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The active role of osteoporosis in the interaction between osteoblasts and bone metastases

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ABSTRACT

Introduction: To minimize the severity of bone metastases and to delay their onset, it is important to analyze the underlying biological mechanisms. The present study focused on the link between OP and metastatic cells, with particular attention to osteoblast behavior.
Methods: Osteoblasts (OB) were isolated from the trabecular bone of iliac crest of healthy (SHAM) and ovariectomized (OVX) adult female rats and co-cultured with MRMT-1 rat breast carcinoma cells as conditioned medium (CM) or alone (CTR) for 24 h, 7 and 14 days and tested for cell viability, morphology and synthetic activity, i.e. C-terminal procollagen type I, alkaline phosphatase, osteoprotegerin, receptor activator for nuclear factor KB ligand and interleukin-8.
Results: Osteoblast morphology showed a reduced organization in the OVX group, in particular in the CM condition. Conversely, the analysis of cell viability revealed significantly higher values in the OVX_{CM} group with respect to the $SHAM_{CM}$ group at all experimental times, whereas the OVX_{CTR} group had significantly lower values at 7 and 14 days in comparison to those of the SHAM group. ALP release was significantly lower in the CM condition than that of CTR at all timepoints, and so was procollagen type I at 7 and 14 days. The RANKL/OPG ratio showed significantly higher values in OVX osteoblasts in comparison with those of the SHAM group, both in CTR and in CM conditions at each experimental time. Finally, OVX_{CM} showed significantly higher values of IL-8 than those of $SHAM_{CM}$ at 7 and 14 days.
Conclusions: The results clearly indicate an influence of the metastatic cells on the osteoblastic physiology at different levels: morphology, viability, release of typical proteins, and also IL-8 as a proinflammatory cytokine, especially marked by osteoporosis. Further investigations might highlight the relationship between osteoblasts and breast cancer cells, which might be useful to improve common drugs used against osteoporosis and bone metastases, by enhancing the bone deposition/tumor progression ratio.

1. Introduction

The skeleton continuously undergoes remodeling: bone is deposited by osteoblasts and reabsorbed by osteoclasts in a coordinated manner [1]. In this finely orchestrated system, the mineralized matrix stores different growth factors, such as insulin-like growth factor (IGFs), transforming growth factor α and β (TGFα and β), fibroblast growth factors (FGF-1 and 2), platelet-derived growth factors (PDGFs), and bone morphogenetic proteins (BMPs), some of which are secreted by osteoblasts during bone deposition and released into the marrow during osteoclast-mediated bone resorption [2]. This microenvironment, as described by the "seed and soil" hypothesis, is an ideal site to grow for breast cancer cells [3]. Biologically, the metastatic process of breast carcinoma in the bone consists of several stages, the first of which requires the ability of invasion and homing, once cancer cells arrest in the bone marrow. The second step is probably the role of growth factors released into the microenvironment [2]. In any case, the relationship between osteoblasts, osteoclasts and immune cells with breast cancer cells is critical for the process of bone metastases [4].

The ability of metastatic cancer cells to produce a variety of cytokines, and other chemical mediators by themselves then triggers a "vicious cycle", which involves the continuous increase of the osteoblast-mediated activation of osteoclasts [5]. In particular, parathyroid hormone-related protein (PTHrP) and TGF-β released by cancer cells have a negative impact on osteoblast function, by increasing the receptor activator of nuclear factor-kB (RANKL) and decreasing osteoprotegerin (OPG) production. As a result the bone synthesis is reduced and the resorption increased and osteoblast differentiation is altered [6].

This observation was also made by Bussard et al. who analyzed *in vitro* some osteoblast-derived cytokines in the presence of metastatic

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breast cancer cells, such as interleukin (IL)-6, monocyte chemotactic protein-1 (MCP-1), vascular endothelial growth factor (VEGF), macrophage inflammatory protein-2 (MIP-2) and KC (hGROα) [7]. These cytokines act in turn as chemo-attractants for cancer cells, which use this way to colonize bone tissue and enhance their survival, but also to enhance osteoclast formation.

Breast cancer commonly causes osteolytic metastases in bone [8,9] and therefore the majority of studies and therapeutic treatments are focused on OC. Nevertheless, the incomplete success of current drugs [7] may suggest a possible impairment of osteoblast function and an osteoblastic role in the persistence of osteolytic metastatic bone disease [10]. It is recognized that the osteoblastic activity associated with metastatic cells starts with a phase of abnormal bone resorption, and only later in the course of the metastatic bone disease is there a shift to a predominantly osteoblastic activity characterized by the formation of abnormal disorganized bone concurrently with a marked reduction in osteoclastic activity [11].

This outcome suggests that osteoblast function is also impaired in osteolytic metastases. However, very little is known about osteoblasts in osteolytic breast cancer metastasis. Mercer et al. claimed that breast cancer cell-conditioned medium blocked OB adhesion and differentiation [12].

Finally, to minimize the morbidity and economic burden associated with bone metastases, it is important to decrease their etiological factors. Although evidence on cancer drug-induced osteoporosis (OP) exists, few studies have also investigated whether OP affects bone metastasis formation [13]. What is the importance of osteoporosis for the onset of metastases? Fili et al. observed that osteolysis combined with osteoporosis not only induces growth factor release from the bone microenvironment, thus perpetuating the 'vicious cycle', but at the same time creates the space needed for tumor growth in bone [8].

In addition, Wu Y-P et al. supported this assumption by hypothesizing an angiogenic effect due to both inflammatory factors related to osteolysis and leptin [14]. Respectively, the inflammatory factors might make blood vessels leakier, thus resulting in an easier hematogenous metastasis to bone and bone marrow, and leptin might regulate VEGF expression and angiogenesis, thus promoting the proliferation of the cancer blood vessel.

The present study focused on the link between OB and metastatic cells in healthy conditions and in OP, with a particular interest in the morphological and synthetic features of osteoblasts. We hypothesized that an investigation into osteoblast behavior might provide a better understanding of the mechanism used by breast tumor cells to colonize the bone, thus implementing the elements of the vicious cycle. To achieve this aim we used an *in vitro* model where osteoblasts derived from healthy and osteoporotic rats were indirectly co-cultured with rat breast carcinoma cells (MRMT-1), by using a conditioned medium.

2. Materials and methods

2.1. Conditioned media preparation

Rat mammary gland carcinoma cells (MRMT-1), established from a cell line maintained by *in vivo* transplantation, were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University 4-1, Seiryo, Aoba-ku, Sendai, Japan). The cells were cultured in flasks, in RPMI 1640 medium (Sigma, MO, USA) supplemented with 10% fetal bovine serum (FBS, heat-inactivated) (Lonza, Verviers, Belgium), 2 mM glutamine, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) (Gibco, INVITROGEN Corporation, Carlsbad, CA). At confluence the cultures were rinsed and serum-free RPMI 1640 added (15 ml per T75 flask, ~1×10^6 cells/cm^2). Twenty-four hours later, the breast cancer cell-conditioned medium was collected, centrifuged, and stored at −80 °C.

2.2. Isolation and culture of osteoblasts

Osteoblasts were isolated from the trabecular bone of the iliac crest of healthy (SHAM) and ovariectomized (OVX) Sprague–Dawley adult female rats (Charles River Calco Lecco, Italy), previously euthanatized from an uncorrelated study (approved by the local Ethical Committee and Italian Ministry of Health). Twelve weeks after bilateral ovariectomy, the osteoporotic condition was confirmed by total body X-ray, microtomography, histology and histomorphometry on iliac crest bone biopsies (data not shown). The bone fragments were washed with serum-free Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, MO, USA), then finely minced with a scalpel and digested with 1 mg/ml collagenase (SIGMA, St. Louis, MO) for 2 h at 37 °C. The enzymatic reaction was stopped by adding an equal volume of medium with 10% FCS and the bone pieces were removed. The harvested cells were washed twice in phosphate buffer saline (PBS) and resuspended in a specific osteoblastic medium (OM): DMEM added with 10% fetal calf serum (FCS, Lonza, Verviers, Belgium), 2 mM glutamine, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), β-glicerophosphate (10^{-8} M) and ascorbic acid (50 μg/ml), placed in culture flasks and incubated at 37 °C in a humidified 95% air/5% CO_2 atmosphere. The cells were detached at subconfluence with 0.05% (w/v) trypsin/0.02% (w/v) EDTA, expanded and used at the second passage for the experiment at a density of 1×10^4 cells/cm^2. SHAM and OVX osteoblasts were refed with different media, which were alternative culture conditions:

- OM, as described above, (CTR);
- 50% OM + 50% breast cancer cell-conditioned medium, (CM) Table 1.

In all cultures media were changed twice a week.

2.3. Alizarin Red-S (ARS) staining

To evaluate calcium deposition in osteoblast cultures before the experimental set-up, SHAM and OVX in OM were washed with phosphate buffer solution (PBS, Euroclone SpA, Milan, Italy), fixed in 4% paraformaldehyde for 15 min, washed twice with deionized water and then stained with Alizarin Red 2% solution (Sigma-Aldrich SRL, Milan, Italy) for 40 min. Thereafter, the cultures were repeatedly washed in deionized water and photographed using an inverted microscope (Nikon Eclipse Ti-S, Nikon Instruments SpA, Italy).

To quantify mineral deposition, a solution of 10% w/v cetylpiridinium chloride in sodium phosphate 10 mM (pH 7) was added to cultures to elute ARS. The developed color was evaluated spectrophotometrically at 540 nm and ARS concentration was obtained by referring to a standard curve.

2.4. Osteoblast viability

To test cell viability at 24 h (T_0), and 1 and 2 weeks after seeding at each culture condition, Alamar blue dye (Serotec, Oxford, UK) was added to each culture well (1:10 v/v) and incubated for 4 h at 37 °C. The reagent is a dye which incorporates an oxidation–reduction (REDOX) indicator that changes color in response to the chemical reduction of growth medium, resulting from cell growth. The absorbance was read spectrophotometrically at 570 and 600 nm wavelengths (for the fully oxidized and reduced forms of reagent) using a MicroPlate reader (BioRad, CA, USA). The calculation of Alamar blue reduction percentage was performed following the manufacturer's instructions. Moreover, at the same timepoints all cultures were stained by a Neutral

Table 1 Experimental groups and culture conditions.

	SHAM	OVX
Osteoblastic medium (OM)	$SHAM_{CTR}$	OVX_{CTR}
Conditioned medium (CM)	$SHAM_{CM}$	OVX_{CM}