

Cytokines Behavior in Multiple Myeloma Patients during Zoledronic Acid Treatment

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Abstract

Objective: Multiple Myeloma (MM) is characterized by uncoupling of bone resorption from bone formation which leads to the predominance of resorption. Bisphosphonates are chemical compounds that selectively concentrate at the interface of the active osteoclasts and the bone resorption surface where they inhibit osteoclast activity. Aim of this study was to describe cytokines behaviour in MM and to evaluate whether zoledronic acid could have an *in vivo* anti-angiogenic property in MM as observed in solid tumours.

Methods: Serum samples from 29 (16 males and 13 females) consecutive MM patients with lytic bone lesions treated with 4 mg of zoledronic acid were tested for platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), tumor necrosis factor (TNF α) and insulin growth factor (IGF-I). Basal cytokine levels were compared with the values observed after 1, 2, 7 and 21 days of treatment, using the Wilcoxon's test for nonparametric-dependent continuous variables.

Results: A significant increase in IL-6 and TNF α was observed on days 1 and 2. As for VEGF, the levels of this cytokine did not change significantly from basal values during the entire period of observation except for a significant increase day 7 ($P=0.0005$). Moreover, PDGF significantly decreased ($P=0.005$) after 2 days from zoledronic acid infusion.

Conclusions: From this study appears that in MM, treatment with zoledronic acid induces a transient reduction in PDGF according with previous studies in solid cancer, while the increase of IL-6 and VEGF could be related through a paracrine mechanism. The anti-myeloma effect of this drug could be driven through this mechanism.

Keywords: Zoledronic acid; Multiple myeloma; Cytokines; Angiogenesis

Introduction

One of the main clinical features of MM is the presence of lytic bone lesion caused by an imbalance between an increased osteoclast activity and a decreased osteoblast activity [1]. The adhesion of MM cells to the bone matrix induces the secretion of several osteoblast activating factors, such as IL-6, interleukin-1 (IL-1), matrix metalloproteinases (MMPs), hepatocyte growth factors (HGF), nuclear factor- κ B ligand (RANKL), VEGF and IGF.

In a condition of inflammation, like in MM, there is also an increased level of TNF α and macrophages inflammatory 1 α (MIP-1 α) which are, in particular, osteoblast suppressor/osteoclast activator. These factors modify the bone marrow microenvironment, upregulating RANKL's receptor required for the activation and differentiation of osteoclasts at the bone lesions sites [1]. As a consequence, osteoclasts activity overcomes the osteoblasts one causing the destruction of bone matrix, leading to the production of these cytokines which have an important role in the biology of malignant plasma cells and in the progression of MM [2,3].

Recently, the mechanism of angiogenesis has been suggested as one of the most powerful pathogenetic mechanisms in the progression of MM. In particular, the presence of low microvessel density in MM, is associated with a more favourable prognosis, while a higher microvessel density is associated with advanced disease. The development of new vessels is modulated by the production of basic fibroblast growth factor (bFGF) and VEGF, which stimulate increase of MM cells and stromal cells through epidermal growth factor (EGF)-receptor [1,3].

Another important cytokine in MM's pathogenesis is PDGF, which is able to promote the chemotactic migration of endothelial cells [1]. PDGF's production is stimulated by different variety of neoplastic cells, as well as, by normal cells such as macrophage and stromal cells [2].

During the last decade, bisphosphonates have become a corner stone in the treatment of bone neoplastic lesions in patients with solid tumours or MM. Chemically bisphosphonate are synthetic analogues of endogenous pyrophosphate. Zoledronic acid, in particular, has a clear mechanism of action in improving bone disease but many hypothetical mechanisms are proposed for its anti-myeloma action. Recently, it has been demonstrated, that in solid tumours, zoledronic acid may play an *in vivo* anti-angiogenic effect through a significant and long-lasting reduction in VEGF serum levels [2].

On the basis of these data, we investigated the modification of different cytokines involved in the pathogenesis of MM in 29 consecutive newly diagnosed MM patients treated with zoledronic

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acid, before anti-neoplastic therapy, with the aim to describe their behaviour, to verify if the same mechanism reported in solid tumors is operational in MM and to investigate its potential antiangiogenic role [2].

Patients and Methods

Patients

Twenty-nine consecutive patients (16 males and 13 females) ages 42-83 years (median age, 58 years) were included in the study. Patients were considered eligible if they had a confirmed diagnosis of MM according to International Myeloma Working Group Criteria [2] and lytic bone lesions.

In addition, patients were required to have at study entry a normal hepatic and renal function, and no acute or chronic infections or inflammatory diseases. Patients were considered ineligible for accrual when they had reported fever (body temperature >38.0°C) during the last week before study entry or had received any radiotherapy, chemotherapy, immunotherapy, or growth factors during the last 4 weeks before study accrual. Patients recently (<1 week) or simultaneously treated with steroids were considered ineligible for the study. Twenty-five patients were in stage III A according to Durie e Salmon's staging system, 4 was in stage II A. All patients underwent to a complete history and a physical examination. Baseline studies included: complete blood count with a full differential; chemistry panel; serum electrophoresis and immune fixation studies, quantitative immunoglobulin assessment; measurements of serum lactate dehydrogenase (LDH), β_2 -microglobulin and protein C reactive (PCR), bone marrow aspirate and, in selected patients, also bone biopsy. Characteristics of the sample are resumed in Table 1.

N. (tot) patients: 29	Median value
Age (years)	58 (min 42-max 83)
Hb (g/dL) (normal range 13.5 -17.5)	12.8 (min 11.6-max 14.1)
Creatinine (mg/dL) (normal range 0.67-1.17)	0.9 (min 0.5-max 2.3)
Calcium (mg/dL) (normal range 8.8-10.2)	10 (min 8.6 – max 10.1)
Bone lesions (1 or more)	4 (min 2- max 10)
Albumin (g/dL) (normal range 3.5-5.4)	2.7 (min 2.01-max 5.4)
M-protein (g/dL)	2.02 (min 0.4-max 5)
β_2-microglobulin (mg/dL) (normal range 0.1-0.25)	0.30 (0.12-0.66)
LDH (U/L) (normal range 0-248)	455 (min 348–max 488)
Protein C reactive (mg/l) (normal range <3)	4.5 (1.44-20.8)
% Plasma cells in bone marrow (Normal value <10%)	35 (min 18–max 90)
Isotype	N / N tot (%)
IgG	15/29 (52%)
IgA	6/29 (21%)
Only light chain	8/29 (27%)
IgD	0/29 (0%)
Biclonal	0/29 (0%)
Stage Durie and Salmon	N / N tot (%)
I	0/29 (0%)
II	4/29 (18%)
III	25/29 (82%)

Table 1: Characteristics of the sample.

Treatment and follow-up investigation

One month before starting chemotherapy for MM, all patients received 4 mg of zoledronic acid (“Zometa”, Novartis) in 250 mL of 0.9% saline over a period of 15 minute as an i.v. infusion. Venous blood samples for cytokine assessment were drawn before the beginning of drug infusion and thereafter at 1, 2, 7 and 21 days after zoledronic acid infusion. After blood drawing, samples were allowed to clot before centrifugation for 10 minutes at 10.000 rpm; thereafter, serum samples were stored at -80°C until testing for PDGF, VEGF, IL-6, TNF α and IGF-I.

Cytokine Analysis

We decide to analyse some of the most important cytokines involved in the pathogenesis and progression of MM. Serum levels of PDGF, VEGF, IL-6, TNF- α and IGF-I were measured using the Quantikine quantitative kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Normal ranges for all tested cytokines were: 3.13-12.5 pg/mL for IL-6; 10.499-29.463 pg/mL for PDGF; 40-258 ng/mL for IGF-I; 62-707 pg/mL for VEGF. As for TNF α values <15.5 pg/mL were considered normal.

Statistical Analysis

Statistical analysis was performed using the SPSS software (SPSS Chicago, 13). Basal cytokine levels were compared with those observed at days 1, 2, 7 and 21 after zoledronic acid infusion using the Wilcoxon's test for nonparametric-dependent continuous variables. Correlation among cytokines was calculated according to the Spearman test.

Results

Cytokines behavior after zoledronic acid single infusion

IL-6: Basal levels of IL-6 were in the normal range (median 3.789 pg/mL; 95% CI: 3.528-6.483). On day 1, we observed a statistically significant increase in IL-6 (median 11.328 pg/mL; 95% CI: 10.583-24.205; P=0.0000), that persisted, also, on day 2 (median 13.796 pg/mL; 95% CI: 11.805-23.981, P=0.0000). Even though from day 7 (median 5.037 pg/mL, P=0.0179) to 21 (median 5.156 pg/mL, P=0.0062) the median values returned within the normal range (Figure 1, panel A).

TNF- α : Before starting zoledronic acid infusion, the levels of TNF- α were always <15.5 pg/mL (median 13.249 pg/mL; 95% CI: 11.618-14.376). However, TNF- α 's level reached a statistically significant increase on days 1 and 2. In particular, on day 1 the median TNF- α value was 16.105 pg/mL, (95% CI: 13.445-17.119; P=0.0001) and on day 2 was 16.366 pg/mL, (95% CI: 12.905-16.461; P=0.0021). Increased level of TNF- α did not persist from day 7 to 21 (Figure 1, panel B).

PDGF: PDGF median level remained within the normal range throughout the study except for day 2, in which we observed a statistically significant reduction in the median value of this cytokine (11.742 pg/mL; 95% CI: 11.982-22.068; P=0.005) (Figure 1 panel C).

VEGF: VEGF value showed a statistically significant increase from the median value observed only on day 7 (333.67 pg/mL; 95%CI: 284.866-466; P=0.0047). VEGF circulating level was within the normal range during the other day of evaluation (Figure1, panel D).

IGF-I: No modification from the median basal value of IGF-1 was observed on days 1, 2,7 and 21 after zoledronic acid (data not shown).

Correlation: A significant positive correlation (P=0.006 by the

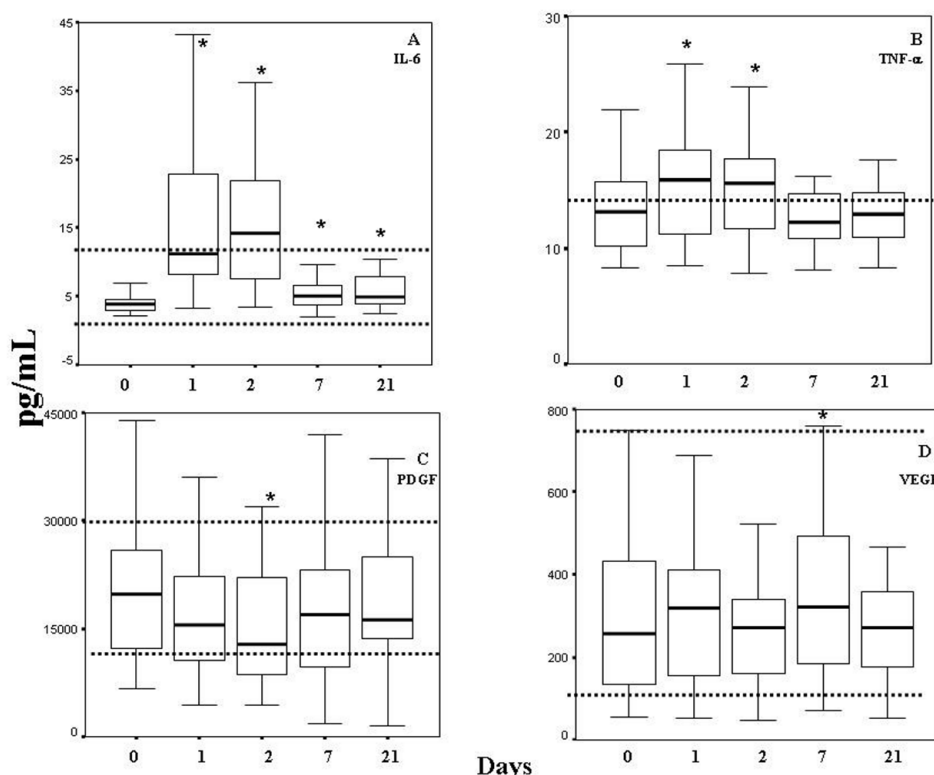


Figure 1: The figure shows the behavior of IL-6 (Panel A), TNF- α (Panel B), PDGF (Panel C) and VEGF (Panel D) levels at days 1, 2, 7 and 21 after Zoledronic acid administration. Horizontal black bar in the boxes represent median value. Bottom and top horizontal bars indicate minimum and maximum values. P values are calculated according to Wilcoxon test for nonparametric-dependent continuous variable. Dotted lines indicate the normal ranges.

Spearman test) was observed only between IL-6 and TNF- α values measured on day 2.

Discussion

Bone marrow neovascularization support the progression in patients with MM, tumor-secreted cytokines activate the bone marrow compartment resulting in the mobilization of circulating endothelial progenitor cells into circulation. After homing to tumor bed in response to chemokine gradients, these cells and macrophages, promote the growth and angiogenesis of tumor by the secretion of proangiogenic factors like cytokines (VEGF, PDGF, metalloproteinases) resulting in the increase of vessel corporation and stabilization and in promoting plasma cell growth and invasion [2]. As known, zoledronic acid is clearly effective at inhibiting the development of myeloma bone disease, but the exact mechanism of its anti-MM action is still unexplained. There are different hypothesis: it has been proposed that because less bone is destroyed following bisphosphonate treatment, there is less volume available for tumor expansion [2].

Similarly, the bone marrow microenvironment has often been perceived as a hospitable environment for tumor growth especially when enriched by growth factors liberated from the bone matrix during bone resorption – the so called ‘seed and soil’ concept. Because treatment with bisphosphonates inhibits bone resorption, this favourable environment would be rendered relatively more inert. It has also been suggested that bisphosphonate treatment can inhibit tumor cell adhesion to mineralized surfaces. Also similar to their effects on osteoclasts, bisphosphonates have been shown to induce pro-apoptotic and anti-proliferative effects on myeloma cells [2].

Anti-angiogenic effects have also been proposed by Croucher et al. [11]. This group demonstrate in murine-MM model that zoledronic acid is able to decrease microvessel density within areas infiltrated by tumor cells, and they cannot exclude the possibility that also blocks production of angiogenic factor like VEGF. More complex interactions have also been proposed by another group that demonstrates that bisphosphonates have the ability to stimulate human $\gamma\delta$ T-cell mediated immunosurveillance [2]. Most recently, sequential administration of doxorubicin followed 24 hours later zoledronic acid infusion has been shown to decrease tumor burden in a mouse model of breast cancer. The authors propose that this effect is mediated by a complex interaction of increased pro-apoptotic factors and decreased cell cycle proteins. About the use of zoledronic acid in solid tumor, Santini et al. [4] reported that in patients with advanced solid cancer and bone metastasis, pamidronate [2] and zoledronic acid [4-10] showed anti-angiogenic properties which were associated with a significant and long-lasting decrease of VEGF serum levels following bisphosphonates infusion. These observations were later confirmed by Ferretti et al. [14] who observed a transient but significant reduction of VEGF and bFGF, 48 hours after the infusion of zoledronic acid in breast cancer patients with bone metastasis.

Although there is some evidence for an anti-angiogenic effect of bisphosphonates in solid organ malignancies, there is no strong evidence for this activity in MM. Furthermore, the role of angiogenesis in MM remains controversial; specifically anti-VEGF agents have proved disappointing. About anti-MM therapy used nowadays, thalidomide and analogues have also been cited as anti angiogenic agents but in more recent times it is their pro-apoptotic properties which have gained more credibility (via interactions with cereblon) [2].

The use of bortezomib, for example, induces respectively, 7% and 31% of complete/partial remission as single agent, while in combination with immunomodulatory drugs like thalidomide or lenalidomide improves the response rate [2]. It has, also, been studied the effect of bortezomib in combination with zoledronic acid, and it has been showed their synergistic activity in inhibiting NF- κ B way, which is overexpressed in several tumors like MM and in which regulates cell cycle progression, inflammation and angiogenesis. In particular, they demonstrated the greater efficacy of zoledronic acid pre-treatment; in fact the partial inhibition of NF- κ B, due to the reduced activation of signalling proteins (such as those of Ras, Rho families) induced by zoledronic acid, could make NF- κ B more vulnerable to bortezomib [2].

An interesting study evaluated 1970 adult patients with newly diagnosis of MM treated: 981 with chemotherapy and zoledronic acid versus 979 with chemotherapy and clodronic acid. Resulting data showed that zoledronic acid reduced mortality by 16% versus clodronic acid and extended median overall survival by 5.5 months; zoledronic acid, also significantly improved progression free-survival by 12% vs. clodronic acid. These findings suggest that zoledronic acid is useful not only in the management of bone lesions but also for potential anti-myeloma benefits [2].

We reported different hypothetical mechanism of anti-MM activity of zoledronic acid, for which there is actually no consensus, for this reason, according with the results found in solid tumors, we explored the contribution of bisphosphonates in anti-MM activity studying its potential anti-angiogenic effect based on modification of cytokines which are pivotal in this pathogenic mechanism [11-18].

We decide to analyze in a pilot study, the following cytokines VEGF, PDGF, IL-6, TNF- α and IGF-I because are important protagonist of the network of cytokines that control growth, progression and dissemination of MM cells.

In our result, as documented also by other investigators we observed a sharp and statistically significant increase in IL-6 and TNF- α median values on days 1 and 2 after zoledronic acid infusion [19,20].

As for IL-6 and TNF sharp increase is concerned, this may be due to the acute effect of zoledronic acid infusion in some patients who, as demonstrated, may suffer of constitutional symptoms such as fever and flu-like symptoms.

IL-6 has been demonstrated to be involved in the proliferation of plasmablastic cells and their differentiation in mature plasma cells, its serum levels is usually increased in patient with MM, and in particular in patients with stage III/II according to Durie and Salmon stadiation, in our experiment its serum value increase especially, after zoledronic acid infusion.

An interesting role is emerged about TNF- α , related with its capacity to induce dissemination of MM cells, in fact after incubation of MM cells precursor with TNF α , monoclonal plasma cells appears in peripheral blood of patients with MM but not from patients with monoclonal gammopathy of undetermined significance (MGUS) [21].

However, differently from what observed in solid tumors [10], a single infusion of zoledronic acid produced a statistically significant decrease of PDGF on day 2, unexpectedly followed by a statistically significant increase of VEGF on day 7. Except of this single occasion, throughout the entire period of study, these two cytokines remained always within the normal range. This unexpected behaviour of PDGF and VEGF indicates that zoledronic acid in MM patients acts differently from what observed in solid tumours.

As known, VEGF is a multifunctional cytokine that stimulates angiogenesis including tumor neovascularization. Although well established in solid tumors, the role of VEGF in bone marrow neo-angiogenesis and paracrine tumor-stromal cell interactions in lympho-hematopoietic malignancies has not been fully elucidated. Some studies evaluated the effects of VEGF on marrow stroma, focusing on the secretion of IL-6 and underlined paracrine interactions between myeloma and marrow stromal cells triggered by VEGF and IL-6. In turn, IL-6 stimulated the secretion of VEGF by myeloma cells, suggesting a paracrine role for VEGF in tumor-stroma interactions in MM [22].

Based on these findings, we could explain the increase of VEGF, as a consequence, of the stimulation by IL-6, (previously increased), and the following reduction as a consequence of response to administration of zoledronic acid. It has been evaluated the role of zoledronic acid in modifying the expression of VEGF-receptor resulting in attenuated response from endothelial cells to VEGF [23].

The biological role of PDGF signaling can vary from autocrine stimulation of cancer cell growth to more subtle paracrine interactions involving adjacent stroma and even angiogenesis. PDGF is a potent bone cell mitogen that stimulates the proliferation of osteoblastic cells, but it may also be involved in the regulation of osteoclastic bone resorption and indirectly induces vascular endothelial cell proliferation and angiogenesis, being the zoledronic acid an inhibitor of resorption, we would attend a reduction has happened [23,24].

In this study, zoledronic acid was able to induce a transient but significant reduction of serum PDGF levels 2 days after the infusion, providing additional interesting preliminary evidence of an *in vivo* antiangiogenetic effect of this molecule. The mechanism of this effect is unknown, but we can deduce that zoledronic acid may elicit several angiogenic-related cytokines patterns and cascades, as demonstrated by preclinical studies. About our study, even if it was a pilot study, there are some limitation based on the low number of patients enrolled and the brief period of monitoring cytokines levels [25].

Our study had, also the aim to validate a method in order to extend the study in a large cohort of patients. We know that the serum levels of cytokines may not reflect the local microenvironmental levels, so further studies are required to define the mechanisms by which zoledronic acid express its anti-myeloma effect by its potential anti-angiogenic activity. In conclusion, we described the modification in serum level of important cytokine in MM. About the anti-angiogenic effect recently demonstrated *in vivo* although the lack of long-lasting reduction of VEGF and PDGF that we noticed differently from what happen in solid cancer [10,20], we could not exclude that zoledronic acid express its anti-myeloma activity by inhibiting VEGF (reduced after day 7) and PDGF, even though zoledronic acid may not have an effect on the cytokine themselves. It is possible that this drug may have an impact on the effect of VEGF and PDGF on signalling that leads to blood vessel development or it also may have an impact on the effect of VEGF and PDGF on myeloma cells directly like hypothesized in some recent study, previously mentioned. For this reason, experimental trial should be addressed to evaluate the real clinical impact in anticancer therapy of antiangiogenic properties of bisphosphonates [26].

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