Advances in Brief

Zoledronic Acid Induces Significant and Long-Lasting Modifications of Circulating Angiogenic Factors in Cancer Patients

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Abstract

Purpose: **The commercial availability of zoledronic acid, a third generation bisphosphonate, prompted us to evaluate the modifications in angiogenic cytokines levels after a single i.v. infusion of this drug.**

Experimental Design: **Thirty consecutive cancer patients with scintigraphic and radiographic evidence of bone metastases were treated with a single infusion of 4 mg of zoledronic acid before any chemotherapy. The patients were prospectively evaluated for circulating levels of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) just before and at 1, 2, 7, and 21 days after zoledronic acid infusion.**

Results: **Basal serum VEGF median levels were significantly decreased at days 2 (−23%), 7 (−28%), and 21 (−34%) after zoledronic acid infusion ($P = 0.0498$, 0.0090, and 0.0011, respectively). Serum PDGF levels were significantly decreased by 25% 1 day after zoledronic acid infusion ($P = 0.0032$). This effect on circulating PDGF levels persisted for 2 days after bisphosphonate infusion ($P = 0.0050$). PDGF levels had returned to values similar to the median basal value at 7 and 21 days. Moreover, a linear regression model with variance analysis showed a significant positive correlation between basal VEGF and PDGF values but not at the following time points. No significant differences were recorded in platelet levels, WBC count, or hemoglobin concentration before and after zoledronic acid single infusion.**

Conclusions: **This study confirms that zoledronic acid could have an *in vivo* antiangiogenic property through a significant and long-lasting reduction in serum VEGF levels.**

Introduction

Bisphosphonates are analogues of endogenous PP_I in which a carbon atom replaces the central oxygen atom and are established as successful agents for the prevention and treatment of postmenopausal osteoporosis (1), corticosteroid-induced bone loss (2), and Paget's disease (3). In recent years, bisphosphonates have also become important in the management of cancer-induced bone disease, and they have now a widely recognized role for patients with multiple myeloma and bone metastases secondary to breast cancer (4–6). Increased osteoclastic bone resorption is among the central mechanisms underlying hypercalcemia of malignancy, and bisphosphonates have been shown to be extremely effective in the management of this complication and have become the treatment of choice (7). Several recent studies suggest that the efficacy of these compounds in the oncological setting, besides the strong antiosteoclastic activity, could also be because of a direct antitumor effect exerted at different levels. It had been postulated that bisphosphonates could exert an antitumor activity by altering the release of growth factors in the bone microenvironment such as transforming growth factor β, insulin-like growth factor I, and other peptides from bone matrix (8). Moreover, other studies suggested that these compounds may also inhibit tumor cell adhesion, invasion, and viability (9, 10) and may induce apoptosis (11, 12) in preclinical models. Furthermore, pamidronate treatment was shown to induce significant amounts of cell death, whereas only zoledronic acid exerted a dramatic effect on cell proliferation (13). Finally, recent evidence suggests that part of the antitumor activity of bisphosphonates may be attributed to an antiangiogenic effect. Most of the knowledge about this effect derives from the studies of Wood *et al.* (14) with the nitrogen-containing bisphosphonate zoledronic acid. The authors reported that zoledronic acid dose-dependently inhibited the *in vitro* proliferation of human umbilical vein endothelial cells induced by FCS, basic fibroblast growth factor, and VEGF (14). Moreover, for the first time in humans, we have shown a significant decrease of circulating VEGF levels in bone metastatic cancer patients receiving a single dose of pamidronate. In this study, VEGF levels reduction was already significant on day 1 after a single pamidronate infusion (90 mg), and the effect was still present on day 7 (15).

VEGF is one of the most potent angiogenic factors. It has a specific mitogenic activity on endothelial cells, and it is apparently devoid of mitogenic activity for other cell types (16). VEGF not only induces proliferation of endothelial cells but also increases vascular permeability and promotes extravasation of proteins from tumor vessels, leading to the formation of a fibrin matrix that makes invasion of stromal cells into the developing tumor possible (17). Inhibition of VEGF has been

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Table 1 Patients' characteristics

Total number	30
Median age (range)	62 (38–81)
Male/female	10/20
Median performance status ECOG score (range)	1 (0–2)
Neoplasm histotype	
Non-small cell lung cancer	4
Breast carcinoma	17
Prostate adenocarcinoma	5
Other primary cancers	4
Bone segments involved by metastases (range)	1–8
Previous chemotherapy	17
Concurrent hormonotherapy	5
Metastases other than bone locations (patients)	
No other locations	12
Lung metastases	3
Liver metastases	7
Lung + liver metastases	4
Other locations	7

Table 2 Number and percentage of patients who developed angiogenic cytokines reduction after single zoledronic acid infusion

	No. of Patients	Percentage
VEGF		
1 day	11	36.7%
2 days	11	36.7%
7 days	14	46.7%
21 days	19	63.3%
PDGF		
1 day	15	50.0%
2 days	16	53.3%
7 days	5	16.7%
21 days	6	20.0%

shown to suppress tumor growth *in vivo* (18). PDGF, compared with VEGF, is a less potent endothelial cell mitogen, but it also stimulates chemotactic migration of endothelial cells (19). PDGF is expressed by a wide variety of malignancies, but some normal cells such as macrophages, stromal cells, and glial cells have also been found to produce PDGF (20).

On the basis of these data, we designed a second study to investigate the potential antiangiogenic role of zoledronic acid in patients with malignancies. To avoid the effect of chemotherapy on the blood levels of tested cytokines, cytokine levels were measured before and at 1, 2, 7, and 21 days after the first infusion of zoledronic acid and before the administration of any chemotherapy.

Materials and Methods

Patients. Thirty consecutive patients (10 males and 20 females), ages 38–81 years (median age, 62 years), with advanced solid cancer and bone metastases, were included in the study (patients' characteristics are shown in Table 1). Patients were considered eligible for the study if they had a histologically confirmed solid neoplasm associated with scintigraphic identification and radiographic confirmation of bone metastases. In addition, patients were required to have at study entry a neutrophil count $\geq 1.5 \times 10^9$/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 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. Hormonotherapy was allowed only if it had started at least 3 months before accrual. All patients received zoledronic acid on an outpatients basis.

Treatment and Follow-Up Investigation. All patients received 4 mg of zoledronic acid (Zometa; Novartis) in 100 ml of 0.9% saline over a period of 15 min as an i.v. infusion starting at 10 a.m. Venous blood for cytokine assessment was drawn into a EDTA anticoagulant tube just before the beginning of drug infusion and again at 1, 2, 7, and 21 days after the zoledronic acid infusion (just before the subsequent infusion). After drawing, the venous blood sample was rapidly centrifuged for 10 min at 10,000 rpm and plasma stored at −80°C until tested for VEGF and PDGF levels. Moreover, WBC and platelet counts, hemoglobin levels, and serum total calcium levels were also determined at the same time points.

Cytokine Analysis. VEGF and PDGF were assayed with the R&D quantitative kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The detection limit of the cytokines were as follows: 62.5 pg/ml for VEGF and 37 pg/ml for PDGF.

Statistical Analysis. Basal cytokine levels were compared with the values observed at 1, 2, 7, and 21 days after zoledronic acid infusion using to Wilcoxon's test for nonparametric-dependent continuous variables. A linear regression model with variance analysis was used to correlate different cytokines levels. A 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. The number an the percentage of patients who developed a reduction of circulating VEGF levels after zoledronic acid infusion are summarized in Table 2. The median VEGF basal value (1222.000 pg/dl; 95% CI: 1115.54–1435.02) showed a non statistically significant decrease of 6.5% 1 day after the single infusion of zoledronic acid (1143.000 pg/dl; 95% CI: 1008.79–1340.29; $P = 0.4950$). However, at 2 days after the zoledronic acid infusion, VEGF levels had decreased additionally to 23% below the basal value (940.000 pg/dl; 95% CI: 857.27–1130.24; $P = 0.0498$). This effect on circulating VEGF levels persisted at 7 days after bisphosphonate infusion, with a median value of 885.000 pg/dl (95% CI: 789.43–1240.87; $P = 0.0090$). Moreover, zoledronic acid induced a significant and long-lasting decrease of VEGF-circulating levels that persisted to at least 21 days after the infusion (800.500 pg/dl; 95% CI: 733.50–1105.03; $P = 0.0011$). These results are summarized in Table 3 and Fig. 1.

PDGF Levels. The number an the percentage of patients who developed a reduction of circulating PDGF levels after zoledronic acid infusion are summarized in Table 2. The median PDGF basal level was 9102.00 pg/dl (95% CI:

Table 3 Cytokines modifications after a single infusion of zoledronic acid

	Median value (pg/dl)	95% CI (pg/dl)	Percentage change *versus* baseline	*P*
VEGF				
Basal levels	1222.000	1115.54–1435.02		
1 day	1143.000	1008.79–1340.29	−6.5	0.4950 (n.s.)
2 days	940.000	857.27–1130.24	−23	0.0498
7 days	885.000	789.43–1240.87	−28	0.0090
21 days	800.500	733.50–1105.03	−34	0.0011
PDGF				
Basal levels	9102.00	8875.04–13029.86		
1 day	6840.00	5609.96–8612.15	−25	0.0032
2 days	6344.00	5701.59–9061.05	−30	0.0050
7 days	10772.00	9142.40–12991.00	+18	0.7410 (n.s.)
21 days	8859.00	7179.08–12780.98	−3	0.8822 (n.s.)

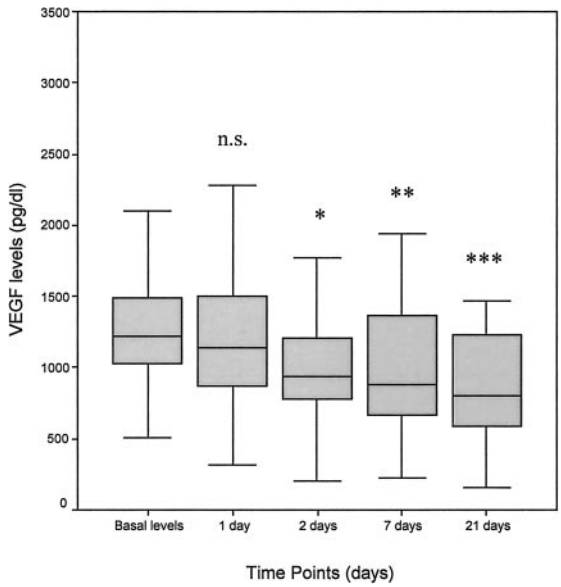

Fig. 1 The figure shows the behavior of VEGF levels at days 1, 2, 7, and 21 after zoledronic acid administration. represents 95 percentiles of all VEGF values. *Horizontal black bar* in the *gray boxes* represent VEGF median value. *Bottom* and *top horizontal bars* indicate minimum and maximum values. *P*s are calculated according to Wilcoxon test for nonparametric-dependent continuous variable: ∗, $P = 0.0498$; ∗∗, $P = 0.0090$; ∗∗∗, $P = 0.0011$; n.s., nonsignificant.

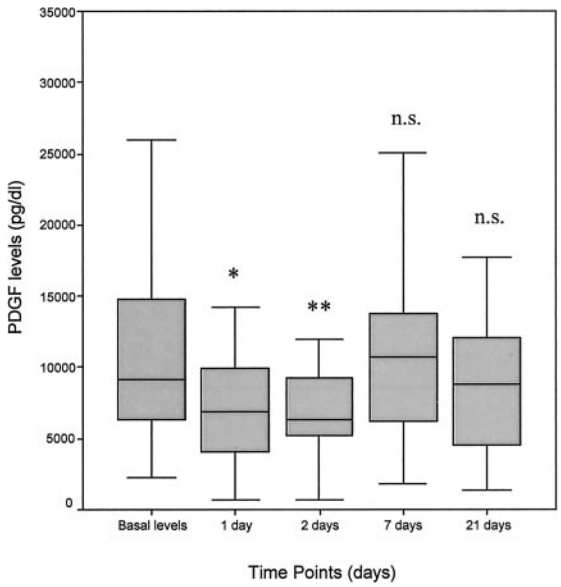

Fig. 2 The figure shows the behavior of PDGF 1, 2, 7, and 21 days after zoledronic acid administration. represents 95 percentiles of PDGF values. *Horizontal black bar* in the *gray boxes* represent PDGF median value. *Bottom* and *top horizontal bars* indicate minimum and maximum values. *P*s are calculated according to Wilcoxon test for nonparametric-dependent continuous variable: ∗, $P = 0.0032$; ∗∗, $P = 0.0050$; n.s., nonsignificant.

8875.04–13029.86). As reported in Table 3 and Fig. 2, these levels significantly decreased 1 day after zoledronic acid infusion to 6840.00 pg/dl (95% CI: 5609.96–8612.15; $P = 0.0032$). This effect persisted at day 2 after infusion, with a median value of 6344.00 pg/dl (95% CI: 5701.59–9061.05; $P = 0.0050$). Circulating PDGF levels returned to values similar to the median basal value at days 7 (10772.00 pg/dl) and 21 (8859.00 pg/dl; *P*s of 0.7410 and 0.8822, respectively).

Cytokines Correlations. A linear regression model with variance analysis showed a significant positive correlation between basal VEGF and PDGF values (β regression coefficient = 0.372; $P = 0.0280$; Table 4 and Fig. 3). As expected, our data did not show a significant correlation between VEGF and PDGF values at the later time points (Table 4).

Secondary Parameters. No significant differences have been recorded in platelet level, WBC count, or hemoglobin concentration before and after zoledronic acid infusion. However, as expected, a statistically significant decrease in plasma total calcium levels was observed after bisphosphonate administration: the median calcium level before zoledronic acid administration was 11.04 mg/dl (range: 10.1–12.8 md/dl), whereas the median value 7 days after zoledronic acid therapy was 8.45 mg/dl (range: 7.4–10.3 mg/dl; $P = 0.0004$). Moreover, 4 of 30 patients showed hypocalcemia 21 days after the infusion of zoledronic acid, and the second administration had to be delayed 1–3 weeks.

The serum calcium concentration did not correlate with any of the circulating cytokines before or after zoledronic acid

Table 4 Correlation between VEGF and PDGF at different time points

Time points	β regression coefficient	*P*
Basal	0.372	0.0280
1 day	0.284	0.070 (n.s.)[a]
2 days	0.275	0.113 (n.s.)
7 days	0.013	0.714 (n.s.)
21 days	0.156	0.541 (n.s.)

[a] n.s., nonsignificant.

infusion. A significant correlation in a linear regression model was noted between basal VEGF level and basal platelet count (β regression coefficient = 4.560; $P = 0.0200$).

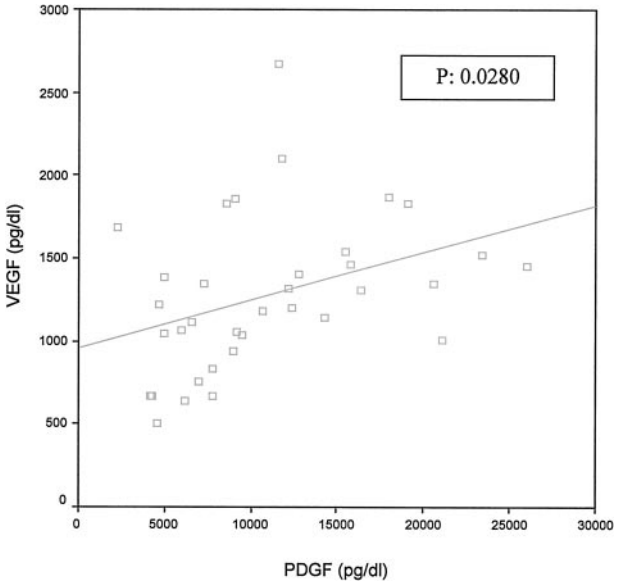

Fig. 3 The figure shows the correlations between basal VEGF and basal PDGF; this correlation was found statistically significant with a *P* of 0.0280. *P*s were calculated using a linear regression model with variance analysis.

Discussion

Similarly to our previous study (15), this study confirms *in vivo* the potential antiangiogenic role of bisphosphonates. However, differently from pamidronate, zoledronic acid induced a more prolonged decrease of serum VEGF levels until 21 days from the infusion. Moreover, zoledronic acid was able to induce also a significant, although transient, decrease of serum PDGF levels.

Although the precise mode of action of bisphosphonates on the clinical evolution of neoplastic disease is still not fully understood, several mechanisms for the antitumor effects have recently been proposed: alteration of bone microenvironment; cytotoxic/cytostatic and proapoptotic effects; reduction of human tumor-cell adhesion to bone matrix; modifications of tumor cells motility and viability; stimulation of γ/δ T cells and innate immunity; and inhibition of tumor angiogenesis. Inhibition of tumor angiogenesis by bisphosphonates is an intriguing hypothesis supported by some evidence in the literature. The study of Wood *et al.* (14) clearly highlights the potential *in vitro* and *in vivo* antiangiogenic properties of zoledronic acid in the preclinical setting. Moreover, recent data by Fournier *et al.* (21) have demonstrated, in a murine model, that zoledronic acid strongly inhibits prostate angiogenesis. Furthermore, the study of Zimering (22) clearly showed, in patients with cancer-associated hypercalcemia, a significant decrease in serum basic fibroblast growth factor levels, a potent tumor angiogenesis factor and normal constituent of bone extracellular matrix, after i.v. bisphosphonate treatment. On the basis of these experimental data, we studied the modifications of recognized circulating angiogenic factors (VEGF and PDGF) induced by a single administration of 4 mg of zoledronic acid before administration of any chemotherapy agent. Our study clearly demonstrates a statistically significant decrease, compared with basal values, in VEGF levels at 1, 2, 7, and 21 days after zoledronic acid infusion. Compared with the effects of pamidronate on serum VEGF levels (15), zoledronic acid clearly induced a longer lasting decrease of this angiogenic cytokine. In particular, 21 days after the first infusion of zoledronic acid, VEGF serum levels remained significantly lower than basal values. 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 (23) and survival in several malignancies (24, 25), with several studies underlining the potential role of VEGF in predicting response of tumor to anticancer therapy (25). As a consequence, the significant and long-lasting decrease of serum VEGF levels after a single infusion of 4 mg of zoledronic acid, reported in this study, may provide preliminary evidence of an *in vivo* antiangiogenetic effect of this bisphosphonate in cancer patients. The mechanism of this effect is unknown, and we have not yet been able to ascertain whether this effect only occurs after the first exposure to zoledronic acid. Previous studies have reported a positive correlation between platelet number and serum VEGF level in cancer patients (26, 27), supporting the hypothesis that platelets may serve the role of storage of VEGF in the circulation. In our study, we demonstrated a significant correlation in a linear regression model only between VEGF basal levels and platelet basal levels. In particular, no significant differences were recorded in platelet levels during the study period. These data confirm that the long-lasting serum VEGF decrease is not related to platelet count modifications but is strictly related to zoledronic action. PDGF expression has been shown in a number of different solid tumors from glioblastomas to prostate carcinomas (28). In these various tumor types, 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 (28). 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 (29). In this study, zoledronic acid was able to induce a transient but significant reduction of serum PDGF levels 1 and 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 surmise that

zoledronic acid may elicite several angiogenic-related cytokines patterns and cascades, as demonstrated by preclinical studies (14, 21). The literature data clearly show the potential and impressive antineoplastic properties of bisphosphonates and shed new light on biological applications of these compounds in the clinical setting. The new generation, heterocylic bisphosphonate, zoledronic acid, through its effects on osteoclasts, osteoblasts, tumor cells, cytokine, and growth-factor production, may interrupt the bone destruction and may represent a new class of drug with antitumour potential. In conclusion, this study confirms that not only pamidronate but also zoledronic acid induces an impressive, significant, and lasting modification of the angiogenic cytokine network.

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