1 017.7	c & d – p = 0.023279
Range	471.7 – 4 673.8	471.7 – 4 673.8	579.1 – 4 673.8	548.8 – 4 673.8	433.1 – 2 474.1	d & e – p = 0.002315
						a & e – p = 0.0001
TGF-β1						b & c – p = 0.027779
Mean ± SD	36.4 ± 17.8	39.5 ± 19.4	30.6 ± 10.5	35.6 ± 18.7	45.4 ± 16.5	a & e – p = 0.0063
Median	33.9	40.1	30.9	32.6	41.4	c & e – p = 0.000270
Range	3.4 – 98.5	5.1 – 98.5	9.2 – 62.3	3.4 – 73.5	19.6 – 82.5	d & e – p = 0.030390
bFGF	n_1 = 119	n_1 = 62	n_1 = 26	n_1 = 31	n_1 = 18	b & e – p = 0.013734
Mean ± SD	6.0 ± 13.4	5.6 ± 8.5	6.0 ± 17.4	6.6 ± 16.9	2.0 ± 3.2	d & e – p = 0.027917
Median	2.1	2.1	1.5	2.6	0.8	
Range	0.0 – 107.9	0.0 – 48.9	0.0 – 106.7	0.0 – 107.9	0.0 – 15.7	a & e – p = 0.0496

n = number of individuals investigated, n_1 = number of individuals with detectable cytokines
values for VEGF, HGF and bFGF – in pg/mL, TGF-β1 – in ng/mL

Table 3
Serum levels of VEGF, HGF, TGF-β1 and bFGF in patients at different stages of MM

Cytokine/receptor	all MM patients n = 162 (a)	stage IA n = 34 (b)	stage IIA n = 28 (c)	stage IIB n = 7 (d)	stage IIIA n = 68 (e)	stage IIIB n = 25 (f)	Normal control group n = 31 (g)	Statistically significant comparison
VEGF								
Mean ± SD	333.1 ± 318.4	241.4 ± 171.4	301.7 ± 245.3	226.5 ± 123.7	375.7 ± 380.3	397.4 ± 368.6	329.0 ± 250.2	
Median	244.5	232.9	219.6	226.5	241.2	329.1	274.8	
Range	22.6 – 2 065.3	23.6 – 582.5	60.0 – 971.9	72.6 – 414.5	22.6 – 2 065.3	64.5 – 1 779.1	31.2 – 1 114.2	
HGF								b & f - p = 0.013233
Mean ± SD	1 859.4 ± 1 296.5	1 538.8 ± 1 134.6	1 673 ± 1 273.7	2 412.5 ± 1 527.8	1 768.0 ± 1 152.9	2 519.8 ± 1 586.0	1 063.3 ± 483.5	c & g – p = 0.025120
Median	1 319.5	1 225.8	1 157.3	2 560.7	1 312.8	2 088.2	1 017.7	d& g – p = 0.030380
Range	471.7 – 4 673.8	572.4 – 4 673.8	471.7 – 4 673.8	609.3 – 4 673.8	548.8 – 4 673.8	656.3 – 4 673.8	433.1 – 2 474.1	e &g – p = 0.000851
								f & g – p = 0.000156
								a & g – p = 0.0113
TGF-β1								a & e – p = 0.0057
Mean ± SD	36.4 ± 17.8	32.9 ± 15.3	36.4 ± 15.2	28.7 ± 19.4	38.2 ± 20.8	35.9 ± 13.9	45.4 ± 16.5	b & g – p = 0.006795
Median	33.9	31.3	35.7	18.4	33.6	37.1	41.4	c & g – p = 0.041950
Range	3.4 – 98.6	6.0 – 69.6	14.3 – 67.6	13.1 – 68.3	3.4 – 98.6	10.5 – 55.1	19.6 – 82.5	d & g – p = 0.018605
								e & g – p = 0.039856
bFGF	n_1 = 119	n_1 = 22	n_1 = 20	n_1 = 4	n_1 = 56	n_1 = 17	n_1 = 18	b & e – p = 0.039983
Mean ± SD	6.0 ± 13.4	4.1 ± 6.6	6.3 ± 19.3	1.0 ± 1.1	8.0 ± 15.0	4.1 ± 4.8	2.0 ± 3.2	d & e – p = 0.23583
Median	2.1	0.8	2.2	0.6	3.3	2.1	0.8	e & g – p = 0.001286
Range	0.0 – 107.9	0.0 – 26.9	0.0 – 106.7	0.0 – 2.5	0.0 – 107.9	0.0 – 15.4	0.0 – 15.7	a & g – p = 0.0115

n = number of individuals investigated, n_1 = number of individuals with detectable cytokines
values for VEGF, HGF and bFGF – in pg/mL, TGF-β1 in – ng/mL

Table 4
Serum levels of VEGF, HGF, bFGF and TGF-β1 in MM patients correlated with clinical and laboratory parameters. (m ± s) (values of VEGF, HGF and bFGF in pg/mL, TGF-β1 – in ng/mL)

Cytokine	VEGF 1	HGF 2	TGF-β1 3	bFGF 4	Statistical significance
Haemoglobin					1. NS
> 10 g/dL	303.2 ± 285.1	1 450.3 ± 1 059.9	37.3 ± 16.6	5.2 ± 12.5	2. p = 0.000013
< 10 g/dL	364.2 ± 350.7	2 270.7 ± 1 379.4	34.7 ± 19.0	6.9 ± 14.2	3. NS
					4. NS
Creatinine					1. NS
> 2 mg/dL	360.0 ± 336.6	2 496.3 ± 1 549.6	34.4 ± 15.2	3.5 ± 4.5	2. p = 0.008089
< 2 mg/dL	326.0 ± 315.5	1 690.3 ± 1 171.3	36.5 ± 18.4	6.6 ± 14.6	3. NS
					4. NS
Albumins					1. NS
> 30 g/L	295.7 ± 244.2	1 769.8 ± 1 229.5	34.6 ± 16.1	5.3 ± 11.3	2. NS
< 30 g/L	448.7 ± 470.0	2 084.7 ± 1 447.5	40.7 ± 22.0	8.5 ± 18.3	3. NS
					4. NS
Calcium					1. NS
(a) > 11 mg/dL	322.5 ± 259.9	2 440.4 ± 1 436.9	33.8 ± 13.4	4.9 ± 5.5	2. a vs b - p = 0.036
(b) 9-11 mg/dL	324.7 ± 313.2	1 771.0 ± 1 229.5	35.4 ± 18.8	5.8 ± 12.6	b vs c - p = 0.039
(c) < 9 mg/dL	357.6 ± 362.6	1 746.7 ± 1 315.2	38.8 ± 17.0	7.1 ± 17.4	3. NS
					4. NS
M-component					1. b vs c – p = 0.019
(a) IgG	330.0 ± 327.3	1 797.1 ± 1 262.9	36.1 ± 17.7	6.3 ± 12.5	2. NS
(b) IgA	262.9 ± 256.9	2 357.8 ± 1 531.5	36.4 ± 19.6	3.3 ± 4.7	3. NS
(c) Light chain	408.6 ± 329.8	1 771.5 ± 1 189.4	39.0 ± 16.7	7.4 ± 20.1	4. NS
CRP					1. p = 0.000011
> 5 mg/L	446.6 ± 392.7	2 032.5 ± 1 392.8	38.2 ± 19.3	6.8 ± 14.0	2. NS
< 5 mg/L	222.9 ± 162.3	1 667.7 ± 1 162.2	33.5 ± 15.6	5.2 ± 12.7	3. NS
					4. NS
β2m					1. p = 0.000492
> 2.5 mg/L	391.3 ± 359.2	1 965.9 ± 1 361.1	38.4 ± 19.5	6.7 ± 15.8	2. NS
< 2.5 mg/L	217.1 ± 170.1	1 609.5 ± 1 104.0	31.6 ± 12.9	4.7 ± 5.9	3. NS
					4. p = 0.044624
LDH					1. a vs b - p = 0.004
(a) > 425 U/L	419.6 ± 363.1	1 959.6 ± 1 381.6	37.6 ± 18.5	8.3 ± 19.6	b vs c - p = 0.044
(b) 210-425 U/L	234.3 ± 157.5	1 643.8 ± 1 084.3	34.5 ± 17.3	4.4 ± 5.9	2. NS
(c) < 210 U/L	426.6 ± 464.4	2 183.1 ± 1 547.7	37.3 ± 17.9	5.9 ± 11.4	3. NS
					4. NS
Bone disease					1. p = 0.002620
0-1 stage	209.7 ± 164.6	1 828.9 ± 823.0	44.3 ± 19.2	4.6 ± 6.6	2. NS
2-3 stage	387.7 ± 325.5	1 968.9 ± 1 298.5	38.9 ± 19.2	4.8 ± 6.6	3. NS
					4. NS

tions of HGF were found in our 2 cases of PCL (2538.6 and 4673.8 pg/mL). In NS myeloma this concentration was low (690.7 pg/mL).

The TGF-β1 level in healthy controls was higher than in MM patients (mean – 45.4 *versus* 36.4 ng/mL) ($p = 0.006$). Analyzing the MM group, the highest values of this cytokine were found in phase I of the disease (mean: 39.5 ng/mL), next, in phase III (mean: 45.4 ng/mL) and the lowest in the plateau phase (mean: 30.6 ng/mL). The difference between phase I and II was statistically significant ($p = 0.03$). A similar relationship was found in the analysis of the TGF-β1 levels in different stages of MM. In advanced stages (IIIA and IIIB), the level was higher (mean in IIIA: 38.2 ng/mL, in IIIB: 35.9 ng/mL) than in stage IA (mean: 32.9 ng/mL), although this difference was not statistically significant. A correlation between TGF-β1 and clinical and laboratory parameters was found only in patients with high β2m levels: the values of TGF-β1 were higher (mean: 37.6 ng/mL) than in the patients with low levels of β2m (mean: 29.8 ng/mL) ($p = 0.04$). Other parameters analyzed did not influence the level of this cytokine

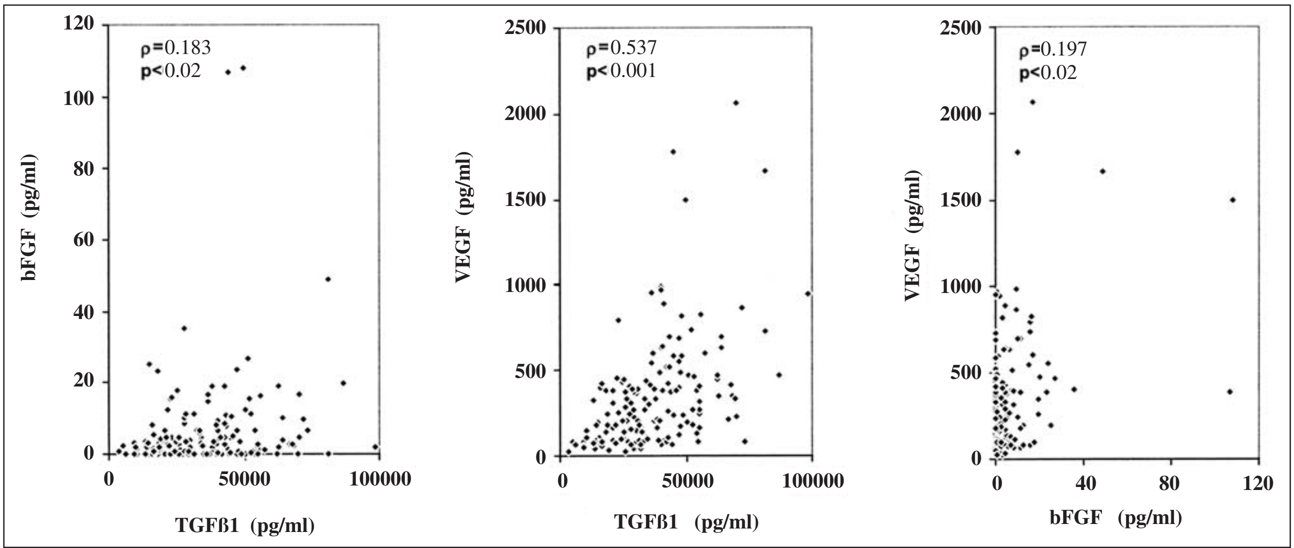

Figure 1
Correlation between serum levels of VEGF, TGF-β1 and bFGF.

(Table 4). In WM patients, the mean level of TGF-β1 was 21.9 ng/mL (range: 10.4 – 41.0). Similar concentrations were found in the NS myeloma patient (22.0 ng/mL) and PCL cases (14.3 and 22.5 ng/mL).

A serum concentration of bFGF was detectable in 119 out of 162 patients (73%) and in 18 out of 31 healthy controls (58%). The mean level of this cytokine was statistically significantly higher in the patient group (mean: 6.0 pg/mL *versus* 2.0 pg/mL) ($p = 0.01$). The level of this cytokine was higher in MM patients in stage IIIA (mean: 8.0 pg/mL), in comparison with those in stage IA (mean: 4.1 pg/mL) ($p = 0.04$). We failed to find any correlation between the value of bFGF and the clinical as well as laboratory parameters depicting the advancement of the disease (Table 4). In WM patients, detectable levels were found in 4 out of 5 cases (80%), with the mean value of 9.0 pg/mL. In the NS myeloma case, the level of bFGF was low (1.6 pg/mL) and in PCL patients was contrasting (3.2 and 15.4 pg/mL).

In patients with monoclonal protein of the IgD class diagnosed in phase III, we found rather low values of angiogenic cytokines (VEGF: 59.2 pg/mL, HGF: 843.8 pg/mL, TGF-β1: 9.2 ng/mL and bFGF: 0.0 pg/mL).

Serial examinations of cytokines analyzed in different phases of MM showed VEGF level raised in active phases of the disease (level in phase I > phase II – 12/18 patients, III > II – 4/4, III > I – 6/18). Similar results were obtained on analyzing other cytokines (HGF – I > II – 11/18, III > II – 2/4, III > I – 6/18, TGF-β1 – I > II – 9/18, III > II – 3/4, III > I – 13/18, bFGF – I > II – 8/18, III > II – 2/4, III > I – 9/18). The correlation between serum levels of cytokines analyzed showed a positive relationship between TGF-β1 and bFGF ($\rho = 0.183$, $p < 0.02$), VEGF and TGF-β1 ($\rho = 0.537$, $p < 0.001$) as well as VEGF and bFGF ($\rho = 0.197$, $p < 0.02$) (Figure 1).

The concentration of VEGF, TGF-β1 and bFGF correlated with the number of platelets in MM patients ($\rho = 0.713$, $p < 0.001$; $\rho = 0.753$, $p < 0.001$; $\rho = 0.188$, $p < 0.02$ respectively) (Figure 2). A positive correlation was also found between the level of β2m and VEGF, bFGF as well as TGF-β1 levels ($\rho = 0.304$, $p < 0.001$; $\rho = 0.051$, $p < 0.02$; $\rho = 0.213$, $p < 0.006$ respectively) (Figure 3). We did not find any correlation between the concentration of angiogenic cytokines measured and Ig class except HGF and M component of the IgG type ($\rho = 0.279$, $p < 0.004$).

The concentration of the cytokines analyzed did not influence the overall survival of patients except VEGF and FGF in 82 newly diagnosed myeloma cases. A VEGF concentration lower than the median value correlated with the probability of longer survival in comparison with patients with higher levels ($p < 0.04$). A serum concentration of FGF higher than its median value predicted longer survival than lower values ($p < 0.04$), (Figure 4).

DISCUSSION

The aim of our study was to evaluate the serum concentration of four angiogenic cytokines and to correlate it with disease activity and progression, as well as selected clinical and laboratory parameters. We estimated the serum levels of VEGF, HGF, TGF-β1 and bFGF, which form a group of cytokines acting positively on the growth of microvessels in MM tumours. The levels of VEGF, HGF and TGF-β1 were detectable in the serum of all 167 patients and all 31 healthy controls. Detectable levels of FGF were found in 73% of patients and 58% of controls. Most authors report the detection of angiogenic cytokines in serum of the vast majority of patients with plasma cell disorders [41-43], other haematological malignancies [44], cancers [45] and autoimmune disorders [46].

In our study, the concentration of VEGF in MM patients was similar to that found in healthy controls. However, higher values of VEGF were found in patients with elevated serum concentrations of β2m and abnormal values of LDH (> 425 U/L and < 210 U/L), as well as advanced stages of bone disease. We did not find a significant increase of VEGF in advanced stages and the progressive phase of MM. We have also stated that low VEGF values in newly diagnosed MM patients may predict longer survival. Our results are in line with earlier

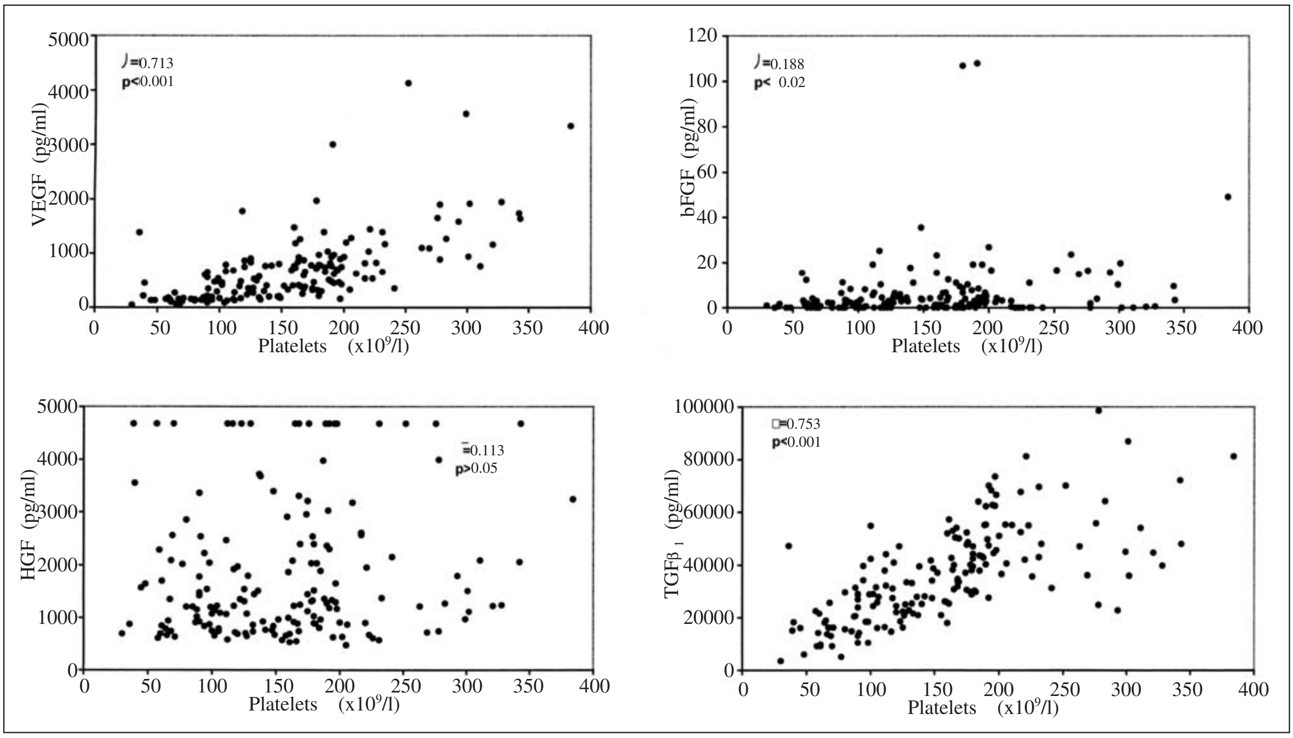

Figure 2
Correlations between serum levels of VEGF, HGF, bFGF and TGF-β1 and platelet numbers.

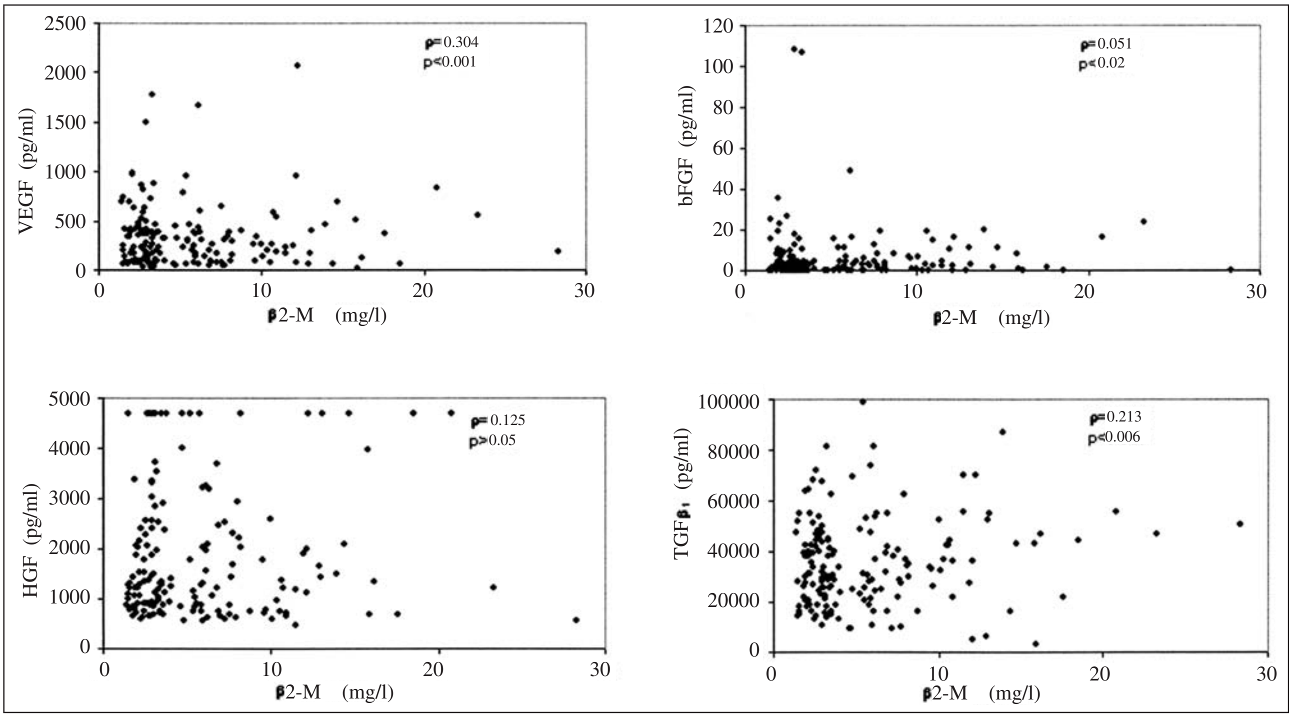

Figure 3
Correlations between serum concentrations of angiogenic cytokines (VEGF, HGF, bFGF and TGFβ$_1$) and serum β$_2$-microglobulin concentrations in MM patients.

observations. Sezer *et al.* suggested that values for VEGF depended on the stage of MM, but the authors failed to prove the significantly elevated value of this cytokine in comparison with the control group. There was also no difference between the concentrations of VEGF in the different stages of MM [41]. Other authors confirm the association of the VEGF concentration with some markers of active disease. Di Raimondo *et al.* measured VEGF using plasma of peripheral blood and bone marrow to avoid involving the amount of VEGF released from platelets. The authors found greater amounts of VEGF in bone marrow plasma, in previously treated

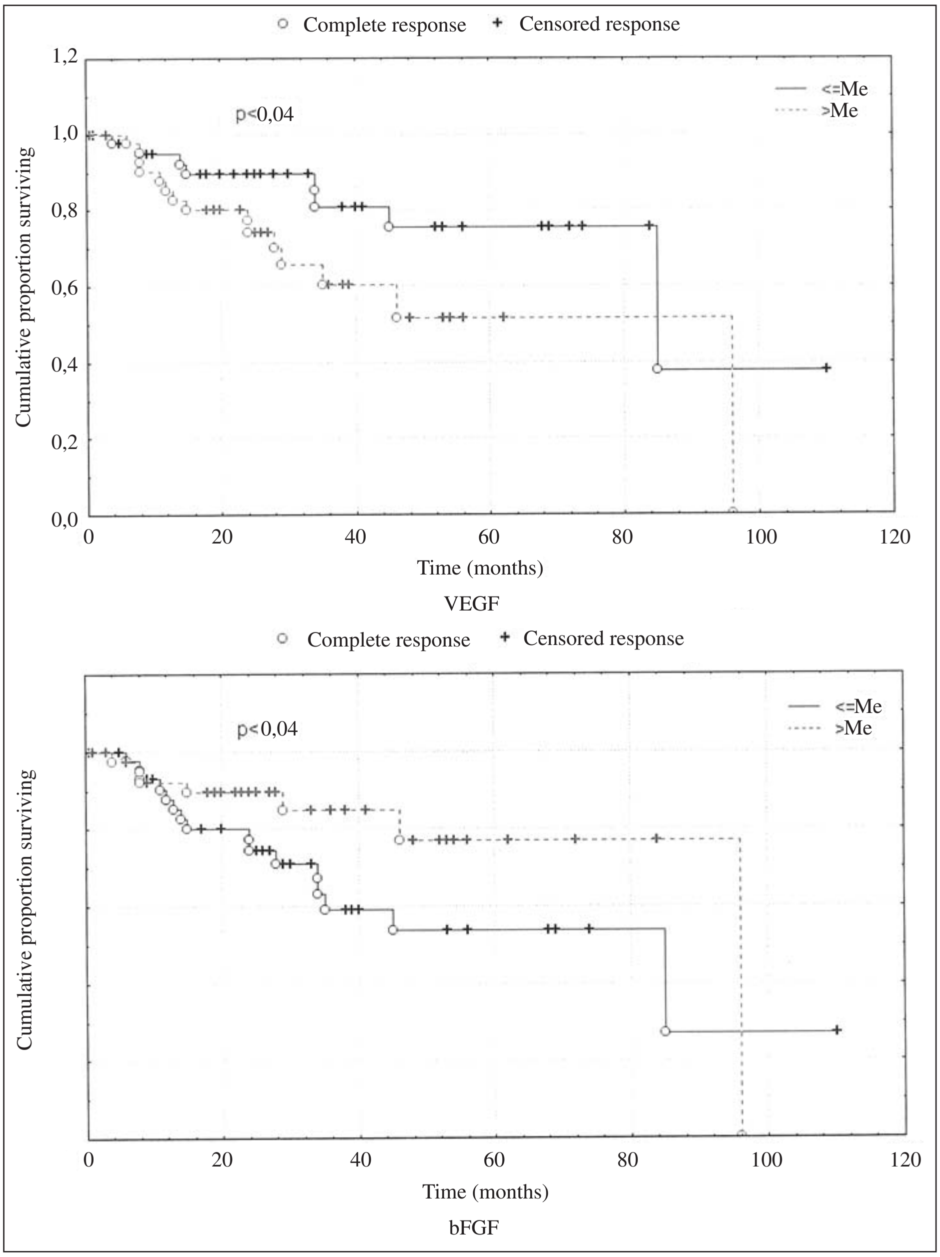

Figure 4
The probability of survival for newly diagnosed MM patients with high and low (> median and < median) concentrations of VEGF and bFGF.

patients and in advanced stages of MM. VEGF correlated with markers of disease activity such as CRP and β2m [42].

HGF is regarded as another important factor involved in the pathogenesis of MM and related bone disease. In our study, the concentration of this cytokine was significantly higher in MM patients than in controls. The results obtained indicate that the HGF level is elevated in MM patients, especially in the advanced phases and stages of disease. MM patients in stage IIIB had higher levels of HGF than those in stage IA. There were also higher levels in phase I (at diagnosis) and in phase III (in progression) than in phase II (plateau phase). Significantly elevated values of HGF were found in MM patients with anaemia, hypercalcaemia and renal failure which complicated the disease. Seidel *et al.* evaluated the HGF level in a large group of 398 MM patients and found a significant difference between the HGF concentration in MM patients and the controls, as in our series. The level of this cytokine was elevated in about 40% of MM patients at diagnosis. HGF seemed to be a significant prognostic factor, because the group of patients with low values lived statistically longer than others with higher levels of HGF. In serial samples, HGF levels were higher at diagnosis and relapse than at response. The same authors studied the concentration of HGF and syndecan-1 in the marrow compartment of MM patients and found that it was much higher than in healthy controls and controls with other diseases [20, 43].

We have reported that the TGF-β1 levels in healthy controls were higher than in MM patients. The reason for these high serum levels of VEGF and TGF-β1 in controls is that platelet α – granules contain large quantities of these cytokines, which may be released from activated platelets during the collection of serum samples. We found a significant correlation between the concentration of VEGF and TGF-β1 (also bFGF) and the number of platelets in those patients analyzed. The same observations were noted by others [41]. Elevated serum values for these cytokines are also reported in patients with atherosclerosis and diabetes [47], so the fact that the control group consisted of persons in older age groups may be another reason for the results obtained. The highest values for TGF-β1 in MM patients we found in phase I and III, as well as stage III of the disease and the lower values in the plateau phase and stage I. Significant elevation of TGF-β1 was found in patients with high serum values of β2m. Kyrtsonis and colleagues also measured the serum levels of TGF-β1 in MM patients. In controls and thrombocytopenic patients, the concentration of this cytokine was very low. The normal range was observed in patients with normal polyclonal immunoglobulins and the higher values were characteristic of the MM patients with immunoparesis. Serum levels of TGF-β1 fluctuated in the same patient at different times but not in relation to remission or relapse. No correlation with disease stage, Ig subtype or with known prognostic factors for MM (CRP, β2m, IL-6) was found [24]. Borset *et al.* reported the additive effect of HGF and TGF-β1 on the proliferation of cells in culture [17]. Brown *et al.* found normal serum levels of TGF-β1 in most patients studied. Cytoplasmic TGF-β1 was increased in plasma cells during progressive disease [48]. Our results suggest that the TGF-β1 concentration is strongly dependent on the platelet number, and that the group of MM patients should be analysed separately, and not compared with healthy persons. The other option for further investigation of this cytokine is to use platelet-poor plasma.

Serum bFGF was detectable in 73% of patients and 58% of controls in our study. The concentration of this cytokine was significantly higher in the patients group than in healthy donors. The highest values were found in patients with advanced stage and progressive disease. However, we failed to find any correlation between the value of bFGF and clinical or laboratory parameters. Unexpectedly, we found that values of bFGF higher than the median measured at the beginning of the disease were predictive of longer survival than lower values. Sezer *et al.* stated that patients with MM, especially in advanced stages, have much higher values of bFGF than MGUS patients and healthy controls. According to these authors, the angiogenic cytokine level analysed decreases after successful chemotherapy. There was no decrease in the level of this cytokine in non-responders [41]. The role of bFGF in MM is still unclear. This cytokine was regarded as the predictor for thalidomide therapy, but opinion is divided [31, 32]. Shoham *et al.* reported that bFGF sustains the stroma suppressive effect for plasmacytoma growth [49]. Diamond *et al.* stated that patients with MM had significantly lower serum concentrations of FGF than patients with MGUS [50].

We also studied the interactions between the levels of angiogenic cytokines analyzed and found positive correlations between FGF and TGF-β1, VEGF and TGF-β1, as well as VEGF and FGF. According to Di Raimondo *et al.* VEGF and bFGF there was no correlation between, while HGF showed a weak correlation with VEGF and a stronger one with bFGF. The authors did not find higher values of these cytokines in patients with extramedullary or solitary plasmacytoma [42]. Similar observations were noted on the basis of our studies.

In conclusion, the present study is the first report analyzing the serum concentration and clinical significance of four proangiogenic cytokines in a large group of patients with MM, and related disorders. We have found that each of peptides evaluated has a specific correlation with the clinical course of multiple myeloma and with each other, although their precise role and interactions need further research.

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