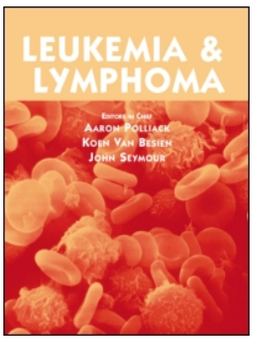

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LETTER TO THE EDITOR

Soluble RANKL production by leukemic cells in a case of chronic lymphocytic leukemia with bone destruction

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Receptor Activator for Nuclear Factor κ B Ligand (RANKL) is a member of the TNF-α superfamily normally produced by osteoblasts and stromal cells, which activates its receptor RANK present on osteoclasts and osteoclast precursors, thus favoring their differentiation and activity. An aberrant expression of RANKL was previously reported in a proportion of B cell malignancies such as Chronic lymphocytic leukemia (CLL),[1] multiple myeloma (MM)[1] and follicular lymphoma.[2] Signaling via RANKL in CLL and in MM cells induce the release of cytokines involved in disease pathophysiology, including IL-6, IL8 and TNF-α,[1] whereas release of soluble RANKL (sRANKL) was observed in MM cells and was not detected in CLL cells.[1] In line with this, bone destruction is a prominent feature of MM but a rare complication in CLL. We here present a case of CLL with lytic bone lesions displaying an aberrant release of sRANKL by the malignant cells.

The patient (JL), a 72-year-old male with a background of dyslipemia and hypertension, consulted in 2009 at the Hematology Department of our Hospital for leucocytes (19.4×10^9/L with 82% of lymphocytes). Immunophenotyping of peripheral blood leucocytes showed an abnormal lymphoid population $CD5^+$, $CD23^+$, $CD79b^{(-)}$, $FMC7^{(-)}$ and $CD20^{+/-}$, confirming the diagnosis of CLL (Matutes score 5/5). The expression of the prognostic markers ZAP-70 and CD38 on circulating CLL cells was evaluated by flow cytometry and was found to be negative for ZAP-70 (2%) and positive for CD38 (80%). The laboratory studies revealed normal values for lactate dehydrogenase (LDH) (315 UI/L) and β2-microglobulin levels of 3.2 μg/mL. The patient had normal serum immunoglobulin levels and a normal pattern of the albumin, alpha, beta and gamma globulins fractions. The serum protein electrophoresis analysis of our patient showed, as previously reported for some CLL patients,[3–5] a small monoclonal component (0.5 g/dL). Immunoglobulin levels, albumin and globulinś fractions, as well as the monoclonal component remained stable in all the peripheral blood analysis performed since the diagnosis until the present. The analysis of IGHV gene was performed as previously described [6] and showed a mutated status (92% homology with the germline), displaying the IGHV3-7*03, D2-8*01 and J4*02 rearrangement. The patient was diagnosed with a RAI I, Binet A CLL and was regularly controlled without presenting any significant change until 2011 when he required hospitalization for a severe back pain. Both X-ray and MRI showed extensive lytic bone lesions. A bone biopsy was performed and its microscopic examination revealed a diffuse infiltrate (Figure 1a) composed of small mononuclear cells positive for CD20, CD23, CD5 and Bcl-2, with a low Ki67 expression (Figure 1b). The bone biopsy was negative for the CD138 staining and no plasma cell infiltration was

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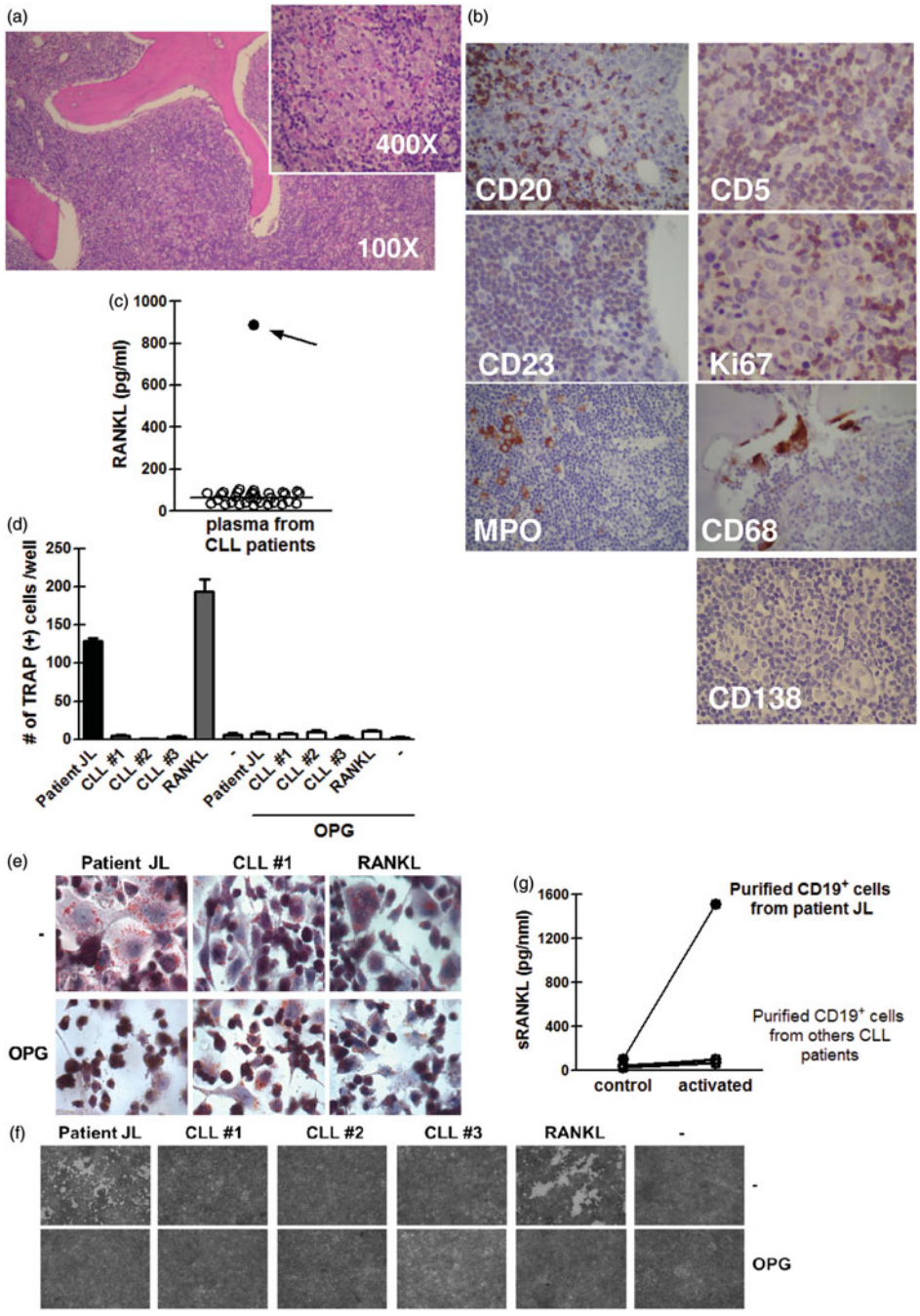

Figure 1. (a) Hematoxylin and eosin staining from vertebral biopsy 100× and 400×. (b) Immunohistochemical staining of the bone lesion with CD20, CD5, CD23, Ki-67, MPO, CD68 and CD138. (c) sRANKL quantification in plasmas from patient JL (black circle highlighted by an arrow) and 30 CLL patients without bone involvement (open circles). sRANKL was evaluated in duplicate by ELISA. (d) The osteoclast formation assay was performed by inducing THP-1 monocytic cell line to undergo osteoclastogenesis as previously reported.[13]. THP-1 cells were also cultured in the presence of 10% of plasma from patient JL, plasmas from other 3 CLL patients or with RANKL (50 ng/mL) as a positive control. The experiment was performed in the absence or presence of the RANKL-decoy receptor OPG (50 ng/mL). After 5 days, osteoclastogenesis was determined by the generation of multinucleated TRAP-expressing cells. Bars express the mean ± SEM of duplicates. (e) Representative digital images from the generation of multinucleated TRAP-expressing cells were taken by light microscopy. 100× magnification shows a representative multinucleated TRAP-expressing cell differentiated with plasma from patient JL. (f) Functional activity of osteoclast-like cells induced by plasma from patient JL was determined by their ability to resorb dentin.[13]. To this aim, THP-1 cells were cultured on dentin discs under the same conditions described above. After 5 days, cells were removed, and dentin resorption was determined by light microscopy. (g) $CD19^{+}$ cells were purified from peripheral blood mononuclear cells from Patient JL and five other CLL patients without bone involvement by negative selection using anti-CD14, CD3 and CD56 mAb and magnetic beads. Afterwards, 3×10^{6} $CD19^{+}$ cells were stimulated with CpG or left untreated for 3 h, washed and cultured in RPMI 1640 + 10% FCS medium for 3 days. Then, the supernatant of each culture was collected and sRANKL was determined by ELISA.

found (Figure 1b). Interestingly, the presence of large multinucleated cells expressing myeloperoxidase and CD68 suggested an osteoclast-like cell appearance (Figure 1b). Cytogenetic and fluorescent *in situ* hybridization (FISH) analysis of peripheral blood lymphocytes were carried out as formerly reported.[7] The following karyotype was observed: 47,XY,+12 (4)/46,XY (16); trisomy 12 was confirmed by FISH (59% of cells). The remaining locus specific probes (D13S319 (13q14), ATM (11q22), TP53 (17p13) and IGH Dual Color Break Apart (14q32) DNA probes (LiVE-Lexel, Argentina) had a normal pattern of signals. Peripheral blood mononuclear cells and plasma samples were also collected and properly stored for further studies.

Osteoclasts play a key role in bone resorption and originate from the fusion of precursors belonging to the monocyte/macrophage lineage in the bone marrow.[8,9] RANKL induces the differentiation of osteoclast precursor cells into mature osteoclasts [10,11] and it also induces the activation [10] and survival [12] of mature osteoclast promoting bone resorption. Thus, the presence of lytic bone lesions in patient JL prompted us to study plasma levels of sRANKL. We decided to evaluate plasma levels of RANKL in our CLL patient with bone destruction (patient JL) and in 30 CLL patients without bone involvement. Samples of all patients were obtained after informed consent in accordance with the Declaration of Helsinki and with Institutional Review Board approval from the National Academy of Medicine, Buenos Aires. As shown in Figure 1c, sRANKL levels were more than 10 times higher in plasma from patient JL (888 pg/mL) compared with the rest of samples (mean ± SE, 64 ± 5 pg/mL). To test whether sRANKL levels in plasma was sufficient to induce osteoclast formation we cultured THP-1 monocytic cell line with 10% plasma from patient JL, plasma from three patients without bone involvement or sRANKL as a positive control. The experiment was performed with or without osteoprotegerin (OPG), the RANKL decoy receptor, and osteoclastogenesis was evaluated by the generation of multinucleated TRAP-expressing cells, as previously reported.[13] As shown in Figure 1d plasma from patient JL, but not from the other CLL patients, increased the number of $TRAP^+$ multinucleated cells, a process that was completely abrogated by OPG treatment. Representative micrographs are shown in Figure 1e. Osteoclast-like cells differentiated from THP-1 cells were functional as shown by their capacity to resorb dentin (Figure 1f). Additionally, the resorption of dentin was blocked when the experiments were performed in the presence of OPG (Figure 1f). Taken together, these results suggest that the high expression of sRANKL in plasma from patient JL may contribute to bone destruction through activation of osteoclastogenesis.

Finally, in order to determine whether leukemic cells were able to secrete sRANKL, $CD19^+$ cells were purified from peripheral blood mononuclear cells from patient JL and five other CLL patients without bone involvement. Then, purified $CD19^+$ cells were cultured for 72 h with or without CpG in order to induce their activation, which was confirmed by the upregulation of CD80 and CD86 evaluated by flow cytometry (not shown). In accordance with a previous report,[1] we found that control cultures of leukemic cells from CLL patients without bone involvement did not produce sRANKL (Figure 1g), while control cultures of purified $CD19^+$ cells from patient JL were able to secrete small but quantifiable amounts of sRANKL (102 pg/mL). *In vitro* activation of purified $CD19^+$ cells from CLL patients without bone involvement induced the secretion of small amounts of the molecule, while the activation of purified CLL cells from patient JL aberrantly enhanced their secretion of sRANKL (Figure 1g). Altogether these findings suggest that the secretion of sRANKL by the leukemic clone may account for its high plasma concentration and may have contributed to the development of his lytic bone lesions. It remains to be determined if malignant cells from other CLL patients with bone destruction can also abnormally secrete sRANKL.

The patient was treated with Pamidronate (6 cycles, 90 mg/monthly) and radiotherapy (36 Gy) achieving an important pain reduction in the back area and lowering the lymphocyte levels. The lytic lesions stabilized. He remained on observation and in 2013 his peripheral blood lymphocyte counts raised with a lymphocyte doubling time less than 6 months. At that moment, cytogenetic analysis revealed a clear clonal evolution with the following karyotype: 47,XY,+X,−8,+12 [cp18]/46,XY (2), and FISH study showed trisomy 12 (42.3% of cells) and a small clone with deletion 17p13 (7.5% of cells). The patient completed the 4th cycle of Rituximab/Bendamustine obtaining a partial response according to the IWCLL-sponsored Working Group criteria.[14] At the end of treatment the patient had a normal hemogram, no organomegaly, while bone marrow analysis revealed a diffuse infiltrate with a nodular pattern composed of small mononuclear cells positive for bcl-2, CD20, CD23, CD5 and Ki67 and negative for CD10, CD138, CD3 and CD68, showing that CLL cells still persist in this niche. Also, the lytic lesion remained stable after BR treatment as assessed by MRI. The patient has a

stable disease since then with no need of treatment until the present.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at http://dx.doi.org/10.3109/10428194.2016.1151506.

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