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Increased angiogenesis and enhanced bone formation in patients with IGM monoclonal gammopathy and urticarial skin rash: new insight into the biology of the Schnitzler syndrome

Running title: Angiogenesis, bone formation and Schnitzler syndrome

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Abstract
Schnitzler syndrome is a rare plasma cell disorder with unclear pathogenesis. We evaluated the circulating levels of four major angiogenic cytokines (VEGF, angiogenin, angiopoietin-1 and -2) and six bone remodeling markers (sRANKL, osteoprotegerin, dickkopf-1, CTX, osteocalcin and bone-specific alkaline phosphatase-bALP) in 13 patients with Schnitzler syndrome. At diagnosis, patients had elevated angiogenic cytokines. The mean VEGF levels were almost 3.5-fold higher in Schnitzler syndrome compared to controls, while 10/13 patients had higher VEGF than the upper controls’ value. Successful treatment led to a significant reduction of VEGF. Patients with Schnitzler syndrome had increased bone formation (high bALP, osteocalcin and osteoprotegerin) which was not balanced by an increase of bone resorption (normal CTX and sRANKL). These data support the value of VEGF for the diagnosis (a new minor criterion?) and follow-up of Schnitzler syndrome, while the uncoupling of bone remodeling in favor of bone formation justifies the presence of bone densification.

Key words: Schnitzler syndrome, VEGF, angiogenesis, bone formation.
Introduction

Schnitzler syndrome is a rare plasma cell disorder characterized by the presence of a monoclonal IgM immunoglobulin in association with a chronic urticarial skin rash and at least 2 of the following minor criteria: intermittent fever, arthralgia or arthritis, bone pain, enlarged lymph nodes, splenomegaly and/or hepatomegaly, increased neutrophil counts, increased ESR and abnormal bone findings with imaging evidence of osteosclerosis.\textsuperscript{1,2}

The pathogenesis of the syndrome is unclear, with only one described case of spontaneous remission.\textsuperscript{3} The presence of high levels of interleukins (ILs) in patients with Schnitzler syndrome suggests that this plasma-cell disorder may be an acquired auto-inflammatory disease due to an unregulated secretion of cytokines via interaction of a clonal product (the M-protein?) with a key component of the IL-1 pathway. This is further supported by the successful therapy of the syndrome with anakinra,\textsuperscript{6} a synthetic analog of the endogenous IL-1 receptor antagonist, or with immuno-suppressant agents.\textsuperscript{7,8} However, the increased levels of the above inflammatory cytokines are not sufficient to explain several disease features, such as bone densification.\textsuperscript{9} Bone remodeling has never been evaluated in a series of patients with Schnitzler syndrome and there are very limited data in the literature for the coupling of bone formation and resorption in this entity. Furthermore, although angiogenesis is implicated in the pathogenesis of other plasma cell disorders, including multiple myeloma (MM)\textsuperscript{10} and POEMS syndrome,\textsuperscript{11} there is no information on the role of angiogenesis in Schnitzler syndrome. Therefore, the aim of this study was to evaluate angiogenic cytokines and bone remodeling in a large series of patients with Schnitzler syndrome in an attempt to better understand the biology of the disease.
Design & Methods

Patients

We studied 13 patients (12M/1F, median age 55 years, range: 39-79 years) with a well characterized Schnitzler syndrome, who were diagnosed, treated and followed in Hôpital Saint-Louis, Paris (France) and in Alexandra Hospital, Athens (Greece), between 1989 and 2009.

Serum had been collected from all patients at the time of diagnosis and at the time of best response to treatment and was stored at -80°C till the date of measurement. Time of best response was defined as the time of the best control of symptoms (mainly urticarial rash, bone pain and fever). All patients had given written informed consent for the sampling and storage of their serum for research purposes.

For the evaluation of bone involvement at the time of diagnosis, patients had a complete skeletal survey, using conventional radiography, while 11/13 patients had also a technetium bone scintigraphy.

Measurement of angiogenic cytokines and bone remodeling markers

The following circulating angiogenic cytokines were evaluated at diagnosis and at the time of best response to therapy: i) vascular endothelial growth factor (VEGF); ii) angiogenin; and iii) angiopoietin-1 and -2. For the evaluation of bone remodeling, the following serum indices were measured at diagnosis: i) the osteoclast regulators, soluble receptor activator of nuclear factor kappa-B ligand (sRANKL) and osteoprotegerin (OPG); ii) the osteoblast inhibitor dickkopf-1 (Dkk-1); iii) the bone resorption marker C-telopeptide of collagen type-1 (CTX) and iv) the bone formation markers, bone-specific alkaline phosphatase (bALP) and osteocalcin. Angiogenic
cytokines and bone markers were measured using commercially available ELISA kits, according to manufacturer instructions, as previously described\textsuperscript{12-14}.

The above molecules were also evaluated in 24 gender- and age-matched healthy subjects (22M/2F, median age 55 years, range: 30-80 years) who served as controls. Each healthy control was examined to ensure that there was no evidence of bone disease: the presence of osteoporosis was excluded by DXA measurements, while the presence of osteoarthritis was excluded using plain radiography. Furthermore, all healthy individuals were taking no medication that could alter their normal bone turnover during the last six months, had no infections or autoimmune disorder at the time of sampling, had normal liver and renal function and were taking no medication for hypertension or any heart disease.

The research protocol was approved by the institutional review board of Alexandra Hospital.

\textit{Statistical analysis}

Wilcoxon Signed Ranks test was used to test for differences within groups. Pearson’s (r) coefficient of correlation was used for correlation between variables.
Results and Discussion

Patients' clinical features
All patients presented with urticaria and monoclonal IgM-kappa protein (median M-peak 0.79 g/dl; range 0.1-2.36 g/dl), while the other clinical characteristics of the patients were typical for patients with Schnitzler syndrome\(^2\) [Figure 1]. Four (30.7%) patients had documented sclerotic bone lesions in plain X-rays. All 11 patients who were evaluated with a technetium bone scintigraphy had high uptake in at least one site.

At diagnosis, 11/13 patients received various symptomatic therapies, including low dose corticosteroids, that were ineffective. Two patients at diagnosis and 6 after initial symptomatic therapy were treated with the quinolone antibiotic pefloxacin, an established therapy for Schnitzler syndrome\(^1\)\(^5\). Five of these 8 patients had complete response of the disease with pefloxacin. Seven patients were treated with anakinra, including 3 who did not respond to pefloxacin. All of them had a complete and sustained control of the disease.

Circulating angiogenic cytokines in Schnitzler syndrome
At diagnosis, patients with Schnitzler syndrome had elevated VEGF (809±598 pg/ml vs. 263±257 pg/ml; \(p=0.001\)) and angiogenin (221±96 ng/ml vs. 169±33; \(p=0.013\)) compared to healthy controls, while they had decreased angiopoietin-1 (18.7±9.1 ng/ml vs. 25.4±10.9 ng/ml; \(p=0.048\)) and angiopoietin-1/angiopoietin-2 ratio
(13.9±8.7 vs. 63.4±102.1; p=0.005) [Figure 2, A-D]. More specifically, 10/13 patients had higher VEGF values than the upper value of the controls, while 6/13 patients had higher values of angiogenin than the upper value of the controls. After successful treatment with pefloxacin or anakinra, VEGF levels decreased (387±207 pg/ml) compared to baseline values (p=0.04; Figure 2E). There were no significant modifications in the levels of the other angiogenic cytokines after successful therapy. With a median follow-up time of 10 years, 3 (23%) patients with symptomatic disease requiring therapy developed Waldenström’s macroglobulinemia (WM) at 5, 7 and 20 years post diagnosis. Interestingly, these patients had decreased levels of angiopoietin-1 (10.2±7.5 ng/ml) compared to all others (21.2±8.2 ng/ml; p=0.04) at diagnosis and reduced angiopoietin-1/angiopoietin-2 ratio (5.3±4.6 vs. 16.5±8.0; p=0.04; Figure 2F). Patients with other plasma cell dyscrasias, like MM11 and POEMS12,16 have also increased circulating VEGF. Indeed in POEMS syndrome the increased level of circulating VEGF is considered as a minor criterion for the disease, while its levels have been used for the follow-up of the patients16. Furthermore, in other IgM-monoclonal gammopathies, such as in WM or in IgM-MGUS the circulating VEGF levels have been found to be 2-2.7-fold higher than that of healthy controls17. In our study, the mean levels of VEGF were almost 3.5-fold higher in patients with Schnitzler syndrome compared to healthy controls, while 10/13 patients had higher VEGF values than the upper VEGF value of the controls. Additionally, VEGF was significantly reduced post successful therapy with anakinra or pefloxacin. Based on these data, we suggest that VEGF may be used as a valuable marker or even as a minor criterion for the diagnosis of Schnitzler syndrome. However, more patients with the syndrome should be studied before the establishment of VEGF as a minor criterion of the disease.
The increased circulating VEGF in Schnitzler syndrome could be related to the IL-1 dysfunction which is present in this disease. It has been reported that IL-1 activates inflammatory cells to produce endothelial cell activating factors, such as VEGF and thus to promote angiogenesis. Moreover, inhibition of IL-1 completely abrogated angiogenesis and reduced VEGF levels by 85% in an in vitro model. Although this is a hypothesis that has to be confirmed, the reduction of VEGF in all our patients who were treated with anakinra (an IL-1 receptor antagonist) further supports this hypothesis.

In our study, patients with Schnitzler syndrome had also a reduced ratio of angiopoietin-1/angiopoietin-2. Angiopoietin-1 and angiopoietin-2 are ligands for the Tie-2 receptor, which is present on endothelial cells and endothelial progenitor cells. Angiopoietin-2 functions to block the angiopoietin-1/Tie-2 signaling, which serves to inhibit endothelial cell activation. Thus angiopoietin-2 facilitates endothelial activation in response to inducers of angiogenesis, such as VEGF. Reduced ratio of angiopoietin-1/-2 indicates high angiogenic activity and is also present in myeloma, where it correlates with advanced disease and poor prognosis. The reduced angiopoietin-1/-2 ratio in our patients confirms that angiogenesis is elevated in Schnitzler syndrome and is implicated in its biology. Another interesting finding of our study is the correlation between low angiopoietin-1/-2 ratio and progression of Schnitzler to WM. Although, the number of our patients who progressed to WM was low (3/13; 23%), but similar to that reported in the literature (15%), and the progression happened several years post diagnosis, this result may suggest that low angiopoietin-1/-2 ratio at diagnosis may indicate a predisposition for evolution of the gammopathy towards an overt WM. However, this has to be confirmed prospectively in larger number of patients.
Markers of bone remodeling in Schnitzler syndrome

At diagnosis patients with Schnitzler syndrome had increased serum levels of bALP (mean±SD: 36.5±15.0 IU/L vs. 26.8±7.1 IU/L; p=0.049), osteocalcin (20.5±18.6 ng/ml vs. 7.4±3.5 ng/ml; p<0.001), Dkk-1 (49.9±13.0 pmol/L vs. 30.9±11.1 pmol/L; p<0.001) and OPG (6.7±1.3 pmol/L vs. 3.5±1.8; p<0.001) compared to healthy controls [Figure 3]. There were no differences between patients and controls regarding sRANKL (0.28±0.22 pmol/L vs. 0.23±0.12 pmol/L; p=0.465) and CTX (0.32±0.25 ng/mL vs. 0.29±0.16 ng/mL; p=0.521).

The four patients, who had documented sclerotic lesions in the conventional radiographic skeletal survey, had significantly higher bALP (59.6±11.8 IU/L vs.32.8±6.1 IU/L; p=0.04) and OPG (9.2±2.3 pmol/L vs. 5.7±0.8; p=0.03) compared to all others. In all patients, there was a strong positive correlation between OPG and VEGF (r=0.676 and p=0.016). No other significant correlations were observed between bone remodeling markers and angiogenic cytokines.

All studied patients with Schnitzler syndrome had a high uptake in a technetium bone scintigraphy, while 30% of patients had sclerotic lesions with conventional radiographic skeletal survey; both suggestive of increased osteoblast function. This was further confirmed by the elevation of both markers of bone formation, bALP and osteocalcin that are produced directly by activated osteoblasts. On the other hand, there was no increase in bone resorption, as assessed by normal CTX and sRANKL levels, while OPG (the decoy receptor of RANKL) was remarkably elevated. These data suggest that there is an enhanced osteoblast function in Schnitzler syndrome, which is not balanced by an elevation of bone resorption, leading to bone densification and osteosclerotic lesions. Other IgM-gammopathies, i.e. WM and IgM-MGUS have also altered bone remodeling but the elevation of bone formation is
not as evident as that observed in Schnitzler syndrome. The cause for this increased osteoblast function is obscure. IL-1 and IL-6 that participate in the pathogenesis of Schnitzler syndrome\textsuperscript{2,4} are well-known stimulators of osteoclast function\textsuperscript{20,21}, while IL-1 directly inhibits osteoblast activity\textsuperscript{22}. During the last years, it became evident that angiogenesis enhances osteogenesis and VEGF is able to increase bone formation through modulation of angiogenesis\textsuperscript{23}. In our cohort of 13 patients, we found a strong correlation between VEGF and osteoprotegerin, suggesting that elevated VEGF may be, at least partially, responsible for elevated bone formation in Schnitzler syndrome. In this study, we also found an elevation of the osteoblast inhibitor Dkk-1. Dkk-1 is a Wnt signaling inhibitor, which is up-regulated by osterix, an osteoblast-specific transcription factor required for bone formation\textsuperscript{24}. Thus the elevation of Dkk-1 in Schnitzler syndrome may reflect a balance effect on the increased activity of the osteoblasts.

Four patients who were treated with anakinra had also a bone scintigraphy at a median of 6 months post-treatment. All patients showed a dramatic reduction of osteoblastic lesions but none of them showed a complete disappearance of the lesions.

In conclusion, our analysis shows altered circulating angiogenic cytokines in Schnitzler syndrome reflecting increased angiogenic activity. Furthermore we document enhanced bone formation with no alterations of bone resorption; this result explains the presence of sclerotic bone lesions in this entity. Successful therapy with either anakinra or pefloxacin is associated with reduction of the major angiogenic cytokine VEGF. These data supports the value of VEGF for the diagnosis and follow-up of patients with Schnitzler syndrome and suggest that VEGF may be used as a minor criterion for the diagnosis of the disease.
Authorship and Disclosures

ET designed the study, performed all laboratory parameters of the study, analyzed the data, and wrote the paper. JPF, BA and MAD performed data collection, analyzed the data and followed-up the patients. BA, JCB, EK and MR followed-up the patients. DB collected patients’ data. DC and EK performed the statistical analysis.

All authors have provided comments on the drafts of the paper and approved the final draft for submission to Haematologica.

The authors declare no competing financial interests.

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Legends to Figures

**Figure 1.** Clinical features of patients with Schnitzler Syndrome except of chronic urticarial rash and IgM monoclonal protein.

**Figure 2.** At diagnosis patients with Schnitzler syndrome had increased circulating levels of VEGF (A) and angiogenin (B) compared to healthy controls and decreased angiopoietin-1 (C) and angiopoietin-1/angiopoietin-2 ratio (D) suggesting increased angiogenic activity in Schnitzler syndrome. After successful treatment with pefloxacin or anakinra, VEGF levels decreased (387±207 pg/ml) compared to baseline values (p=0.04, E). Three patients who progressed to overt WM had decreased levels of angiopoietin-1/angiopoietin-2 ratio at diagnosis compared to all other patients (5.3±4.6 vs. 16.5±8.0; p=0.04, F).

**Figure 3.** At diagnosis patients with Schnitzler syndrome had elevated serum bALP (A), osteocalcin (B), OPG (C) and Dkk-1 (D). The increase of bone formation markers (bALP, osteocalcin and OPG) in combination with normal bone resorption is responsible for the bone densification observed in Schnitzler syndrome.
Figure 1.
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