IL-7 Modulates Osteoclastogenesis in Patients Affected by Solid Tumors

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ABSTRACT: **High levels of interleukin-7 (IL-7) have been associated with bone loss due to its stimulatory osteoclastogenic activity. Osteolytic patients' peripheral blood mononuclear cells (PBMCs) differentiate into osteoclasts without adding stimulating factors. Now, we investigated the potential role of IL-7 in the spontaneous osteoclastogenesis occurring in these patients. We identified significant differences in serum IL-7 levels between patients with/without bone metastases, suggesting that IL-7 might be effective as a clinical marker of disease progression. In patients' PBMC cultures we demonstrated that IL-7 stimulates osteoclastogenesis by inducing TNF-α release by T and B cells. These findings add further details to the disclosure of the mechanisms controlling bone metastases in solid tumors.**

KEYWORDS: **bone metastasis; osteoclast; IL-7**

INTRODUCTION

Interleukin-7 (IL-7) is a pleiotropic immune regulatory protein predominantly produced by stromal cells and by cells at the inflammatory sites.[1] IL-7 regulates peripheral T cell homeostasis by modulating the expansion of peripheral T cell populations in states of T cell depletion.[2] The production of IL-7 by some solid tumors suggests its potential impact on the process of tumorigenesis,[3] but it is unclear how IL-7 is involved in solid tumor development and progression. IL-7 is also involved in the control of osteoclast (OC) differentiation, which is mainly regulated by M-CSF, RANKL, and tumor necrosis factor

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(TNF)-α. It has been recently demonstrated that in patients affected by solid tumor with osteolysis, peripheral blood mononuclear cells (PBMCs) spontaneously differentiate into OC.[4] The aim of this work was to study the role of IL-7 in spontaneous osteoclastogenesis, occurring in patients affected by solid tumor with bone metastases.

MATERIALS AND METHODS

Patients and Cell Cultures

Samples from peripheral blood were obtained from 50 male patients affected by solid tumors and 50 healthy controls, matched for age and sex. We analyzed patients with newly diagnosed lung, kidney, bladder, prostate, or colon cancer. We excluded cancer patients previously treated with chemo/hormonotherapy and with preexisting bone pathologies. PBMCs were cultured with/without rhIL-7 at different doses (0.5–1-2,5–10 and 15 ng/mL), R&D Systems (Abingdon, UK). Culture supernatants were collected on days 5 and 10, when medium was refreshed. For some experiments, patient PBMCs were cultured with different doses of anti-IL7 (50–100–300 ng/mL), R&D Systems or with increasing concentration of a neutralizing anti-TNF-α antibody and osteoprotegerin, PeproTech (London, UK). Cultures were stopped after 15 days, mature OCs were identified as $TRAP^{+}$ (Tartrate-resistant acid phosphatase, Sigma Aldrich, St. Louis, MO, USA) multinucleated cells. Monocytes, T and B cells were isolated from PBMCs by MACs microbeads, Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

Enzyme-Linked Immunosorbent Assay (ELISA)

The amount of IL-7 in cell culture supernatant and serum was determined by ELISA kit (R&D Systems, Abingdon, UK). TNF-α (Biosource, Nivelles, Belgium) and RANKL (Biomedica Gruppe, Wien, Austria) ELISA kit was performed on culture supernatants.

Quantitative Analysis of IL-7 Gene Expression

Total RNA was extracted from T and B cells and the first-strand cDNA synthesis was performed as previously described.[4] Quantitative analysis of IL-7 was performed with real-time quantitative polymerase chain reaction (RQ-PCR) using β-actin as housekeeping control, according to the previously published procedure.[5]

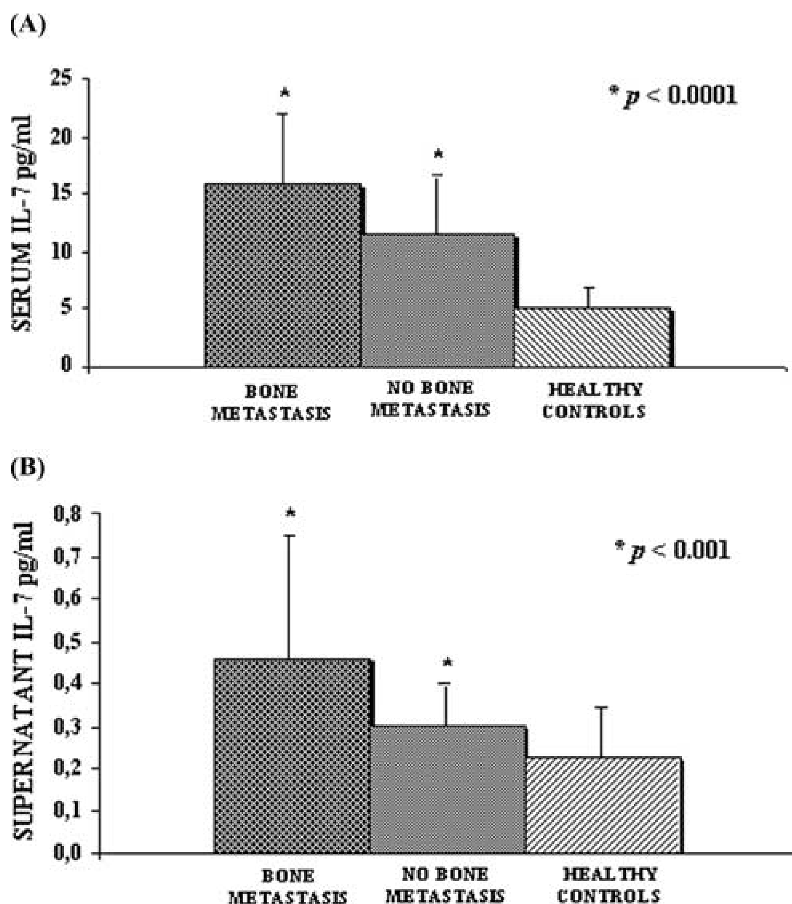

FIGURE 1. IL-7 levels in sera and cell culture media. Bone metastatic patients had significantly higher serum and supernatant levels of IL-7 compared to patients without bone metastasis and healthy controls (**A**, **B**, respectively).

Statistical Analyses

Statistical analyses were performed with the Statistical Package for the Social Sciences (spssx/pc) software (SPSS, Chicago, IL, USA). The results were considered statistically significant for $P < 0.05$.

RESULTS AND DISCUSSION

We tested serum level of IL-7 in 50 patients affected by solid tumors with/without osteolytic metastases and found the highest IL-7 levels in

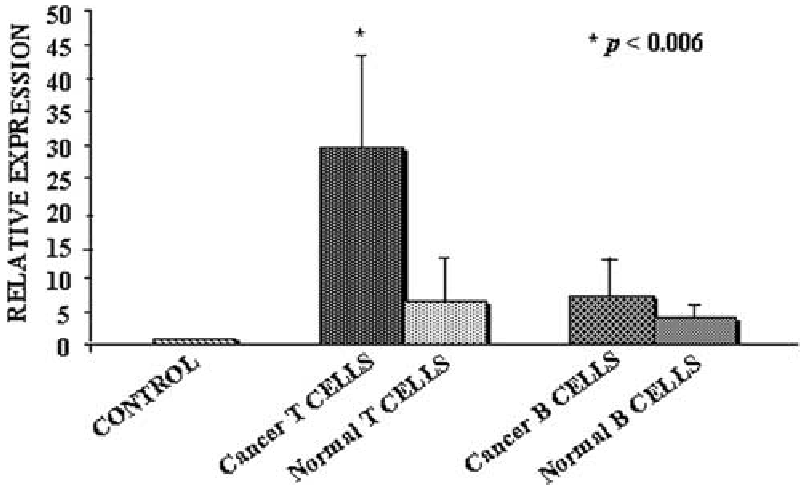

FIGURE 2. Quantitative analysis of IL-7 expression in T and B cells. Both T and B cells from healthy controls expressed less IL-7 than patients. B cells derived from cancer patients expressed significantly higher levels of IL-7 compared to T cells. The control bar represents the negative control: IL-7 expression in H522 cell line.

osteolytic cancer patients, followed by patients without bone lesions, and then healthy controls, $P < 0.0001$ (FIG. 1A). To investigate whether IL-7 is present in PBMC cell cultures, we measured it and showed that IL-7 levels were significantly higher in patients' than in healthy controls' cell cultures, $P < 0.001$ (FIG. 1B).

To identify the source of IL-7 in PBMC cultures we analyzed IL-7 expression in T and B cells by quantitative analysis with real-time PCR. We showed that both the cellular types expressed more IL-7 than the normal counterpart, but B cells have a higher expression than T cells, $P < 0.006$ (FIG. 2). This last result was confirmed by dosing IL-7 in supernatants of monocyte plus T and B cell co-cultures (data not shown).

IL-7 increased OC differentiation compared to basal condition (FIG. 3A, C) in both patients and healthy controls (FIG. 3B, D, respectively). The main stimulation was at 2.5 ng/mL of IL-7, $P < 0.001$, while at 15 ng/mL, IL-7 did not have a stimulatory effect on osteoclastogenesis (FIG. 3E). By adding a neutralizing anti-IL-7 antibody on osteolytic patients' PBMC, we observed a dose-dependent inhibition of spontaneous osteoclastogenesis, $P < 0.01$ (FIG. 3F).

Since literature data link IL-7 to TNF-α and RANKL-dependent osteoclastogenesis,[6–8] we measured their levels in culture media. In detail, we demonstrated that IL-7 supports OC formation by inducing the TNF-α production in both PBMC and co-culture of monocyte plus T or B cell culture media (FIG. 4). We hypothesized that TNF-α levels reached maximal values in the PBMC cultures probably due to a cooperative release of TNF-α by monocytes,[9] T and

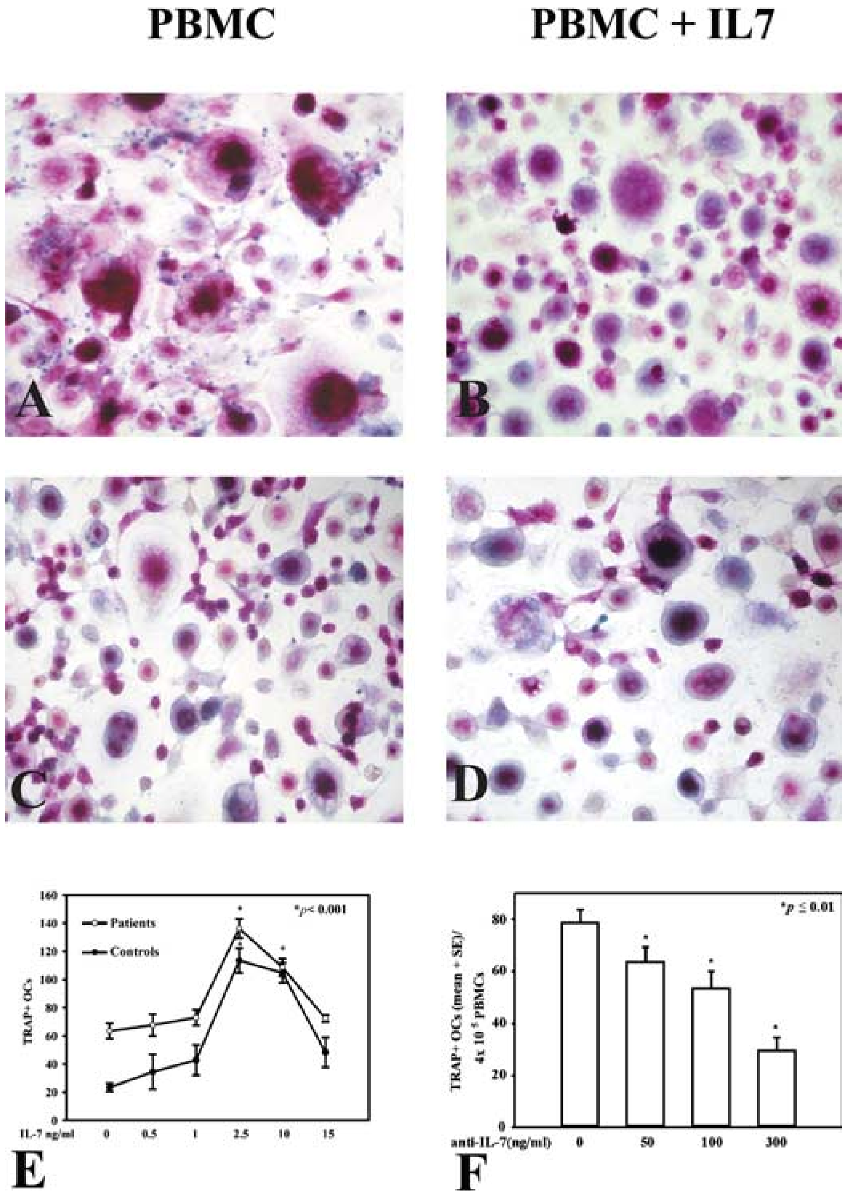

FIGURE 3. Effect of IL-7 on osteoclastogenesis *in vitro*. In both bone metastatic patients (**A**) and healthy controls (**C**), the number of OCs (TRAP$^+$ multinucleated cells) were increased after stimulation with IL-7 (**B**, **D,** respectively). Specifically, osteoclastogenesis significantly increased at 2.5 and 10 ng/mL of IL-7 compared to the unstimulated condition (**E**). The anti-IL-7 antibody added in cell culture determined a dose-dependent inhibition of spontaneous osteoclastogenesis in patients (**F**).

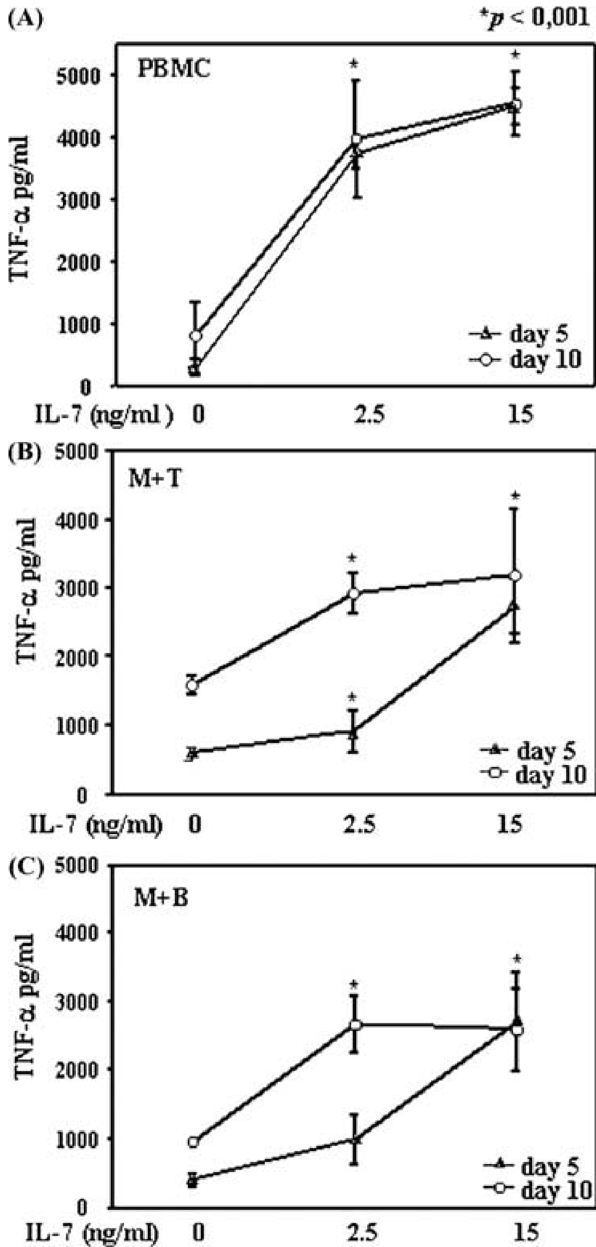

FIGURE 4. IL-7 upregulates TNF-α release in supernatants. Patients' and healthy controls' PBMCs and monocytes plus T or B cells were cultured in the absence or presence of IL-7. TNF-α levels increased in a dose-dependent manner in PBMC cultures (**A**), while in monocytes plus T or B cells TNF-α levels showed the peak value at 2.5 ng/mL of IL-7 (**B**, **C**, respectively).

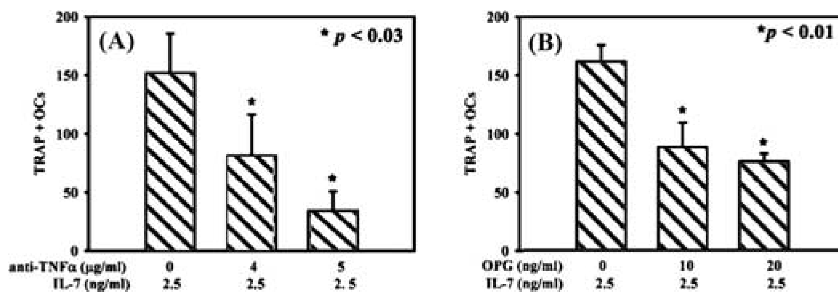

FIGURE 5. Anti-TNF-α and OPG inhibit IL-7-induced osteoclastogenesis. The addition of both OPG and anti-TNF-α antibody caused a dose-dependent inhibition of IL-7-induced osteoclastogenesis, in detail the anti-TNF-α antibody (**A**) caused a strong osteoclastogenesis inhibition compared to OPG (**B**).

B cells stimulated by IL-7. We observed a little and not statistically significant dose-dependent increase of RANKL after IL-7 stimulation, even though OPG caused a dose-dependent inhibition of IL-7-induced osteoclastogenesis. Nevertheless, the osteoclastogenesis inhibition was major with the anti-TNF-α antibody compared to the OPG treatment (FIG. 5). This observation suggests that in our model TNF-α has a stronger role in OC formation than RANKL, but both factors may synergize in promoting osteoclastogenesis.

In conclusion, we identified a key role of IL-7 in the formation of solid tumor bone metastasis since we demonstrated that IL-7, produced mainly by B cells in cell culture, directly sensitizes T cells to release pro-osteoclastogenic factors, such as TNF-α and RANKL, and enhances spontaneous osteoclastogenesis. This work represents the first experimental step to set an IL-7 cut-off value, representative of a warning threshold for clinicians, allowing them to follow the bone metastatic disease both at diagnosis and during treatments.

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