Pamidronate Induces Modifications of Circulating Angiogenetic Factors in Cancer Patients

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ABSTRACT

Purpose: **Recently, new experimental data suggested that, besides inhibiting osteoclasts, bisphosphonate may also have an antitumor effect. Antiangionetetic activity is one of the possible mechanisms of anticancer activity attributed to bisphosphonates. The purpose of this study was to evaluate the modifications in angiogenic cytokines levels after pamidronate infusion.**

Experimental Design: **Twenty-five consecutive cancer patients with bone metastases treated monthly with disodium pamidronate infusion were evaluated prospectively for circulating levels of vascular endothelial growth factor (VEGF), γ-IFN, interleukin (IL)-6, and IL-8 at different time points: just before and after 1, 2, and 7 days after pamidronate infusion.**

Results: **Basal VEGF levels decreased significantly 1, 2, and 7 days after pamidronate infusion. γ-IFN and IL-6 levels increased 1 day after the infusion but rapidly decreased after 2 days. Moreover, our data showed a statistically significant negative correlation between VEGF and γ-IFN levels ($P < 0.0001$) and a positive correlation between VEGF and IL-8 ($P = 0.04$).**

Conclusions: **This study confirms that pamidronate could have antiangiogenic properties through a significant and lasting decrease of VEGF serum levels.**

INTRODUCTION

Bisphosphonates are potent inhibitors of bone resorption; therefore, they are used widely and successfully for treating or preventing malignant hypercalcemia, tumoral osteolysis, Paget's disease, and osteoporosis (1, 2). Despite this wide use, the mechanism of their action is not yet fully understood. In contrast, it has been shown that pamidronate, a second generation potent aminosubstituted bisphosphonate, produces potent and specific inhibition of bone resorption by suppressing the accession of osteoclast precursors into bone and their transformation into mature osteoclasts (3, 4). Moreover, recent data suggest that besides inhibiting osteoclasts activity, bisphosphonate may also have an antitumor effect. In fact, the apoptotic and antiproliferative effect of bisphosphonates on osteoclasts may be exerted also on macrophages and tumor cells (5–7). This antiproliferative mechanism of bisphosphonates has been attributed to a cytotoxic/cytostatic or proapoptotic effect (6, 7), a reduction of human tumor-cell adhesion to bone matrix (8), a γ/δ T-cell stimulation (9), and an inhibition of tumor angiogenesis (10, 11).

One of the most potent and specific angiogenic factors in cancer is the VEGF.[2] Its clinical importance for tumor growth is supported by the demonstration that most tumors produce VEGF and that the inhibition of VEGF-induced angiogenesis significantly inhibits tumor growth *in vivo* (12). Moreover, VEGF expression has been shown to be an independent prognostic factor related to survival in several malignancies (13). On the basis of these data, we designed a study to investigate the potential antiangiogenic role of pamidronate in patients with malignancies. To avoid the effect of chemotherapy on the blood levels of cytokines recognized to play a role in tumor angiogenesis, the levels of these cytokines were studied just before and 1, 2, and 7 days after the first infusion of pamidronate before the administration of any chemotherapy.

We present here the results of this study.

MATERIALS AND METHODS

Patients. Twenty-five consecutive patients (13 males and 12 females), aged 49–77 years (median age, 65), with advanced solid cancer and bone metastases, were included in the study (Table 1). Patients were eligible for the study if they had histologically confirmed solid neoplasm associated with scintigraphic and radiographic identification of bone metastases. In addition, patients were required to have a neutrophil count $> 2 \times 10^9$/liter, a platelet count $> 100 \times 10^9$/liter, normal hepatic and renal function, and no acute or chronic infections or inflammatory diseases. Patients were ineligible for the study when they had had fever (body temperature $> 38°C$) 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. In addition, patients treated recently or simultaneously with steroids were considered ineligible for the study. In all cases, pamidronate was administrated on an outpatients basis.

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[2] The abbreviations used are: VEGF, vascular endothelial growth factor; IL, interleukin; CI, confidence interval.

Table 1 Patients' characteristics

Total number	25
Median age (range)	65 years (49–77)
Male/female	13/12
Median performance status ECOG[a] score (range)	2 (1–3)
Neoplasm histotype	
Non-small cell lung cancer	6
Breast carcinoma	11
Prostate adenocarcinoma	5
Bladder urothelial carcinoma	2
Unknown primary cancers	1
Bone segments involved by metastases (range)	7 (2–14)
Previous chemotherapy	12
Metastases other than bone locations (patients)	
No other locations	13
Lung metastases	5
Liver metastases	5
Lung + Liver metastases	2
Median basal value of calcium plasmatic concentration (range)	10.23 mg/dl (8.8–12)

[a] ECOG, Eastern Cooperative Oncology Group.

Treatment and Follow-up Investigation. All patients received 90 mg of disodium pamidronate (Aredia, Novartis) in 500 ml of 0.9% saline over a period of 2 h as i.v. infusion starting at 9.00 a.m. Venous blood for cytokines assessment was drawn in an EDTA-anticoagulated tube just before the beginning of drug infusion and 1, 2, and 7 days after pamidronate infusion. After drawing, venous blood was rapidly centrifuged for 10 min at 10,000 rpm and plasma stored at −80°C until tested for VEGF, γ-IFN, IL-6, and IL-8 levels. Moreover, WBCs and platelets count, hemoglobin levels, and total calcium plasmatic levels were also determined at the same times.

Cytokine Analysis. Plasma levels of IL-6, IL-8, and γ-IFN were measured with PharMingen OptEIA sets according to the instructions of the manufacturer (BD PharMingen, San Diego, CA). VEGF was assayed with the R&D quantikine kit following the instructions of the manufacturer (R&D Systems, Minneapolis, MN). The detection limits of the cytokines were as follows: IL-6 $<$ 4.7 pg/dl; IL-8 $<$ 3.9 pg/dl; γ-IFN $<$ 7.8 pg/dl; VEGF $<$ 31.2 pg/dl.

Statistical Analysis. Basal values of tested cytokines were compared with values observed after 1, 2, and 7 days from pamidronate infusion according to Mann-Whitney *U* test for nonparametric independent variables. A linear regression model with variance analysis was used to correlate different cytokines levels. Two-tailed *P* was considered significant when $<$0.05. SPSS software (version 10.00; SPSS, Chicago, IL) was used for statistical analysis.

RESULTS

VEGF Analysis. Median VEGF basal value (745 pg/dl; 95% CI: 684.8–986.26) showed a significant ($P = 0.019$) decrease 1 day after single pamidronate infusion (602.49; 95% CI: 424.06–753.25). Moreover, 2 days after pamidronate infusion, an additional decrease in VEGF levels was recorded (556.84; CI: 358.3–640.71; $P = 0.001$). This effect on VEGF circulating levels persisted also 7 days after bisphosphonate infusion, with a median value of 556.88 (CI: 442.49–684.28). These results are summarized in Table 2 and Fig. 1.

Table 2 Cytokines modifications after a single infusion of Pamidronate

	Median value (pg/dl)	95% CI (pg/dl)	*P* (Mann-Whitney *U* test)
VEGF			
Basal levels	745.00	654.80–986.26	
1 day	602.49	424.06–753.25	0.019
2 days	556.84	358.3–640.71	0.001
7 days	556.88	442.49–684.28	0.03
γ-IFN			
Basal levels	12.00	11.25–18.65	
1 day	21.28	18.43–28.65	0.003
2 days	11.53	11.70–18.39	n.s.
7 days	9.725	10.17–16.48	n.s.
IL-6			
Basal levels	9.81	6.8–12.2	
1 day	14.16	11.8–16.8	0.007
2 days	11.71	7.1–12.6	n.s.
7 days	11.93	9.1–13.5	n.s.

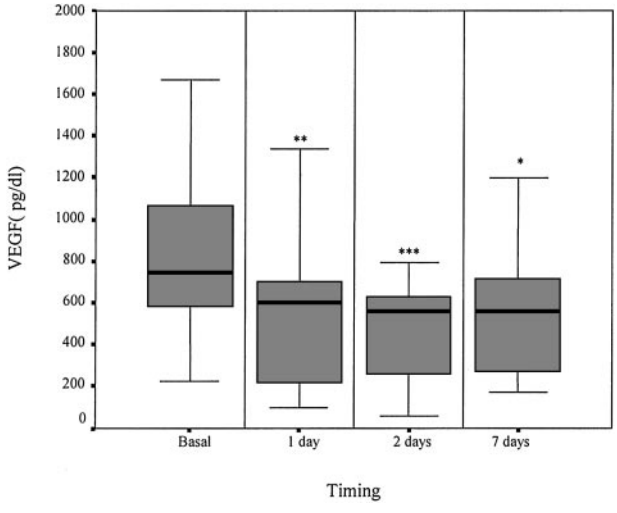

Fig. 1 The behavior of VEGF levels 1, 2, and 7 days after pamidronate infusion. *Gray boxes*, 95 percentiles of all VEGF values. *Horizontal black bar* in the *gray boxes*, VEGF median value. *Bottom* and *top horizontal bars*, minimum and maximum values. *P*s are calculated according to Mann-Whitney *U* test for nonparametric independent continuous variable: ∗ = *P*, 0.03; ∗∗ = *P*, 0.019; ∗∗∗ = *P*, 0.001.

γ-IFN Levels. The median value of γ-IFN basal levels was 12 pg/ml (95% CI: 11.25–18.65). As reported in Table 2 and Fig. 2, these levels increase significantly 1 day after pamidronate infusion to 21.28 pg/dl ($P = 0.019$), returning to levels similar to the median basal values after 2 and 7 days ($P = 0.791$ and $P = 0.557$, respectively).

IL-6 Levels. A significant increase in IL-6 levels was observed 1 day after pamidronate infusion (median IL-6 basal value: 9.81 pg/dl; 95% CI: 6.8–12.2 and median IL-6 value after 1 day: 14.16 pg/dl; 95% CI: 11.8–16.8; $P = 0.007$). From these values, the IL-6 level decreased to 11.71 pg/dl (95% CI: 7.1–

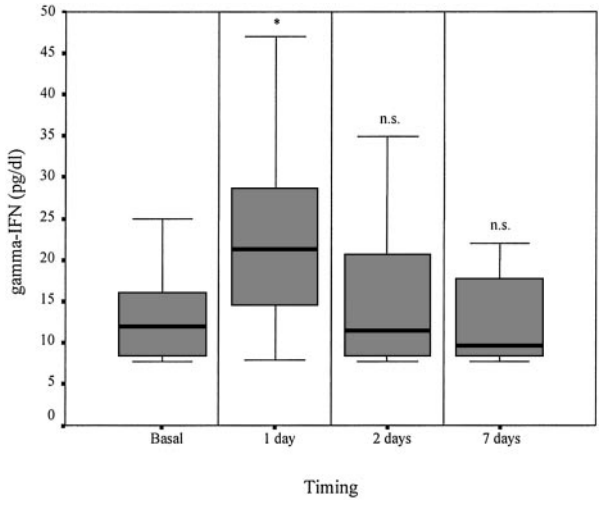

Fig. 2 The behavior of γ-IFN levels 1, 2, and 7 days after pamidronate infusion. *Gray boxes,* 95 percentiles of all γ-IFN values. *Horizontal black bar* in the *gray boxes,* γ-IFN median value. *Bottom* and *top horizontal bars,* minimum and maximum values. *P*s are calculated according to Mann-Whitney *U* test for nonparametric independent continuous variable: ∗ = *P,* 0.003; *n.s.,* nonsignificant.

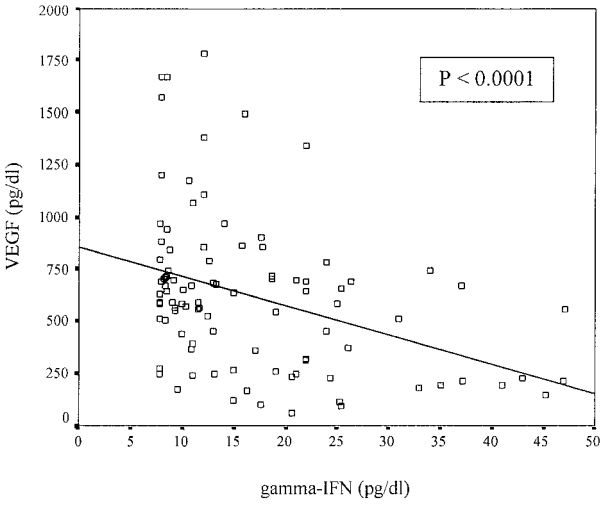

Fig. 3 The correlations between VEGF and γ-IFN; this correlation was found statistically significant with a $P < 0.0001$. *P*s were calculated using a linear regression model with variance analysis.

12.6) 2 days after the infusion ($P = 0.263$) and persisted on values nonsignificantly different from the basal ones ($P = 0.362$), 7 days after pamidronate infusion.

IL-8 Levels. No significant modifications in IL-8 levels were found at any time points after pamidronate infusion (median IL-8 basal value: 16.88 pg/dl; 95% CI: 15.02–55.9 pg/dl; median IL-8 value after 1 day: 20.06 pg/dl; 95% CI: 11.64–54.31 pg/dl; median IL-8 value after 2 days: 11.80 pg/dl; 95% CI: 11.15–40.63 pg/dl; median IL-8 value after 7 days: 14.52 pg/dl; 95% CI: 8.96–59.05 pg/dl).

Cytokines Correlations. A linear regression model with variance analysis showed a significant negative correlation between VEGF values and γ-IFN values ($P < 0.0001$; Fig. 3) and a significant positive correlation between VEGF and IL-8 ($P = 0.040$). Our data didn't show a significant correlation between VEGF and IL-6 ($P = 0.663$; Table 3).

Table 3 Main correlations between VEGF and other cytokines

	β regression coefficient	*P*
γ-IFN	−14.139	<0.0001
IL-6	0.569	n.s. (0.663)
IL-8	2.530	0.040

Secondary Parameters. No significant differences were found in platelet levels, WBCs count, and hemoglobin concentration before and after pamidronate infusion. However, as expected, a statistically significant decrease in total calcium plasmatic levels was obtained after disphosphonate administration. In particular, the median calcium level before pamidronate administration was 10.23 mg/dl (range: 8.8–12 md/dl), whereas the median value 7 days after pamidronate therapy was 9.23 mg/dl (range: 8.4–10.7 mg/dl; $P = 0.002$). Calcium plasmatic concentration did not correlate with any of the circulating cytokines before and after pamidronate infusion.

A significant correlation in a linear regression model was recorded between VEGF basal levels and platelet levels (β regression coefficient: 3.31; $P = 0.03$).

DISCUSSION

Bisphosphonates have been used successfully for many years in the treatment of hypercalcemia and to reduce skeletal-related complications of metastases. However, there is recent evidence to suggest that an antitumor effect may also play a role (5). In literature, three adjuvant clodronate trials in high risk breast cancer patients have been reported. The first, an open-label controlled trial (14), reported a reduction in osseous and nonosseous recurrences and an increase in disease-free and overall survival with 2 years of clodronate. A second open-label trial (15) of similar size involving lymph node-positive breast carcinoma patients showed no effect on the rate of bone metastasis relapse and a deleterious effect on incidence of relapse of nonosseous metastases within 3 years of clodronate. A third placebo-controlled trial with 1079 patients reported, in an interim analysis, a reduction in osseous metastases during treatment with 2 years of clodronate but no effect on nonosseous metastases or survival (16). Despite these results, the way of bisphosphonates action on clinical evolution of neoplastic disease is still not fully understood; although, recently, several mechanisms of antitumor effect have been proposed: (*a*) cytotoxic/cytostatic effect; (*b*) proapoptotic effect (1, 6, 7); (*c*) reducing human tumor-cell adhesion to bone matrix (8); (*d*) γ/δT cells and innate immunity stimulation (9); and (*e*) tumor angiogenesis inhibition (10, 11). Tumor angiogenesis inhibition by bisphosphonates is an intriguing hypothesis supported by some evidences in literature (10, 11). Particularly, the new bisphoshonate zoledronate showed inhibitory effects on endothelial cell proliferation *in vitro* and on basic fibroblast growth factor-induced angiogenesis *in vivo* (11). On the basis of these data, we tried to analyze the modifications of recognized circulating angiogenic factors (VEGF, γ-IFN, and IL-8) and cytokines of acute phase (IL-6) induced by a single

administration of 90 mg of disodium pamidronate before the administration of any chemotherapy agent. As reported previously in literature (2, 8), our study clearly underlines that pamidronate induces, after 1 day from the infusion, an IL-6-mediated acute phase reaction. This reaction is short and reversible and probably mediated by stimulation of transient production of IL-6 from macrophages and monocytes. Moreover, our study clearly demonstrates a statistically significant decrease, compared with basal values, in VEGF levels after 1, 2, and 7 days from pamidronate infusion. VEGF is one of the most potent and specific angiogenic factors of cancer-induced angiogenesis. VEGF expression has been shown to be an independent prognostic factor related to tumor progression (17) and survival in several malignancies (13, 18), and several studies have underlined a potential role of VEGF in predicting response of tumor to anticancer treatments (19). As a consequence, the significant and lasting decrease of VEGF serum levels after a single infusion of 90 mg of pamidronate, reported in the present study, may provide preliminary evidence of an *in vivo* antiangiogenetic effect of bisphosphonates in cancer patients. The mechanism of such effect elicited by bisphosphonate is not known and is not possible to confirm whether this effect is only seen after the first exposure to pamidronate.

Previous studies have reported a positive correlation between platelet number and serum VEGF level in cancer patients. These data confirm the hypothesis that platelets may serve the role of storage of VEGF in the circulation (20–22).

In our study, we report also a significant and short increase of IFN-γ serum levels after 1 day by a single infusion of pamidronate. IFN-γ is a pleiotropic cytokine endowed with potent immunomodulatory effects and secreted by activated CD4 and CD8 T cells. The real role of serum IFN-γ increase is not known, although there are some evidences in the literature supporting an antiangiogenic action by IFN-γ through an inhibition of endothelial proliferation (23, 24). This hypothesis is confirmed indirectly by the significant negative correlation between serum levels of VEGF and IFN-γ observed in our study. IL-8 is a cytokine produced by mononuclear cells that is involved in polymorphonuclear neutrophil leukocyte and T lymphocytes recruitment and activation. Moreover, there are some evidences that IL-8 and VEGF promote tumor angiogenesis, cancer growth, and metastasis and are coexpressed by human head and neck squamous cell carcinomas and a variety of other malignancies (25–27). As a consequence, the significant positive correlation between VEGF and IL-8 serum levels observed in our study seems to confirm this hypothesis.

Despite the small size and heterogeneous population, the obtained results are noteworthy. However, they require confirmation by additional investigations.

In conclusion, the study confirms that pamidronate induces significant and lasting modifications of angiogenic cytokines pattern. Experimental trial should be addressed to assess the real clinical impact in anticancer therapy of antiangiogenic properties of bisphosphonates.

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