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RESEARCH

The Significance of non Correlation Between Interleukin-8 Serum Levels with Bone Marrow Microvascular Density in Patients with Myeloma Multiple

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Abstract In multiple myeloma (MM), angiogenesis plays a substantial role in disease progression. Interleukin-8 (IL-8), a pro-inflammatory chemokine with potent pro-angiogenic properties, has been implicated in the pathophysiology of MM. The aim of the study is to measure serum levels of IL-8 in MM patients and to correlate them with markers of angiogenesis, such as circulating levels of platelet derived growth factor-AB (PDGF-AB) and angiogenin (Ang), and bone marrow microvascular density (MVD). Fifty-three newly diagnosed MM patients, 23 of them, who reached plateau phase after effective treatment and 20 healthy controls, were studied. Serum levels of PDGF-AB, Ang and IL-8 were measured by ELISA, whereas bone marrow MVD was estimated by immunohistochemical staining of vessels with anti-CD31. All measured parameters were higher in MM patients compared to controls and in increased disease stages. They all also significantly decreased in plateau phase. IL-8 correlated positively with Ang and PDGF-AB, but not with MVD. The circulating levels of IL-8, PDGF-AB and Ang are elevated in patients with MM. The lack of correlation between IL-8 with MVD suggests that its levels represent the inflammatory element of MM disease and the participation in angiogenesis process is rather complex with multifactorial mechanisms.

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Introduction

Multiple myeloma (MM) is a debilitating B cell neoplasia, being part of a diseases' spectrum ranging from monoclonal gammopathy of undetermined significance to plasma cell leukemia. The molecular basis for the progression of MM is quite complex. Both genetic aberrations in MM cells and evolving interactions between different cell types can occur. Malignant plasma cells are believed to rely heavily on their interactions with elements of the surrounding microenvironment, including osteoclasts, osteoblasts, endothelial cells (EC), macrophages and bone marrow stromal cells [1].

Angiogenesis, the formation of new blood vessels, is a constant hallmark of MM bone marrow. It can be evaluated by measuring microvascular density (MVD), using immunohistochemically CD31 stained paraffin-embedded bone marrow biopsies and counting the mean number of vessels per area in each sample [2]. Various cytokines have been implicated in the angiogenic process. Among them, platelet derived growth factor-AB (PDGF-AB) has been reported to be a potent stimulator of angiogenesis in many solid tumors and hematological malignancies, including MM [3]. Angiogenin (Ang) is another potent angiogenic factor and an acute phase protein, activating EC, while it is also secreted by proximal tubular epithelial cells under hypoxic conditions and may modulate vascular remodeling in the renal interstitium [4]. IL-8 is a chemokine targeting neutrophils and lymphocytes. It plays a significant role in inflammatory and tumor associated angiogenesis and furthermore, in tumor progression [5]. Moreover, IL-8 induces proliferation and chemotaxis of MM and other tumor cells, such as melanoma [6]. It has been shown that stromal IL-

8 production is induced by interleukin-1β (IL-1β), parallels with MM disease activity and correlates with bone marrow angiogenesis [5, 7]. We have already shown in the past that serum levels of IL-8 have been correlated with the known direct angiogenic cytokine hepatocyte growth factor (HGF), but not with vascular endothelial growth factor (VEGF) neither MVD [8].

The purpose of this study was to evaluate serum concentrations of IL-8, of other angiogenesis-related cytokines, such as PDGF-AB and Ang, and bone marrow MVD, at diagnosis, various stages and after effective treatment in MM patients, in order to estimate the role of IL-8 in the angiogenic process.

Materials and Methods

Patients

Fifty-three newly diagnosed MM patients (26 male and 27 female; median age 69 years, range 36–89 years) were enrolled in the study. Patients with renal or liver impairment, previous or current other forms of malignancy, other bone marrow diseases, HIV, other uncontrolled infectious diseases, use of immunomodulatory drugs or incapability to consent, were excluded from the study. According to international staging system (ISS), 14 were in stage I, 18 in stage II and 21 in stage III of the disease. The types of monoclonal proteins were: IgG for 31 patients, IgA for 16 patients and light chain disease for 6 patients. None of the patients had received any kind of myeloma-related therapy prior to examination. Twenty-three of them, who reached the plateau phase after effective treatment, were also re-evaluated. Twenty, age and sex matched, healthy volunteers were used as controls. The study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent for the study was obtained from all subjects, prior to their inclusion in the study.

Methods

Serum samples were collected from patients and controls, aliquoted into separate vials, stored at −70 °C, and assayed at the end of the study, in order to avoid inter-assay variability. Serum levels of PDGF-AB, Ang and IL-8 were measured by the solid-phase sandwich enzyme-linked immunosorbent assays (ELISA), employing monoclonal anti-PDGF-AB, Ang and IL-8 (Quantikine R&D System Minneapolis MN, USA), according to manufacturer's instructions.

Bone marrow biopsies were performed during local anesthesia, from posterior iliac crest. They were fixed in 10 % formalin, decalcified in 10 % EDTA for 48 h and embedded in Paramat extra. Initially, hematoxylin and eosin stained 3 μm thick sections were examined by light microscope. Blood vessels were highlighted by immunostaining EC with a monoclonal antibody to CD31. MVD was assessed in a blinded manner, by the simultaneous assessment of two experienced pathologists, as has already been described in the past [9]. Briefly, slides were scanned at 100x magnification to determine three "hot spots" (the areas with the highest number of microvessels). After the identification of the hot spots in each specimen, individual microvessels were counted at 400x magnification for systematic examination. The measurements were performed in a surface area, of each hot spot, of 0.0625 mm^2, corresponding to the projection of the eyepiece graticule. Both pathologists agreed on what constituted a single microvessel before any vessel was included in the count. Microvessels were defined as red stained EC, single or clustered, in tubes or nests, clearly separated from adjacent microvessels, with or without a lumen. Large vessels or vessels in the periosteum or bone were excluded. The mean of the two independent counts was considered to be the final measurement for each counting field and hot spot, with an overall interobserver variation close to 10 %. The mean microvessel count from three hot spots was calculated and expressed as vessels/0.0625 mm^2.

Statistical Analyses

All measured parameters are expressed as mean ± SD. Statistical comparison between MM and control group was made using the non-parametric Mann-Whitney test. The non-parametric Kruskal-Wallis test and the one-way analysis of variance (ANOVA) evaluated differences between disease stage groups. Comparisons between values obtained before and after treatment were evaluated with the paired samples *t*-test or Wilcoxon signed rank test. Correlation between the measured parameters was studied with Pearson's correlation (r). A 5 % significance level was applied.

Table 1 Mean ± SD values of interleukin-8 (IL-8), platelet derived growth factor-AB (PDGF-AB), angiogenin (ANG) and microvessel density (MVD) in multiple myeloma patients and controls

	IL-8 *(pg/ml)*	**PDGF-AB** *(pg/ml)*	**ANG** *(pg/ml)*	**MVD** *(/0.0625 mm^2)*
Controls	28.7±7.6	214.9±129.7	220.7±146.7	2.2±0.7
Patients	37.7±13.5	1962.2±1380.0	726.1±329.9	8.8±4.0
p-value	*<0.009*	*<0.001*	*<0.001*	*<0.001*

Table 2 Mean ± SD values of interleukin-8 (IL-8), platelet derived growth factor-AB (PDGF-AB), angiogenin (ANG) and microvessel density (MVD) in disease stages, according to ISS

	IL-8 *(pg/ml)*	**PDGF-AB** *(pg/ml)*	**ANG***(pg/ml)*	**MVD** *(/0.0625 mm²)*
Stage I	30.4±5.4	1104.7±496.7	382.4±144.7	6.8±3.0
Stage II	36.7±9.1	1978.8±1393.4	749.9±302	7.5±2.9
Stage III	43.5±17.6	2571±1495	934.7±250.8	11.2±4.1
p-value	*<0.01*	*<0.006*	*<0.001*	*<0.001*

Results

Mean (± SD) values of analyzed parameters in pre-treatment patients and controls are shown in Table 1. All measured parameters were higher in MM patients compared to healthy controls ($p<0.009$ for IL-8, $p<0.001$ for the other cases). The values of analyzed parameters in pre-treatment patients according to ISS are shown in Table 2. All values were higher in increased disease stages ($p<0.01$ for IL-8, $p<0.006$ for PDGF-AB and $p<0.001$ for Ang and MVD) (Fig. 1 for IL-8).

In the pre-treatment group, serum levels of IL-8 correlated positively with Ang ($r=0.299$ $p<0.03$) (Fig. 2) and PDGF-AB ($r=0.334$ $p<0.01$), but not with MVD. Furthermore, PDGF-AB correlated with Ang ($r=0.599$ $p<0.0001$) and MVD ($r=0.290$ $p<0.03$) and Ang correlated with MVD ($r=0.475$ $p<0.0001$).

Data from the patients after effective treatment are shown in Table 3. MVD and serum levels of IL-8, PDGF-AB and Ang were significantly decreased in the plateau phase ($p<0.001$ for all cases). It is of importance that post-treatment values of IL-8 were inferior to the minimum levels of the control group.

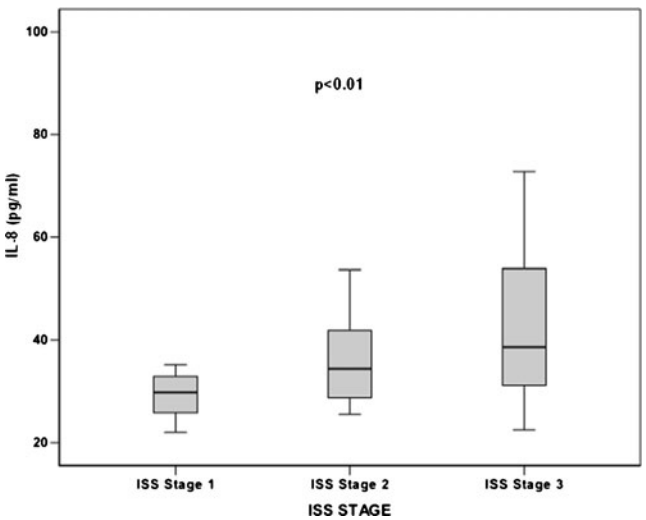

Fig. 1 Serum levels of interleukin-8 (IL-8), in pre-treatment multiple myeloma patients, according to international staging system (ISS)

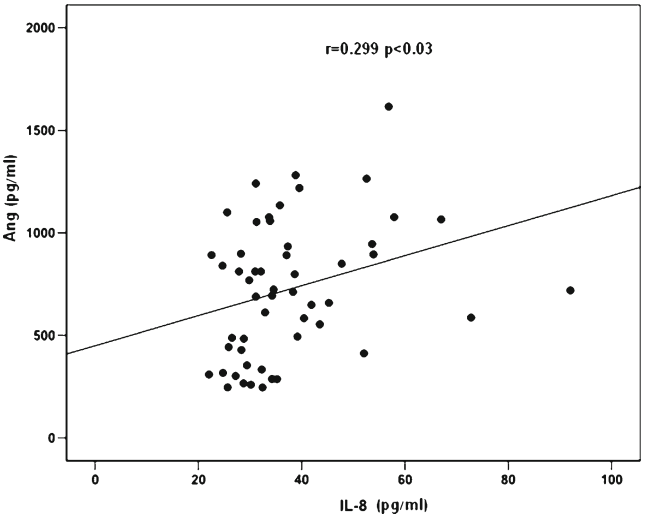

Fig. 2 Correlation between interleukin-8 (IL-8) and angiogenin (Ang) in multiple myeloma patients

Discussion

IL-8 is an angiogenesis-related chemokine, which, along with the rest of CXC chemokines, governs the homing and differentiation of hemapoietic stem cells [10]. It is also a chemotactic and growth factor for several tumors [8, 11] and plays an important role in inflammation and wound healing [12]. IL-8 is produced by various cell types, including macrophages, neutrophils, EC and several tumor cells, such as myeloma plasma cells, and acts through binding to its receptors CXCR1 and CXCR2 [13]. Furthermore, IL-8 has also a significant role in tumor progression and angiogenesis [14, 15]. Bone marrow stromal cells and EC from patients with MM was found to secrete IL-8, which in turn may recruit neutrophils *in vivo* and subsequently release angiogenic factors to recruit EC [16–18]. Moreover, IL-8 has been shown to increase the proliferation of tumor cells, and binding to CXCR1 and CXCR2 expressing endothelial cells to prolong their survival and to enhance their ability to form tubules, supporting the theory that the pro-angiogenic

Table 3 Mean ± SD values of interleukin-8 (IL-8), platelet derived growth factor-AB (PDGF-AB), angiogenin (ANG) and microvessel density (MVD) before and after treatment

	IL-8 *(pg/ml)*	**PDGF-AB** *(pg/ml)*	**ANG** *(pg/ml)*	**MVD** *(/0.0625 mm²)*
Pre-treatment	38.2±12.9	1879.2± 1305.1	687.4± 284.4	8.6±3.9
Post-treatment	27.6±7.1	651.9± 226.4	362.7± 194.2	2.8±0.9
p-value	*<0.001*	*<0.001*	*<0.001*	*<0.001*

effects of IL-8 are due to activation of both malignant and EC [19, 20]. Levels of IL-8 have been found elevated in plasma of patients with advance disease stage and were higher in non-complete remission (CR) than CR patients after therapy [21, 22]. In our study we have shown that IL-8 serum levels are significantly higher in MM patients compared to normal controls. We also found that pre-treatment IL-8 levels differ significantly according to disease stage. Thus patients in stage III had significantly higher levels of IL-8 compared to those at stage I and II. These findings confirm previous data which showed that stromal IL-8 production parallels MM disease activity [7].

Patients with hematological malignancies have increased bone marrow MVD, supporting the notion that bone marrow angiogenesis plays a role in the pathogenesis and progression of these tumors. Previous studies showed that bone marrow angiogenesis—hence MVD—is increased in newly diagnosed MM compared to monoclonal gammopathy of undetermined significance, normal controls and patients with amyloidosis [23]. In our study we confirmed that MVD was significantly increased in newly diagnosed MM patients, compared to controls, also in advanced stages of the disease compared to stage I and decreased following treatment.

PDGF-AB is a growth factor implicated in enhanced proliferation and migration of pericytes, stabilizing the newly formed vasculature [24]. It has been implicated in angiogenesis process and evolution of MM [3]. Our results confirm those data, since their levels were found to be paralleled with disease activity and decreased in plateau phase. Furthermore, their levels correlated with IL-8 and MVD.

Many recent reports underscored the role of Ang in tumor angiogenesis and growth [25]. Ang can activate tissue plasminogen activator, with subsequent generation of plasmin, which in turn degrades laminin and fibronectin of the basic membrane. The destruction of basic membrane is prerequisite for the migration of EC during neovascularization [26, 27]. Ang is overexpressed in many epithelial and hematological malignancies and being associated with poor prognosis [28]. It has also been used as a molecular target for the treatment of various cancers [29]. Our results, similarly with PDGF-AB, showed that their levels were in parallel with disease activity and decreased in plateau phase. Moreover, their levels correlated with IL-8, MVD and PDGF-AB as well.

On the other hand, no correlation was found between IL-8 with MVD, in accordance to previous reports [8, 22], where a further positive correlation was found with HGF but not with VEGF. In the present study, we extend this observation, showing further positive correlations of IL-8 with both direct angiogenic cytokines Ang and PDGF-AB. On the other hand, the lack of correlation between serum levels of IL-8 and MVD was in contrast with other previous data, where in a small group of patients, those with high MVD, estimated by anti-CD34 immunohistochemical expression, had significantly higher stromal cell IL-8 production [7]. Our results are based on a bigger sample of patients, where the correlation analysis was performed in the entire group of them. We believe that the lack of correlation between IL-8 and MVD, nor the major angiogenic cytokine VEGF, could be attributed by the fact that IL-8 is a pro-inflammatory chemokine with direct and indirect pro-angiogenic properties. Contrary to other cytokines with direct effects on angiogenesis, such as VEGF, PDGF-AB and Ang, IL-8 is a multifunctional chemokine, with direct action both on myeloma and EC, and additionally, multiple indirect actions on bone marrow microenvironment. Therefore, one could suggest that elevated IL-8 expression may represent the inflammatory element of the disease that can enhance the angiogenic process in both direct and indirect manners. Furthermore, its elevated levels have been related with poor prognosis in MM and there have been various therapeutic models using inhibition of IL-8 action with promising results [11, 20].

In conclusion, the circulating levels of IL-8, PDGF-AB and Ang are elevated in patients with MM. Their correlation with disease activity implicates that they have an important role in MM pathogenesis. On the other hand, the lack of correlation between IL-8 with MVD suggests that its participation in the angiogenic process is rather complex, where inflammation, cellular stress and tumor presence may regulate its production and stimulation of endothelial cells. Nevertheless, IL-8 levels might be used in future prognostic models and provide evidence that this chemokine could be a molecular target for MM drug development.

Conflict of Interest The authors declare that they have no conflict of interest.

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