Osteoblast-conditioned medium promotes proliferation and sensitizes breast cancer cells to imatinib treatment

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Abstract

Inhibition of platelet-derived growth factor receptor (PDGFR) signaling restricts the growth of human breast cancer in the bone of nude mice. We hypothesized that osteoblast-secreted substances may alter the response capacity of breast cancer cells to the PDGFRs tyrosine kinase inhibitor imatinib mesylate. We found that osteoblast-conditioned medium (OCM) increases the proliferation rate of the estrogen receptor negative (ER−) MDA-MB-231 and of the ER+ MCF-7 human breast cancer cell lines and the growth-promoting effect on ER+ cells is independent from estrogen. OCM significantly improved the dose- and the time-dependent sensitivity of the tumor cells to the anti-proliferative effect of imatinib. We also found that MDA-MB-231 and MCF-7 cells express the two PDGFRs subtypes, PDGFR-α and PDGFR-β, and OCM treatment increases the expression of the PDGFRs. Furthermore, imatinib inhibited the phosphorylation rate of its target tyrosine kinase receptors. We conclude that bone microenvironment, through osteoblast-secreted substances may cause estrogen-independent proliferation of breast cancer cells by a mechanism mediated by the induction of PDGFRs expression. The enhanced sensitivity of OCM-treated breast cancer cells to imatinib would justify investigation on the efficacy of imatinib in bone breast cancer metastasis.

Endocrine-Related Cancer (2007) **14** 61–72

Introduction

Receptor tyrosine kinases have been proposed as potential targets for anti-tumor therapy. Imatinib mesylate (also known as STI571 or Gleevec, and hereafter called imatinib) belongs to the group of new drugs classified as signal transduction inhibitors and has been approved as an effective treatment for chronic myeloid leukemia (CML; Capdeville *et al.* 2002). Imatinib inhibits Bcr–Abl kinase activity, causing apoptosis in Philadelphia+ cells and inducing cytogenetic remissions in the majority of CML patients (Capdeville *et al.* 2002). Additional tyrosine kinases are inhibited by imatinib: c-kit, the receptor for kit ligand (KL), and the two structurally similar platelet-derived growth factor receptors (PDGFRs), PDGFR-α and PDGFR-β (Capdeville *et al.* 2002, Fletcher 2004).

Results of recent clinical studies have shown that imatinib therapy is well tolerated and leads to remission in patients with c-kit-positive gastrointestinal stromal tumors which contain gain-of-function mutations in c-kit (Heinrich *et al.* 2002). Imatinib has also been reported to inhibit the growth of glioblastoma, dermatofibrosarcoma protuberans, neuroblastoma, Ewing sarcoma, Leydig cell tumor, and small cell lung cancer, all of which may express the PDGF/PDGFR or KL/c-kit autocrine growth loops (Kilic *et al.* 2000, Krystal *et al.* 2000, Sjoblom *et al.* 2001, Merchant *et al.* 2002, Beppu *et al.* 2004, Basciani *et al.* 2005).

There have been few studies on PDGFs/PDGFRs and KL/c-kit in breast neoplasias. KL and c-kit are high in normal mammary gland, significantly lower in *in situ* and almost completely undetectable in invasive breast cancer (Ulivi *et al.* 2004, Yared *et al.* 2004).

Breast carcinoma is known to express PDGF and PDGFR (Coltrera *et al.* 1995) and overexpression of PDGFR-α in breast cancer was associated with tumor progression (Carvalho *et al.* 2005). These data sustained the PDGF/PDGFR system as potential therapeutic target in breast cancer. Accordingly, it has been reported that imatinib inhibits the growth of human epithelial breast cancer cell lines (Brown *et al.* 2004, Roussidis *et al.* 2004, Uziel *et al.* 2005). Moreover, activated PDGFRs are expressed by endothelial and tumor cells in breast cancer tumors growing in the bone of nude mice and imatinib treatment inhibited PDGFR phosphorylation in tumor cells and tumor-associated endothelial cells, coincident with increased apoptosis, reduced proliferation and osteolysis, and lower microvessel density in the tumors (Chelouche-Lev *et al.* 2005). Interestingly, cultured breast tumor cells did not express detectable levels of PDGFRs while both PDGFR-α and PDGFR-β were detected in cells growing in bone, suggesting that expression of the receptors can be upregulated by the organ microenvironment (Chelouche-Lev *et al.* 2005). These data indicate that bone metastasis of breast cancer may have a favorable response to imatinib. Therefore, we assessed the ability of osteoblast-conditioned medium (OCM) to influence the sensitivity to imatinib of human breast cancer cells. Here, we show that conditioned medium from cultured human osteoblasts stimulates the growth of estrogen receptor-positive (ER+) and ER− breast cancer cells, causes upregulation of PDGFRs expression, and enhances their susceptibility to the imatinib treatment suggesting that the communication between osteoblasts and breast cancer cells modulates the sensitivity of the tumor cells to imatinib.

Materials and methods

Cell lines and culture conditions

ER+ noninvasive MCF-7, ER− invasive MDA-MB-231 human breast cancer cell lines, and a human fetal osteoblast cell line, hFOB1.19 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The carcinoma cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) high glucose (EuroClone, Milano, Italy) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL, Life Technologies, Inc.) in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. hFOB1.19 cell line was maintained at 33.5 °C in DMEM/F-12 (Sigma-Aldrich), 10% charcoal-stripped FCS, 100 units/ml penicillin, 10 mg/ml

streptomycin, and 300 μg/ml geneticin in a humidified atmosphere of 5% CO_2, 95% air.

Conditioned medium from osteoblasts

To obtain differentiated osteoblasts, hFOB1.19 cells were plated into 100 mm Petri dishes and allowed to grow until 80% confluence. According to the protocol provided by Dr Spelberg and ATCC (Harris *et al.* 1995), the cells were exposed to differentiation medium and transferred to a 39.5 °C 5% CO_2, 95% air-humidified incubator for 3 days. The medium was then replaced with fresh serum-free medium plus 0.2% BSA. After 48 h, the OCM was collected, centrifuged to remove cellular debris, and stored at −80 °C until use.

PDGFs and transforming growth factor-β (TGF-β) assays

The levels of PDGF-A (R&D Systems, Inc., MN, USA), PDGF-B (Ray Biotech Inc., Norcross, GA, USA), and TGF-β (DRG Diagnostics, Marburg, Germany) in OCM were measured by ELISA kits, following the instructions of the manufacturers. The results are expressed as mean ± s.D. of three determinations in triplicate. The minimum detectable dose was 2.07 pg/ml for PDGF-A, 2 pg/ml for PDGF-B, and 1.9 pg/ml for TGF-β.

Cell growth characterization

Breast cancer cells were plated in 60 mm dishes (5×10^5 cells/dish) and incubated in DMEM containing 10% FCS. At 60% confluence, the cells were incubated for 24 h with phenol red-free DMEM and 5% charcoal-stripped FCS and then treated with 10 nM 17β-estradiol (E₂; Sigma), the ER antagonist ICI182,780 (Zeneca Pharmaceuticals, Macclesfield, UK; 1 μM) and OCM (50% final concentration) or their combinations. In a separate set of experiments, the cells were incubated with OCM in the absence or in the presence of anti-PDGF antibody which recognizes both PDGF-A and PDGF-B (Upstate, Charlottesville, NY, USA) or anti-TGF-β (Chemicon International, Inc., Temecula, CA, USA) neutralizing antibodies at 15 and 5 μg/ml concentration respectively. These concentrations are in the range reported previously for neutralization (Gorlach *et al.* 2000, Dunker *et al.* 2001). According to manufacturers' instructions, the antibodies concentrations should neutralize <10 ng/ml PDGF and <1 ng/ml TGF-β in media. After 24 h, the cells were trypsinized and counted. To determine the effect of imatinib on the growth of breast cancer cells and the influence of OCM on the sensitivity of MCF-7 and MDA-MB-231 cells to imatinib, the cells

were seeded at 1×10^4 cells/well in 24-well tissue culture plates and cultured in DMEM supplemented with 10% FCS. When the cells reached 60% confluence, the medium was replaced with medium containing 10% FCS, OCM (50% final concentration), imatinib (provided by Dr Elisabeth Buchdunger, Novartis Pharmaceuticals, Basel, Switzerland), or OCM plus imatinib. MCF-7 cells grown with or without OCM were also exposed to imatinib plus 1 μM ICI182,780. At the end of incubation, the cells were trypsinized and counted.

Apoptosis was studied by terminal dUTP nick end-labeling (TUNEL) labeling (fluorescein *in situ* detection kit, Roche Diagnostics) on 1×10^5 MDA-MB-231 and MCF-7 cells grown in DMEM containing 10% FCS or OCM for 24 h on glass coverslips in the absence or in the presence of imatinib. TUNEL staining was quantitated by assessing a total of 1000 cells per culture and calculating the percentage of positive nuclei under a fluorescence microscope (Olympus, Tokyo, Japan).

Cells proliferation was determined using a BrdU incorporation assay, utilizing a cell proliferation ELISA colorimetric kit (Boehringer, Mannheim, Germany) following the manufacturer's protocol. Breast cancer cells (1×10^3) were added to 96-well plates and allowed to adhere for 24 h in DMEM containing 10% FCS. Then the cells were incubated for 24 h in 10% FCS or 10% FCS mixed in a 1:1 ratio with OCM. The media were then replaced with the same media in the absence or in the presence of various concentrations of imatinib (1–10 μm) and incubated for 12 h. The plates were analyzed at room temperature using a Sunrise Microplate Reader (TECAN, San Jose, CA, USA), with the absorbance set at 450 nm. Each experiment was conducted in triplicate. The data are presented as percentage of controls.

Western blots

Breast cancer cells were seeded in 100 mm plates in DMEM containing 10% FCS. When the cells reached 80% confluence, the cells were incubated for 12 h in 10% FCS or 10% FCS mixed in a 1:1 ratio with OCM. In additional experiments, the cells incubated with OCM, were exposed to anti-PDGF (Upstate) or anti-TGF-β (Chemicon International, Inc.) neutralizing antibodies at 15 and 5 μg/ml concentration respectively. The media were then replaced with the same media with or without imatinib (10 μM). After 3 h, the cells were washed with PBS, harvested, and cell proteins were extracted and separated as described (Basciani *et al.* 2004). Nitrocellulose membranes were incubated at 4 °C overnight with 1:300 dilution of one of the following primary antibodies: anti-PDGFR-α, anti-PDGFR-β, anti-phospho-PDGFR-α (anti-p-PDGFR-α), anti-p-PDGFR-β, and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10 000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. The same blots were stripped and reprobed. Bound antibodies were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Western blot analyses were repeated at least thrice on independent cell lysates with reproducible results. Protein bands were quantified by densitometric analysis using a densitometry computer software (Kodak Digital Science) after normalizing individual bands to the respective β-actin bands.

Reverse transcription and real-time PCR assay

To study the expression for PDGFR-α and PDGFR-β mRNAs, total cellular RNA was isolated from cultured breast tumor cells using the Trizol reagent (Invitrogen) according to manufacturer's procedure. Reverse transcription was performed with 1 μg total RNA as described (Viereck *et al.* 2002). Real-time reverse transcriptase-PCR (RT-PCR) analysis was performed using an ABI Prism 7300 analyzer (Applied Biosystems, Foster City, CA, USA). Amplification was carried out with 50 ng cDNA, in 96-well plates, using SYBR green PCR Master mix (Applied Biosystems) in 25 μl volume. Each sample was analyzed in triplicate. PCR conditions were: 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, and 60 °C for 1 min. Primers were designed using Primer Express software (Applied Biosystems) and were synthesized by Primm (Milano, Italy). The primer sequences were the following: PDGFR-α, fw 5′-CCTTGGTGGCACCCCTTAC-3′, PDGFR-α, rv 5′-TCCGGTACCCACTCTTGATCTT-3′; PDGFR-β, fw 5′-CCAATGAGGGTGACAACGACTA-3′, PDGFR-β, rv 5′-GGCCCTCGTCAGCAACCT-3′. The results were analyzed using Sequence Detection System software (Applied Biosystems). Generation of double-stranded DNA was measured in real time by the increase in fluorescence caused by the spontaneous binding of SYBR green. Target gene C_t values were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; defined to be the house-keeping gene). Data were analyzed using the $2^{-\Delta\Delta C_t}$ method and expressed as fold change compared with control.

Statistical analysis

Student's *t*-test was used for statistical analysis. $P < 0.05$ was accepted as significant.

Results

OCM stimulates the growth of breast cancer cells

We first evaluated the effect of OCM on the growth of ER− MDA-MB-231 and ER+ MCF-7 human breast cancer cell lines cultured in steroid-free medium (Fig. 1). OCM (50% final concentration) increased the growth rate of both MCF-7 and MDA-MB-231 cells (2.2- and 1.5-fold versus control respectively; Fig. 1A and B). The growth of ER+ MCF-7 cells was enhanced significantly by OCM either compared with basal estrogen-free culture conditions or with E_2-treated cells (1.4-fold). The exposure of MCF-7 to OCM and E_2 in combination had an additive effect on cells growth (2.7 versus control and 1.8 versus E_2). The ER antagonist ICI182,780 significantly reduced the cell number of E_2-treated MCF-7 cells. By contrast, ICI182,780 did not affect the growth of OCM-treated cells and only partially inhibited the growth of MCF-7 cells exposed to OCM plus E_2. As expected, the number of ER− MDA-MB-231 cells was unaffected by E_2 either alone or in combination with OCM (Fig. 1B).

Osteoblasts secrete several cytokines and growth factors which could be responsible for the stimulation of breast cancer cells growth. Primary osteoblasts of both human and murine origins have been shown to
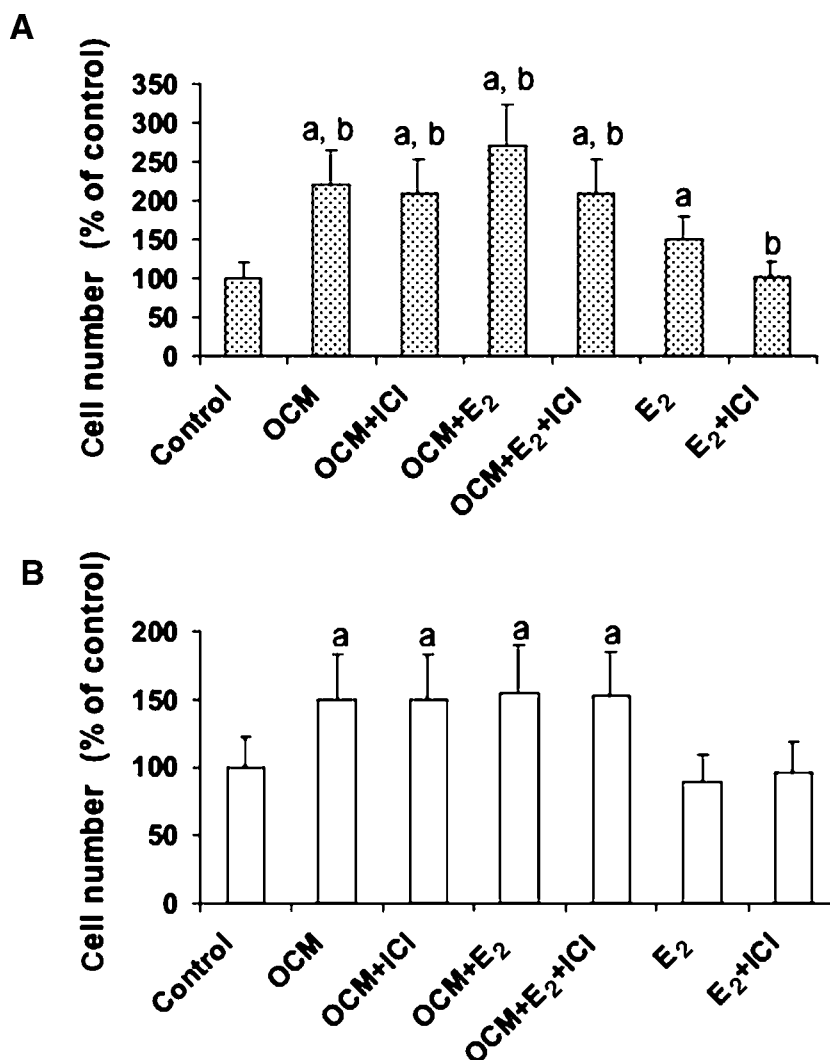
Figure 1 OCM increases the proliferation of MCF-7 (A) and MDA-MB-231 (B) cells. Cells cultured in DMEM containing 5% steroid-free FCS were treated with OCM (50% final concentration), E_2 (10 nM), ICI182,780 (1 μM), or their combinations. After 24 h, cell growth was determined by cell count, as detailed in Materials and methods. Data are presented as percentage of control untreated cells. Bars represent the mean ± s.D. of three independent experiments performed in triplicate. a, Significant versus control; b, significant versus E_2-treated cells.

produce IL-1, IL-6, VEGF, TGF-β (Taichman & Emerson 1998), and PDGFs (Zhang *et al.* 1991). All of these proteins have the ability to induce proliferation of breast cancer cells. We have confirmed here that OCM contains PDGF-A (160.4 ± 16.2 pg/ml, mean $\pm$ S.D. of three determinations in triplicate), PDGF-B (30.2 ± 2.6 pg/ml) and TGF-β (41.6 ± 2.2 pg/ml). However, the addition of neutralizing antibodies against PDGFs and TGF-β either alone or in combination did not reduce the growth-stimulating effect of OCM (data not shown).

These data indicate that there are factors in OCM, which promote the growth of the ER$-$ and ER$+$ breast cancer cells independently from estrogen, PDGFs, or TGF-β stimulation.

OCM sensitizes breast cancer cells to the anti-proliferative and apoptotic effect of imatinib

To determine whether OCM influences the response of breast cancer cells to imatinib, we evaluated the effect of imatinib on the growth of MCF-7 and MDA-MB-231 cells in the presence or in the absence of OCM (Fig. 2). Imatinib induced a concentration-dependent inhibition of MDA-MB-231 and MCF-7 cell growth. The imatinib concentration causing 50% reduction in cell number (IC_{50}) after 24 h of treatment was 7 μM for cells grown without OCM and 2.5 μM for cells grown with OCM (Fig. 2A). The time course of imatinib action was also influenced by OCM. The time for 50% decrease of cell number was reduced from 72 to 24 h for both cell lines cultured with 2.5 μM imatinib in the presence of OCM (Fig. 2B). The concomitant treatment of the ER$+$ MCF-7 cells with ICI182,780 plus imatinib in the absence of OCM caused a small increase of the inhibition of cells growth compared with imatinib alone, in the presence of OCM the curves were almost identical (Fig. 2B). We then evaluated whether the growth-inhibitory effect of imatinib in the presence of OCM was due to apoptosis, reduced proliferation, or both. The addition of 2.5 μM imatinib to MDA-MB-231 and MCF-7 cells for 24 h induced a significant increase in apoptotic cells in basal culture conditions compared with control (Fig. 3A). OCM did not modify the percentage of apoptosis of MDA-MB-231 cells treated with imatinib. On the contrary, OCM produced a significant increase of the apoptotic effect of imatinib on MCF-7 cells. The proliferation of MCF-7 and MDA cells under imatinib treatment was also influenced by OCM (Fig. 3B). In both cell lines, a profound anti-proliferative effect was observed when the cells were grown with OCM compared with basal culture conditions.

Together, these results indicate that there are factors present in OCM which sensitize MCF-7 cells to the apoptotic and to a lesser extent anti-proliferative effect of imatinib, while the major mechanism whereby imatinib affects the growth of MDA-MB-231 cells in the presence of OCM is through reduction of tumor cell proliferation, rather than by induction of tumor cell apoptosis.

OCM increases the expression of PDGFR-α and PDGFR-β and imatinib inhibits the phosphorylation of PDGFRs

We evaluated whether the treatment with OCM was accompanied by modification of PDGFRs and activated PDGFRs expression and investigated the status of receptors and receptors phosphorylation in the presence of imatinib (Fig. 4). In basal culture conditions, PDGFR-α, and to a lesser extent PDGFR-β, were expressed by both MCF-7 and MDA-MB-231 cells. OCM modified the expression of PDGFRs. PDGFR-α levels increased 4-fold in MDA-MB-231 cells and 1.7-fold in MCF-7 cells. PDGFR-β levels were approximately 2-fold greater in OCM-treated cells compared with cells in basal culture conditions (Fig. 4). When breast cancer cells were treated with OCM, the phosphorylation of PDGFRs increased. The levels of phosphorylated PDGFR-α (p-PDGFR-α) and p-PDGFR-β were 3 and 1.6 times greater for MDA-MB-231 cells and 3 and 2 times greater for MCF-7 cells. A profound inhibition of the phosphorylation of the receptors by imatinib, even stronger for the OCM-treated cells, was constantly observed.

These data indicate that the higher proliferation rate of breast cancer cells induced by OCM is, at least in part, due to an increased expression of the PDGFRs and of PDGFR-α in particular and to the higher phosphorylation rate of PDGFRs. We suggest that this effect makes the cells more dependent on PDGFRs activation for growth and survival explaining their higher sensitivity to imatinib treatment.

Several factors produced by the osteoblasts can stimulate the expression of the PDGFRs. Among these, TGF-β upregulates the PDGFRs in mammary carcinoma cells (Jechlinger *et al.* 2006). Furthermore, the PDGFRs ligands found in OCM could be responsible for the increased phosphorylation rate of their cognate receptors. We neither see any modification in the expression of the PDGFRs induced by OCM in the presence of anti-TGF-β antibody nor a reduction in their phosphorylation in the presence of anti-PDGFs antibody (not shown). We conclude that the increased expression and phosphorylation rate of the PDGFRs in
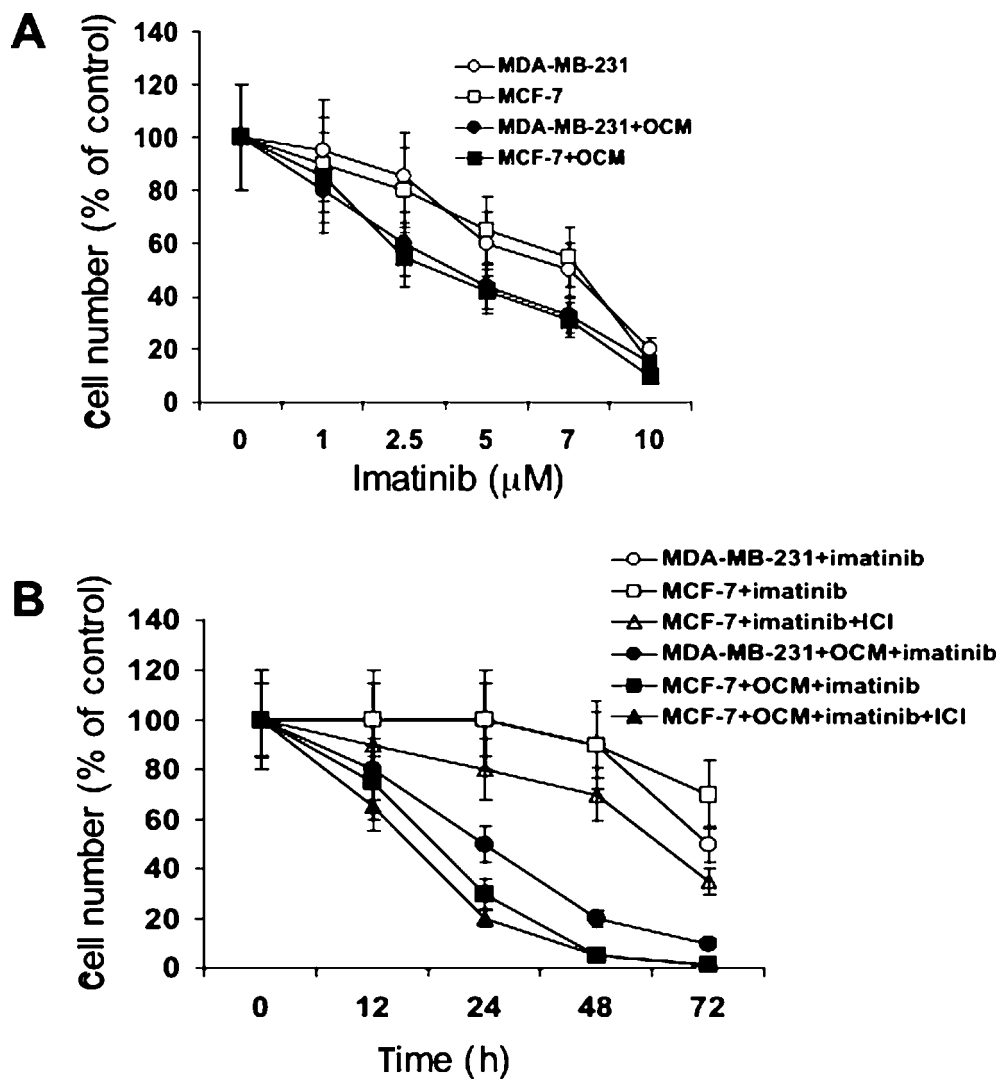
Figure 2 OCM increases the inhibitory effect of imatinib on the growth of MCF-7 and MDA-MB-231 cells. (A) breast cancer cells were cultured for 24 h in FCS or OCM (50% final concentration) in the presence or in the absence of various concentrations of imatinib. (B) time course of the effect of imatinib (2.5 μM) on the growth of breast cancer cells cultured in the absence or in the presence of OCM (50%, volume for volume), and of imatinib and ICI182,780 (1 μM) on ER+ MCF-7 cells in the absence or in the presence of OCM. Cell number was determined by cell counting. Experiments were performed thrice in triplicate. Data are presented as percentage of control (mean ± S.D.).

breast cancer cells seen after OCM treatment are not mediated by TGF-β and PDGFRs ligands respectively.

OCM increases the transcription of PDGFRs mRNA by breast tumor cells in an estrogen-independent way

As we observed an upregulation of the PDGFR-α and PDGFR-β protein expression in OCM-treated breast cancer cells, we sought to determine whether the *PDGFRs* gene expression can similarly be linked to the treatment with OCM (Fig. 5). We found that PDGFR-α
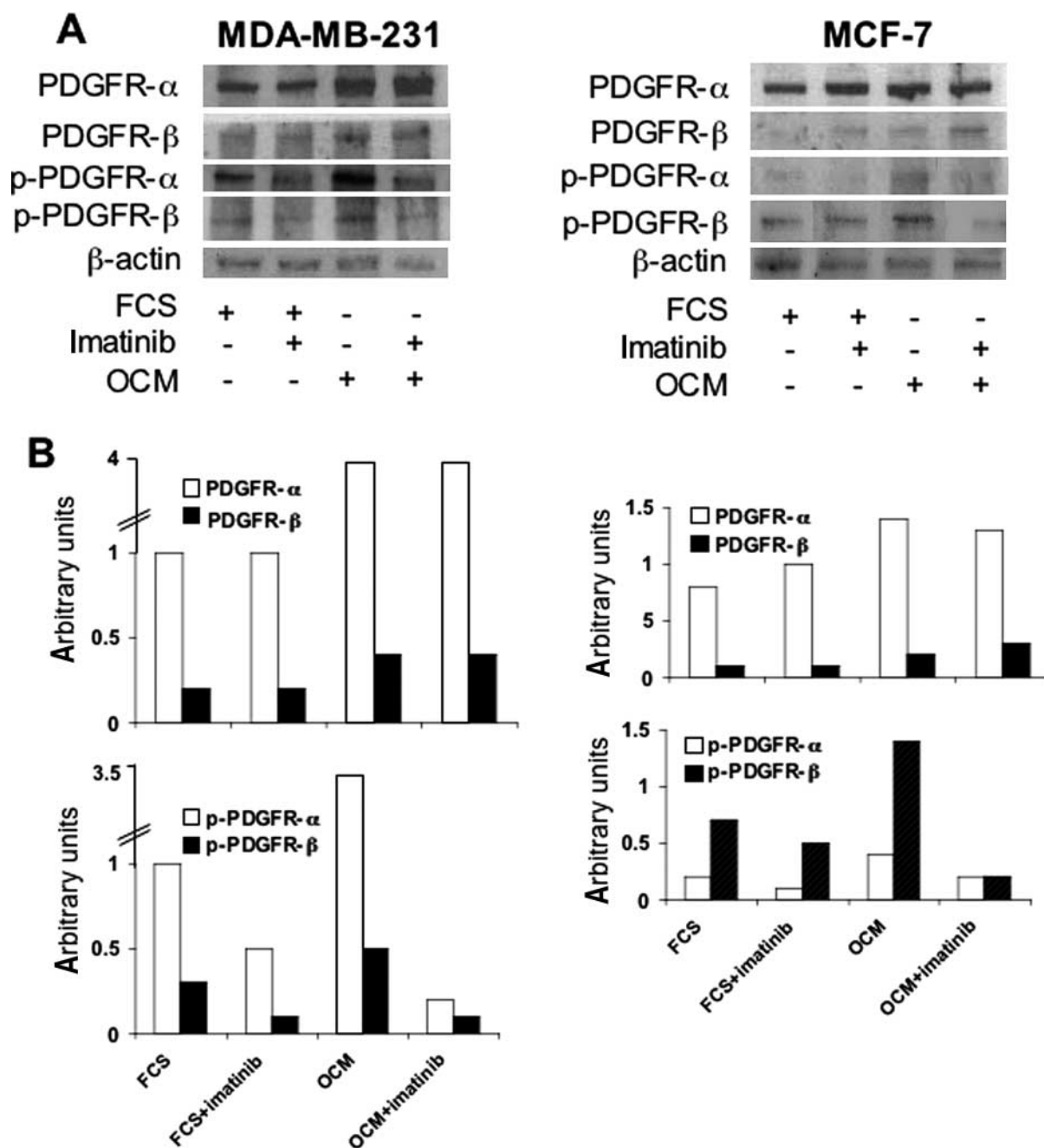
mRNA expression in MDA-MB-231 and MCF-7 cells incubated with OCM was 3.7- and 3.0-fold higher respectively, than in cells cultured without OCM. The PDGFR-β mRNA increased 1.7 times in MCF-7 and 1.8 times in MDA-MB-231 cells cultured with OCM. Previous studies have shown that, in some cell types, estrogens increase the expression of PDGFs and PDGFRs mRNAs, indicating that the PDGFs/PDGFRs system could be a potential mediator of estrogen action (Gray *et al.* 1995). Thus, we determined the effect of E_2 on the PDGFRs mRNAs levels in the ER+ MCF-7 cells in the presence or in the absence of OCM
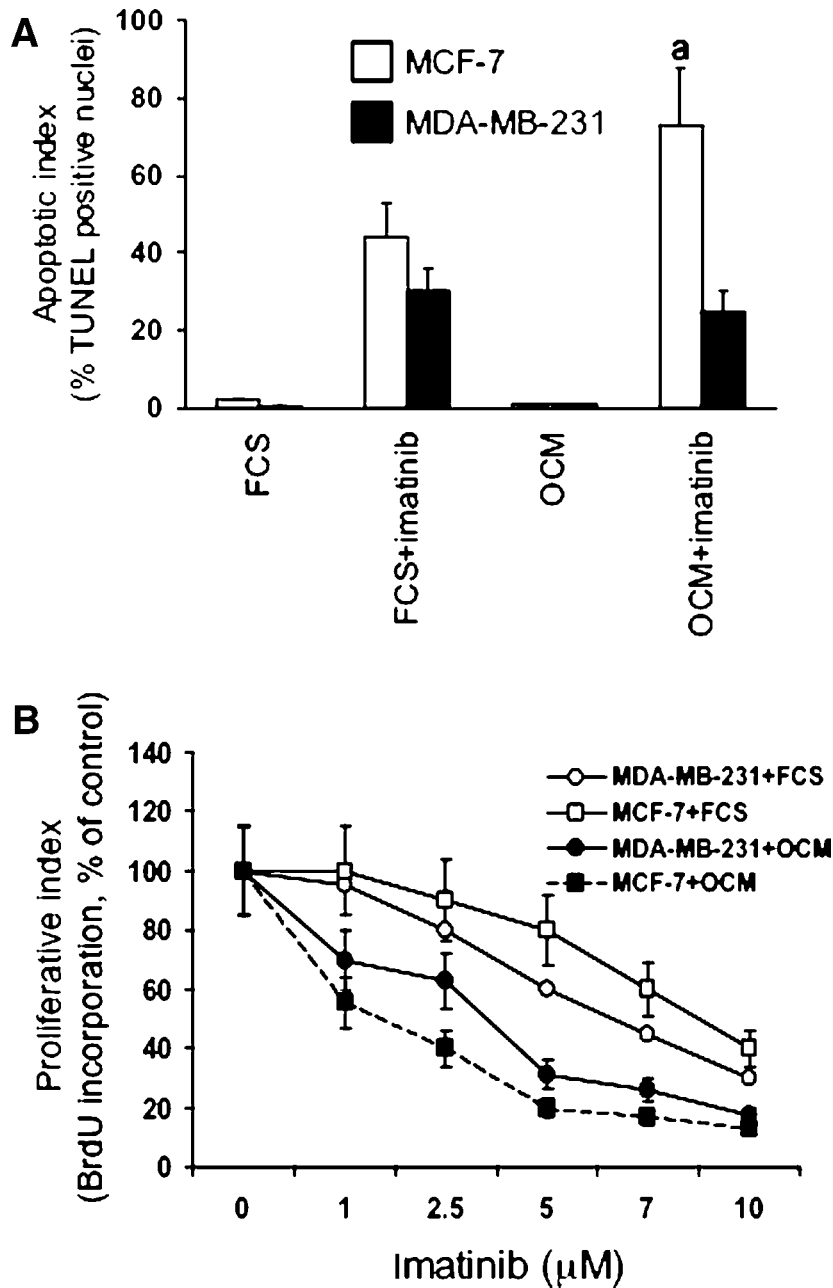
Figure 3 Effect of imatinib on apoptosis (A) and proliferation (B) of MCF-7 and MDA-MB-231 cells in the presence or in the absence of OCM. A, apoptosis was evaluated on MCF-7 (open columns) and MDA-MB-231 (solid columns) cells grown in DMEM containing 10% FCS or OCM (50% final concentration) for 24 h in the absence or in the presence of imatinib (2.5 μM). Data are expressed as percentage of TUNEL-positive nuclei. a, $P < 0.05$ between imatinib-treated cells in the presence and in the absence of OCM. Proliferation was evaluated on MCF-7 and MDA-MB-231 cells incubated for 12 h in 10% FCS or 10% FCS mixed in 1:1 ratio with OCM in the absence or in the presence of various concentrations of imatinib. Data are presented as percentage of BrdU incorporation of control.

(Fig. 5B). The exposure of MCF-7 cells to E_2 produced a significant increase of the expression of the PDGFR-α and PDGFR-β genes that was completely reverted by the ER antagonist ICI182,780. When the MCF-7 cells were treated with E_2 in conjunction with OCM, the levels of the PDGFRs mRNAs were significantly increased compared with E_2 alone. However, in this experimental condition, the inhibition of *PDGFRs* genes expression induced by ICI182,780 was distinctly less pronounced. These data indicate that

Figure 4 OCM upregulates PDGFRs expression and imatinib inhibits the phosphorylation of PDGFRs. (A) MDA-MB-231 and MCF-7 cells were incubated for 12 h with 10% FCS or 10% FCS mixed in a 1:1 ratio with OCM followed by an additional 3-h incubation in the presence or in the absence of imatinib. Immunoblots of MDA-MB-231 and MCF-7 cells protein extracts were probed with anti-PDGFR-α, anti-PDGFR-β, anti-p-PDGFR-α, and anti-p-PDGFR-β antibodies. For normalization, the filters were stripped and reprobed for β-actin. Blots show representative experiments performed at least twice which gave comparable results. (B) densitometric evaluation of the signals shown in (A) normalized to the levels of β-actin, and shown as histograms of arbitrary units.

osteoblast-secreted factors cause an estrogen-independent induction of the *PDGFRs* gene expression and confirm that the PDGF/PDGFR system is a potential mediator of the estrogen action in ER+ breast tumor cells.

Discussion

In this study, we have found that OCM stimulates breast cancer cells growth and sensitizes the cells to the growth-inhibitory effect of imatinib. Our study also presents data
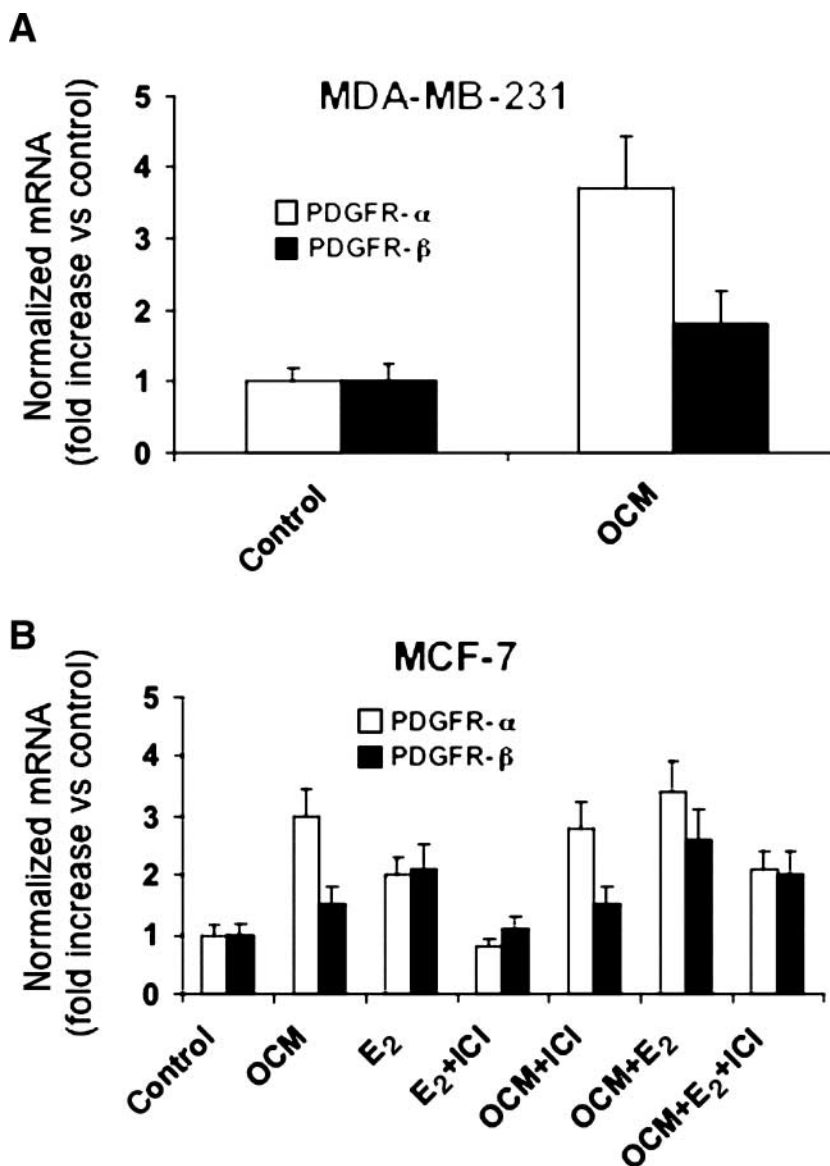
Figure 5 Real-time RT-PCR of MDA-MB-231 (A) and MCF-7 (B) cells PDGFR-α (open columns) and PDGFR-β (solid columns) mRNAs expression. A, MDA-MB-231 cells at 60% confluence were incubated for 24 h with phenol red-free DMEM, 5% charcoal-stripped FCS before the addition of OCM (50% final concentration) for an additional 24 h. MCF-7 cells at 60% confluence were incubated for 24 h with phenol red-free DMEM, 5% charcoal-stripped FCS before the addition of E_2 (10 nM), OCM (50% final concentration), ICI182,780 (1 μM), or their combinations for an additional 24 h. Cells were harvested, total RNA was isolated and real-time RT-PCR was performed with 1 μg total RNA. The data were normalized to GAPDH and expressed as mean±s.d. fold increase of control for three separate experiments performed in triplicate.

indicating that osteoblast-secreted substances increase the expression of PDGFR-α and PDGFR-β and their phosphorylation rate, leading to an imbalance in growth factors dependence of breast cancer cells.

The stimulation of proliferation originating from OCM was exerted both in ER+ and ER− cells, suggesting that OCM promotes breast cancer cell growth regardless of estrogen dependency. This was further confirmed by the fact that the ER antagonist

ICI182,780 did not interfere with the growth induced by OCM on ER+ MCF-7 cells, indicating that osteoblast-secreted substances could rescue the growth of ER+ breast cancer cells from estrogenic control. Our results are consistent with a previous report showing that conditioned medium collected from primary human osteoblasts cultures had a pronounced stimulatory capacity on some human mammary tumor cells (Goren *et al.* 1997).

Owing to the recently recognized ability of bone microenvironment to enhance the anti-tumor effect of imatinib (Chelouche-Lev *et al.* 2005), we sought to determine if osteoblast-secreted substances, besides their growth-promoting effect, contribute to sensitize breast cancer cells to imatinib. In agreement with previous studies (Brown *et al.* 2004, Roussidis *et al.* 2004, Uziel *et al.* 2005), in basal culture conditions, imatinib inhibited the growth of MDA-MB-231 and MCF-7 cells in a dose-dependent manner. Notably, we found that when the cells were grown in OCM-containing media, the IC$_{50}$ and the time course for imatinib action were significantly reduced; it was documented that this growth-inhibitory effect was exerted through a significant reduction of proliferation in MDA-MB-231 and MCF-7 cells and an increase of apoptosis in MCF-7 cells. Furthermore, while in the absence of OCM, the growth-inhibitory effect of imatinib on ER+ cells was reinforced by ICI182,780, in the presence of OCM the growth-inhibiting effect of imatinib was not modified by ICI182,780. These results further confirm that OCM rescues ER+ breast cancer cells from estrogenic control and indicate that osteoblast-secreted substances increase the sensitivity of breast cancer cells to imatinib.

To investigate the mechanisms of the sensitizing effect of OCM to the anti-proliferative effect of imatinib, we determined whether OCM interfered with PDGFRs, target tyrosine kinase receptors for imatinib action. As a first step, we evaluated the expression of PDGFRs proteins in basal culture conditions. In line with previous studies, we found low PDGFRs levels in MCF-7 (Yu *et al.* 1998). Concerning the ER− breast cancer cells, it has been shown that cultured cells did not express PDGFRs, while both PDGFR-α and PDGFR-β were seen in cells growing in bone (Chelouche-Lev *et al.* 2005). We found low levels of PDGFRs in MDA-MB-231 cells by immunoblotting. An increase in the expression of PDGFR-α and PDGFR-β proteins and of their phosphorylation in both ER− and ER+ cancer cells grown in OCM was constantly observed. We have ruled out that these effects might be assigned to TGF-β or PDGF, both produced by the osteoblasts and able to stimulate PDGFRs expression and phosphorylation (Ikuno & Kazlauskas 2002, Crowley *et al.* 2005, Gotzmann *et al.* 2006). Indeed, neutralization of these growth factors with the respective antibodies alters neither the growth-promoting effect of OCM nor the increased expression and phosphorylation rate of the PDGFRs. It is important to stress that PDGFRs and other protein tyrosine kinases can be activated by mechanisms different from receptor stimulation by its

ligand such as cross-activation by other kinases or transactivation. For example, the EGF receptor and the PDGFR are known to interact physically (Liu & Anderson 1999), EGF stimulation has been shown to: increase the tyrosine phosphorylation of PDGFR and the subsequent recruitment of PI-3K to PDGFR (Habib *et al.* 1998), and PDGFR transactivation mediates the trophic effect of angiotensin II (Kelly *et al.* 2004). The increased expression of the PDGFR-α and PDGFR-β proteins, induced by OCM was confirmed at mRNA level, both in ER− and ER+ cells. Interestingly, we found that estrogen treatment enhanced the expression of the *PDGFRs* genes in ER+ cancer cells. This behavior was found in different kinds of ER-expressing tissues (Gray *et al.* 1995, Finlay *et al.* 2004); a correlation was noted between ER and PDGFR expression in breast carcinomas (Coltrera *et al.* 1995). Analogously, to what was observed at protein level, and in line with the lack of inhibition by ICI182,780 on growth induced by OCM in MCF-7 cells, the hyperexpression of *PDGFRs* genes induced by OCM in ER+ cells was not inhibited by ICI182,780, indicating that this effect was attributable to a mechanism not related to ER activation. Further studies are required to identify the osteoblast-secreted substances responsible for PDGFRs overexpression and activation.

Notably, a substantial proportion of tumors in patients with localized disease, and all tumors in patients with metastatic disease become resistant to endocrine therapies (Gururaj *et al.* 2006). Our findings concerning the effect of OCM on the growth of breast cancer cells are consistent with clinical observations and provide experimental evidence that factors secreted by bone stimulate the proliferation of breast cancer regardless of estrogens. Thus, osteoblast-derived factors appear to be in place to substitute estrogens by promoting the growth of metastatic breast cancer cells in bone. Accordingly, it has recently been reported that *PDGFRs* genes are among the identified seven genes involved in anti-estrogen-resistant proliferation of human breast cancer cells (Meijer *et al.* 2006). These findings might contribute in explaining the loss of response to anti-estrogens by breast cancer cells in the bone microenvironment.

Although, in line with previous results, our data seem to indicate that breast cancer cells respond to imatinib and the bone microenvironment sensitizes the cells to imatinib action. In a phase II trial, to evaluate the safety and efficacy of imatinib in patients with unselected metastatic breast cancer, no clinical benefit was observed in this cohort of patients (Modi *et al.* 2005). Despite this, it should be underlined that of the

16 patients enrolled in this study, only 4 patients out of the 13 with testable baseline breast cancer tissues stained positive for PDGFR, and only 3 of these patients were evaluable. Furthermore, the number of patients with metastatic bone disease is not reported. Hence, a selected group of patients with metastatic bone disease positive for PDGFRs expression should be explored.

In conclusion, we demonstrate that OCM, via a hyperexpression of PDGFRs, stimulates breast cancer cell growth and sensitizes breast cancer cells to the anti-proliferative effects of imatinib. These results, coupled with the recently reported anti-osteolytic effect of imatinib (Dewar *et al.* 2006) and with the decreased bone remodeling seen in patients taking imatinib (Berman *et al.* 2006), suggest that imatinib alone or in combination with other breast cancer drugs may have therapeutic value in breast cancer bone metastasis.

Funding

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