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Variability of the Paracrine-Induced Osteoclastogenesis by Human Breast Cancer Cell Lines

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ABSTRACT

Breast cancer frequently metastasizes to the bone, often leading to the formation of osteolytic lesions. This work compares the paracrine-induced osteoclastogenesis mediated by four human breast cancer cell lines, the estrogen-receptor positive T47D and MCF-7 and the estrogen-negative SK-BR-3 and Hs-578T cell lines. Human osteoclast precursor cells were cultured in the presence of conditioned media from the breast cancer cell lines (10% and 20%), collected at different culture periods (48 h, 7 days, and 14 days). Cultures performed in the absence or the presence of M-CSF and RANKL served as negative and positive control, respectively. Results showed that the cell lines differentially expressed several osteoclastogenic genes. All cell lines exhibited a significant osteoclastogenic potential, evidenced by a high TRAP activity and number of osteoclastic cells, expression of several osteoclast-related genes, and, particularly, a high calcium phosphate resorption activity. Differences among the osteoclastogenic potential of the cell lines were noted. T47D and MCF-7 cell lines displayed the highest and the lowest osteoclastogenic response, respectively. Despite the variability observed, MEK and NF-κB signaling pathways, and, at a lesser extent, PGE2 production, seemed to have a central role on the observed osteoclastogenic response. In conclusion, the tested breast cancer cell lines exhibited a high osteoclastogenic potential, although with some variability on the cell response profile, a factor to be considered in the development of new therapeutic approaches for breast cancer-induced bone metastasis. J. Cell. Biochem. 113: 1069–1079, 2012.

KEY WORDS: OSTEOCLASTOGENESIS; BREAST CANCER CELL LINES; CONDITIONED MEDIUM; PERIPHERAL BLOOD MONONUCLEAR CELLS; BONE METASTASIS

Bone tissue is one of the most frequent targets for cancer metastasis, particularly in breast, prostate, lung, thyroid, and renal cancers [Chambers et al., 2002; Mundy, 2002]. In order to create a microenvironment suitable for cancer cell adhesion and propagation, bone tissue has to be previously remodeled. This is achieved by the establishment of functional crosstalks between cancer and bone cells, mediated by the secretion of soluble molecules and cell–cell interactions, which modulates bone metabolic activities [Mundy, 2002; Costa-Rodrigues et al., 2011ac]. In this context, bone metastasis can be divided into three major categories, namely, osteolytic, osteoblastic, or mixed metastasis, all involving the disruption of the bone metabolism [Mundy, 2002].

Due to the osteolytic nature of breast cancer metastasis [Diel et al., 2000; Chambers et al., 2002; Mundy, 2002; Reddi et al., 2003; Rose and Siegel, 2006], several bone complications are common in patients suffering from breast tumors, such as pathological fractures, pain, debilitating neurologic symptoms, and hypercalcemia [Chambers et al., 2002]. Since breast cancer cells do not have the ability to resorb bone, they must disrupt the normal coordinated equilibrium between osteoblastic and osteoclast activities during bone remodeling [Zaidi, 2007; Datta et al., 2008], shifting it towards bone resorption [Chambers et al., 2002; Mundy, 2002; Kakonen and Mundy, 2003]. It is known that breast cancer cells produce many soluble molecules, such as, for example, IL-1, IL-6, among others, that can act on osteoclastic cells, leading to an enhancement of bone resorbing activity [Pederson et al., 1999; Kakonen and Mundy, 2003; Kang et al., 2003; Bendre et al., 2003ab; Rose and Siegel, 2006]. The global effect is usually an extensive bone destruction and the proliferation of cancer cells in the bone tissue [Clohisy and

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Ramnaraine, 1998]. It was observed previously that breast cancer cell lines can modulate the osteoclastogenic response [Ono et al., 1998; Pederson et al., 1999; Thomas et al., 1999; Grano et al., 2000; Hunt et al., 2001; Morgan et al., 2004; Bendre et al., 2005; Gallet et al., 2006; Lau et al., 2007; Nicolin et al., 2008]. These studies involved several cell lines, but the variability of the origins of the osteoclastic cells (mainly from mouse, rat, rabbit, or chicken), experimental protocols and methodologies used to assess osteoclastogenesis, led, in some cases, to contradictory information, which difficult the establishment of general conclusions. Regarding this, it is known that estrogen-responsive breast cancer cells have more aggressive bone metastatic behavior than their estrogen receptor-negative counterparts [Koenders et al., 1991; Solomayer et al., 2000; James et al., 2003]. In this context, the aim of this work is to compare the paracrine-induced osteoclastogenesis mediated by four human breast cancer cell lines, the estrogen-receptor positive T47D and MCF-7 cell lines [Sasser et al., 2007] and the estrogen-negative SK-BR-3 and Hs-578T cell lines [Oh et al., 1993; Liu et al., 2009]. The former three are associated with the development of bone metastasis, while Hs-578T is not usually associated with such skeletal complications [Yin et al., 2003; Park et al., 2007]. In the present work, breast cancer cell lines were characterized for the expression of the two classic osteoclastogenic inducers M-CSF and RANKL, and also for other important osteoclastogenic molecules, such as IL-1β, IL-6, IL-17, TNF-α, and GM-CSF [Boyle et al., 2003; Zhao et al., 2007; Vaananen and Laitala-Leinonen, 2008]. Human peripheral blood osteoclast precursors were treated with conditioned media (CM) from breast cancer cell lines, collected at different culture periods. Cell response was assessed for several osteoclast-related parameters, and for the involvement of MEK and NF-κB signaling pathways, as well as PGE2 production.

MATERIALS AND METHODS

CONDITIONED MEDIA (CM) FROM HUMAN BREAST CANCER CELL LINES

Human breast cancer cell lines, SK-BR-3, MCF-7, Hs-578T, and T47D, were maintained in alpha-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml ascorbic acid. Cultures were performed at 37°C in a 5% CO_2 humidified atmosphere. At about 70% confluence, cells were detached with 0.05% trypsin and 0.5 mM EDTA, and seeded at 5×10^4 cell/cm^2. Cultures were maintained for 24 h, 7 days, and 14 days without any further medium change. After these culture periods, CM were collected, centrifuged at 550*g* for 10 min, aliquoted and stored at −20°C. Cell layers were assessed for total protein content, in order to normalize the amount of each CM to be used as a potential osteoclastogenic inducer.

GENE EXPRESSION OF OSTEOCLASTOGENIC MOLECULES BY BREAST CANCER CELL LINES, RT-PCR

SK-BR-3, MCF-7, Hs-578T, and T47D cell cultures, maintained for 7 days, were characterized by RT-PCR for the expression of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and the osteoclastogenic genes M-CSF, RANKL, IL-1β, IL-6, IL-17, TNF-α, and GM-CSF. For that, RNA was extracted with RNeasy® Mini Kit (Qiagen) according to manufacturer's instructions. The concentration and purity of total RNA in each sample were determined by UV spectrophotometry at 260 nm and by calculating the $A_{260\,nm}/A_{280\,nm}$ ratio, respectively. Half microgram of RNA was reversely transcribed and amplified (25 cycles) with the Titan One Tube RT-PCR System (Roche), with an annealing temperature of 55°C. The amplification conditions were chosen accordingly to preliminary calibration experiments (data not shown), in order to become possible to use the RT-PCR analysis as a quantitative tool. The primers (Sigma Genosys) used are listed on Table I. RT-PCR products were subjected to a densitometric analysis, after separation on a 1% (w/v) agarose gel. Gel analysis was performed with ImageJ 1.41 software and the values obtained were evaluated considering the corresponding GAPDH value of each experimental condition as 100%.

QUANTIFICATION OF IL-1β, IL-6, AND TNF-α PRODUCTION BY BREAST CANCER CELL LINES

Il-1β, IL-6, and TNF-α concentrations were determined in the culture medium (conditioned for 7 days) of each breast cancer cell line, with the corresponding Human Elisa Kits (Abcam), following manufacturer's instructions. After detection, the absorbance of the samples was measured in an ELISA plate reader (Synergy HT, Biotek). Results were expressed as pg/ml.

ISOLATION AND CULTURE OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

Blood was collected from healthy donors with ages comprised between 25 and 35 years old. Peripheral blood mononuclear cells (PBMC) were isolated as described previously [Costa-Rodrigues et al., 2011b]. Shortly, blood was diluted (1:1) with PBS and applied

TABLE I. Primers Used on RT-PCR Analysis of Breast Cancer Cell Cultures

Gene	5′ Primer	3′ Primer
GAPDH	5′-CAGGACCAGGTTCACCAACAAGT-3′	5′-GTGGCAGTGATGGCATGGACTGT-3′
M-CSF	5′-CCTGCTGTTGTTGGTCTGTC-3′	5′-GGTACAGGCAGTTGCAATCA-3′
RANKL	5′-GAGCGCAGATGGATCCTAAT-3′	5′-TCCTCTCCAGACCGTAACTT-3′
IL-1β	5′-ATGGCAGAAGTACCTAAGCTCGC-3′	5′-ACACAAATTGCATGGTGAAGTCAGTT-3′
IL-6	5′-ATGAACTCCTTCTCCACAAG-3′	5′-GTGCCTGCAGCTTCGTCAGCA-3′
IL-17	5′-TAGACTATGGAGAGCCGACC-3′	5′-TACCCCAAAGTTATCTCAGG-3′
TNF-α	5′-TCAGATCATCTTCTCGAACC-3′	5′-CAGATAGATGGGCTCATACC-3′
GM-CSF	5′-CTGAGTAGAGACACTGCTGCTG-3′	5′-TGCCTGTATCAGGGTCAGTGTG-3′

on top of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences). After centrifugation at 400*g* for 30 min, PBMC were collected and washed twice with PBS. On average, for each 100 ml of processed blood about 70×10^6 PBMC were obtained.

PBMC, seeded at 1.5×10^6 cells/cm^2, were maintained in α-MEM supplemented with 30% autologous human serum, 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 2 mM L-glutamine. Cell cultures were performed in the absence or presence of recombinant 25 ng/ml M-CSF and 40 ng/ml RANKL [Costa-Rodrigues et al., 2010] (negative and positive control, respectively), or in the presence of 10% or 20% (v/v) of CM from the human breast cancer cell lines. Cell cultures were incubated for 21 days at 37°C in a 5% CO_2 humidified atmosphere. Culture medium was replaced once a week.

OSTEOCLASTOGENIC RESPONSE OF PBMC CULTURES

Tartarate-resistant acid phosphatase (TRAP) activity. TRAP activity was assessed by the *para*-nitrophenilphosphate (*p*NPP) hydrolysis assay, as described previously [Costa-Rodrigues and Fernandes, 2011]. Briefly, after solubilization of cell layers with 0.1% (v/v) Triton X-100, cellular extracts were incubated with 12.5 mM *p*NPP in 0.04 M tartaric acid and 0.09 M citrate (pH 4.8) for 1 h at 37°C. The reaction was stopped with 5 M NaOH, and the absorbance of the samples was measured at 405 nm in an ELISA plate reader (Synergy HT, Biotek). Results were normalized with total protein content of cultures, determined by Bradford's method [1976], and expressed as nmol/min/$mg_{protein}$.

Histochemical staining of TRAP-positive multinucleated cells. After washing twice with PBS, cell layers were fixed with 3.7% formaldehyde. Following 10 min of incubation, samples were rinsed with distilled water and stained for TRAP with Acid Phosphatase, Leukocyte (TRAP) kit (Sigma), according manufacturer's instructions. Shortly, cells were incubated at 37°C for 1 h, in the presence of naphtol AS-BI 0.12 mg/ml, 6.76 mM tartrate, and 0.14 mg/ml Fast Garnet GBC. Cell layers were washed with distilled water and stained with hematoxylin. Cells multinucleated and TRAP-positive present in each well were counted.

Immunostaining of F-actin cytoskeleton and vitronectin and calcitonin receptors. After being fixed with 3.7% (v/v) *para*-formaldehyde for 15 min, cells were permeabilized with 0.1% (v/v) Triton X-100 for 5 min. Cell layers were stained for actin with 5 U/ml Alexa Fluor® 647-Phalloidin (Invitrogen) and for vitronectin and calcitonin receptors (VNR and CTR, respectively) with 50 μg/ml mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems). Anti-VNR and anti-CTR IgGs detection was performed with 2 μg/ml Alexa Fluor® 488-Goat anti-mouse IgGs. Cultures were observed by Confocal Laser Scanning Microscopy (CLSM).

Expression of osteoclast-related genes by RT-PCR. PBMC cultures were assessed for the expression of GAPDH, the osteoclast functional genes TRAP, cathepsin K (CATK), and carbonic anhydrase 2 (CA2), the differentiation factor c-myc and the activation gene c-src [Zhao et al., 2007]. After extraction with RNeasy® Mini Kit (Qiagen) according to manufacturer's instructions, RNA was analyzed for its concentration and purity in each sample by UV spectrophotometry at 260 nm and by calculating the $A_{260\,nm}/A_{280\,nm}$ ratio, respectively. RNA, 0.5 μg, was reverse transcribed and amplified (25 cycles) with the Titan One Tube RT-PCR System (Roche), with an annealing temperature of 55°C. The amplification conditions were chosen accordingly to preliminary calibration experiments (data not shown), in order to become possible to use the RT-PCR analysis as a quantitative tool. The primers used were purchased from Sigma Genosys and are presented on Table II. RT-PCR products were separated on a 1% (w/v) agarose gel and gel band intensities were analyzed with ImageJ 1.41 software. Values were considered as a percentage of the corresponding GAPDH value of each experimental condition.

Calcium phosphate resorption assay. PBMC cultures were maintained on BD BioCoat™ Osteologic™ Bone Cell Culture Plates (BD Biosciences), for 21 days. After the end of culture period, cells were removed with 6% NaOCl and 5.2% NaCl, following manufacturer's protocol and the remaining calcium phosphate layers were visualized by phase contrast light microscopy. Resorption lacunae were identified and total resorbed area was quantified with ImageJ 1.41 software.

INTRACELLULAR MECHANISMS INVOLVED IN THE OSTEOCLASTOGENIC RESPONSE

When indicated, PBMC cultures were treated with different signaling pathway inhibitors. U0126, a MEK signaling pathway inhibitor, was tested at 1 and 10 μM, because of the contradictory effects attributed to this molecule by different authors, namely, it has been proposed a stimulatory effect of this molecule on osteoclastogenesis, when present at low concentrations [Hotokezaka et al., 2007; Yamashita et al., 2010], while other reports have revealed an inhibitory effect in that process [Yang et al., 2008; Costa-Rodrigues et al., 2011c]. PDTC, a NF-κB signaling pathway inhibitor, was used at 10 and 100 μM, because the lower concentration has been previously described as the IC_{50} on rat osteoclastic differentiation [Hall et al., 1995]. Indomethacin (1 μM) was also included in the study, because it inhibits the synthesis of the osteoclastogenic inducer PGE2

TABLE II. Primers Used on RT-PCR Analysis of PBMC Cultures

Gene	5′ Primer	3′ Primer
GAPDH	5′-CAGGACCAGGTTCACCAACAAGT-3′	5′-GTGGCAGTGATGGCATGGACTGT-3′
TRAP	5′-ACCATGACCACCTTGGCAATGTCTC-3′	5′-ATAGTGGAAGCGCAGATAGCCGTT-3′
CATK	5′-AGGTTCTGCTGCTACCTGTGGTGAG-3′	5′-CTTGCATCAATGGCCACAGAGACAG-3′
CA2	5′-GGACCTGAGCACTGGCATAAGGACT-3′	5′-AAGGAGGCCACGAGGATCGAAGTT-3′
c-myc	5′-TACCCTCTCAACGACAGCAG-3′	5′-TCTTGACATTCTCCTCGGTG-3′
c-src	5′-AAGCTGTTCGGAGGCTTCAA-3′	5′-TTGGAGTAGTAGGCCACCAG-3′

[Kellinsalmi et al., 2007; Kawashima et al., 2009]. PBMC cultures were maintained in the same experimental conditions as those described above, and were assessed for TRAP activity and number of TRAP-positive multinucleated cells.

STATISTICAL ANALYSIS

Data presented in this work are the means of five separate experiments performed with cells from five different blood donors. Three replicas of each condition were made for each experiment. Data were evaluated using a two-way analysis of variance (ANOVA) and no significant differences in the pattern of the cell behavior were observed. Statistical differences found between control and experimental conditions were determined by Bonferroni's method. For values of $P \leq 0.05$, differences were considered statistically significant.

RESULTS

EXPRESSION OF OSTEOCLASTOGENIC MOLECULES BY BREAST CANCER CELL LINES

Breast cancer cell lines, cultured for 7 days, were assessed by RT-PCR for the expression of M-CSF, RANKL, IL-1β, IL-6, IL-17, TNF-α, and GM-CSF (Fig. 1A). In addition, the concentration of IL-1β, IL-6, and TNF-α in the corresponding CM was determined (Fig. 1B).

The four cell lines expressed M-CSF, but Hs-578 and SK-BR-3 cell lines displayed significantly higher levels compared to MCF-7 and T47D cell lines (Fig. 1A). For both Hs-578 and SK-BR-3 cell lines, expression levels of M-CSF were much higher than those observed for the other tested genes. RANKL and IL-1β were expressed at lower levels than M-CSF, but higher than the other genes. MCF-7 cell line did not reveal any RANKL expression. IL-1β was expressed by all cell lines, but T47D cells revealed higher values. IL-6, IL-17, and TNF-α expression was detected at very low levels. Hs-578T did not express IL-6, and TNF-α was only expressed by both SK-BR-3 and T47D cells. GM-CSF expression was not detected.

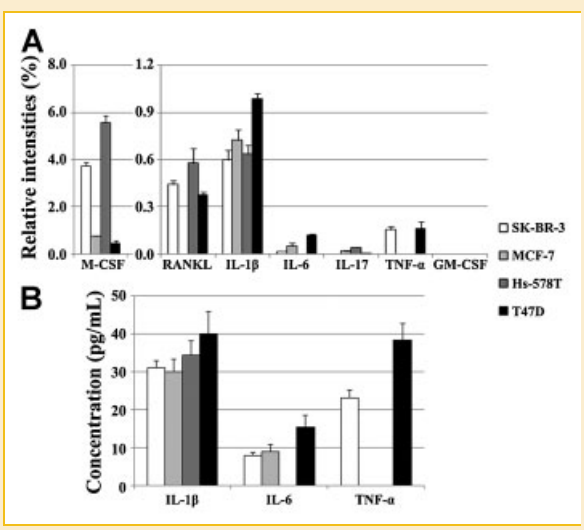


Fig. 1. RT-PCR assessment of human breast cancer cell lines for the expression of M-CSF, RANKL, IL-1β, IL-6, IL-17, TNF-α, and GM-CSF (**A**) and quantification of IL-1β, IL-6, and TNF-α present in the conditioned media (7 days) of each breast cancer cell line (**B**). RT-PCR band intensities were determined and normalized to the corresponding GAPDH values. Results are the mean of five independent analysis.

Concentration of IL-1β was higher in the culture medium of Hs-578 and, specially, T47D, although in both cases it did not reach statistical significance, compared to the remaining two cell lines (Fig. 1A). IL-6 was not detected in the CM from Hs-578T, and the higher concentration was observed on CM from T47D. Finally, TNF-α was only found in the CM from two cell lines, namely, SK-BR-3 and T47D, with a higher concentration in the latter one.

OSTEOCLASTOGENIC BEHAVIOR OF PBMC CULTURES

TRAP activity. Number of TRAP-positive multinucleated cells. Figure 2 shows the TRAP activity of PBMC cultures maintained in the absence or presence of recombinant M-CSF and RANKL (negative and positive control, respectively), or supplemented with CM from SK-BR-3, MCF-7, Hs-578T, and T47D cell lines (10% or 20%), cultured for 48 h, 7 days, or 14 days. PBMC cultures performed in the absence of M-CSF and RANKL displayed low TRAP activity. Supplementation with the growth factors resulted in a significant increase (~3-fold) and maximal levels were attained at day 14, remaining approximately constant during the 3rd week. Compared to the negative control, the presence of CM from the breast cell lines also resulted in increased TRAP activity, in selected experimental conditions, but significant differences were observed regarding the different cell lines. Compared to the positive control, CM-treated cultures presented a longer lag phase in the production of the enzyme, and, at day 14, TRAP activity was lower. However, enzyme levels increased with culture time and maximal values were always attained at day 21.

CM from SK-BR-3 cell line, collected from 48 h and 7-day cultures, used at 10%, induced the higher response; the highest values were found in PBMC cultures supplemented with CM from 7-day SK-BR-3 cultures (about 62% higher than that on positive control, at day 21). CM from 14-day cultures revealed a low ability to stimulate TRAP activity.

Regarding the CM from MCF-7 cell line, the highest TRAP activity was found on PBMC cultures supplemented with CM from 7-day cultures, used at 10%; at day 21, levels were slightly higher than those on the positive control.

PBMC cultures supplemented with CM from Hs-578T cell line displayed very low levels of TRAP activity at day 14. However, levels increased sharply during the 3rd week in the presence of CM from 7-day Hs-578T cultures, used at 10% or 20% (at day 21, TRAP activity was about 40–44% higher than that in positive control).

CM from 48 h T47D cultures, at 10% or 20%, elicited the highest values of TRAP activity in PBMC cultures, being about 64–72% higher (at day 21) than the one found on the cultures treated with M-CSF and RANKL.

After 14 and 21 days of culture, the profile observed for the number of TRAP-positive multinucleated cells in PBMC cultures was similar to that found for TRAP activity, either in control conditions or in the presence of CM from the breast cancer cell lines (Fig. 3). Results showed that the CM from 7-day cultures of the cell lines SK-

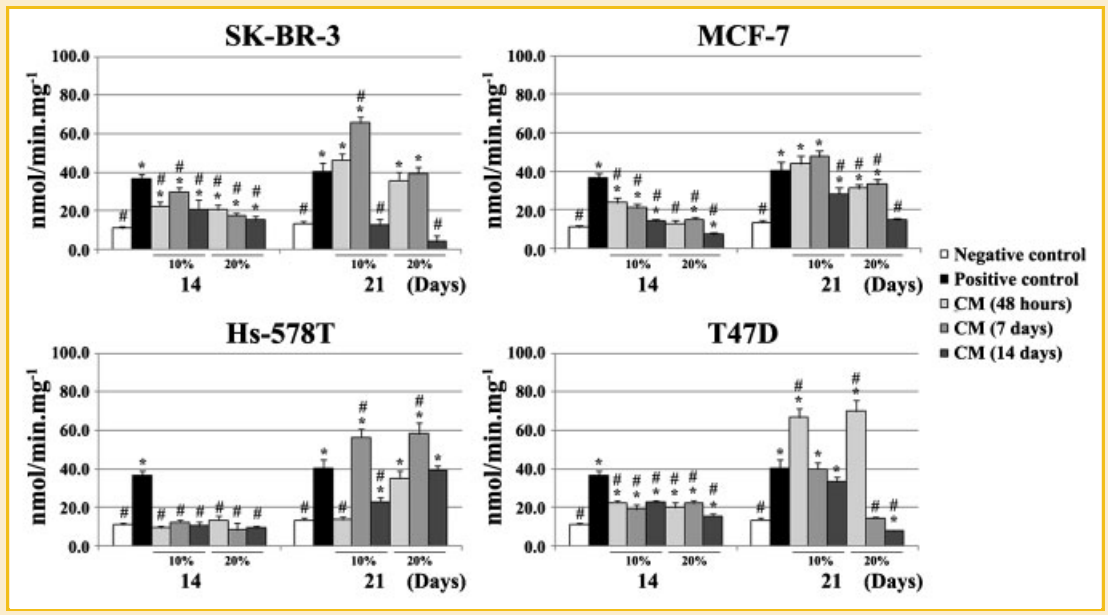


Fig. 2. TRAP activity of PBMC cultures performed in the absence or presence of recombinant M-CSF and RANKL (negative and positive control, respectively), or in the presence of 10% or 20% of conditioned medium (CM) from human breast cancer cell lines, collected after 48 h, 7 days, and 14 days of culture. *,#Significantly different from negative and positive control, respectively. Results are the mean of five independent experiments from five different donors.

BR-3, MCF-7, and Hs-578T, at 10%, and the CM from 48 h T47D cell cultures, also at 10%, elicited the higher TRAP activity and number of TRAP-positive multinucleated cells in PBMC cultures. With this information, PBMC cultures performed in these conditions were further characterized regarding other osteoclast-related parameters, as follows.

CLSM visualization of F-Actin rings and vitronectin and calcitonin receptors. At day 21, PBMC cultures performed in the presence of CM from the breast cancer cell lines displayed cells with well-defined F-actin rings and positive for the presence of VNR and CTR, in a way similar to that seen in the cultures supplemented with M-CSF and RANKL (Fig. 4).

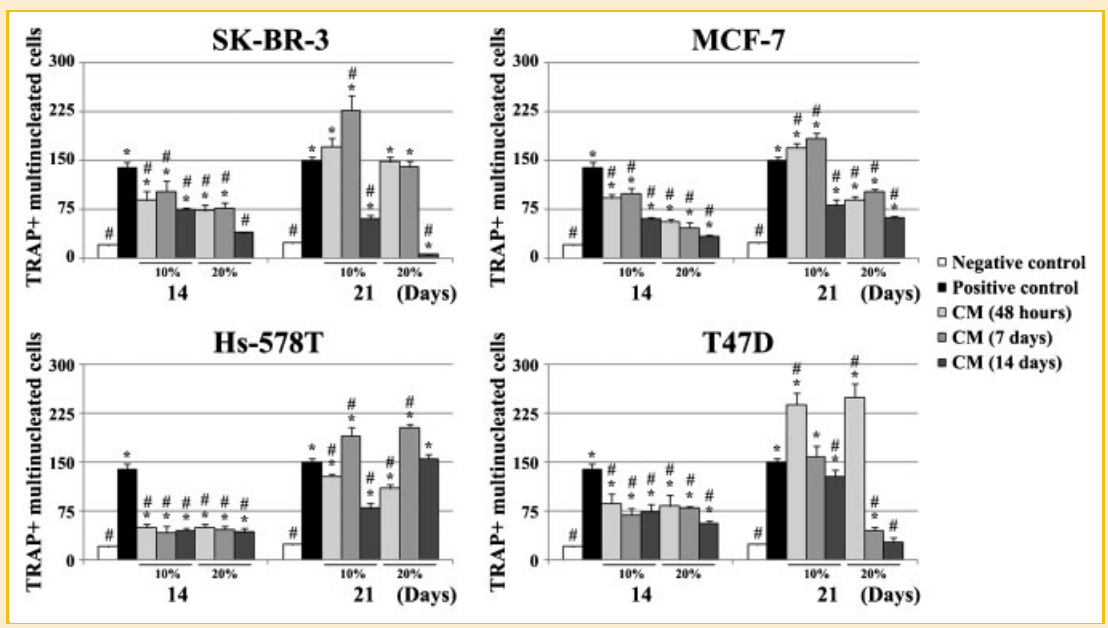


Fig. 3. Histochemical TRAP staining of PBMC cultures maintained in the absence or presence of both M-CSF and RANKL (negative and positive control, respectively), or in the presence of 10% or 20% conditioned medium (CM) from SK-BR-3, MCF-7, Hs-578T, and T47D cell lines, collected after 48 h, 7 days, and 14 days of culture. *,#Significantly different from negative and positive control, respectively. Results are the mean of three independent experiments from five different donors.

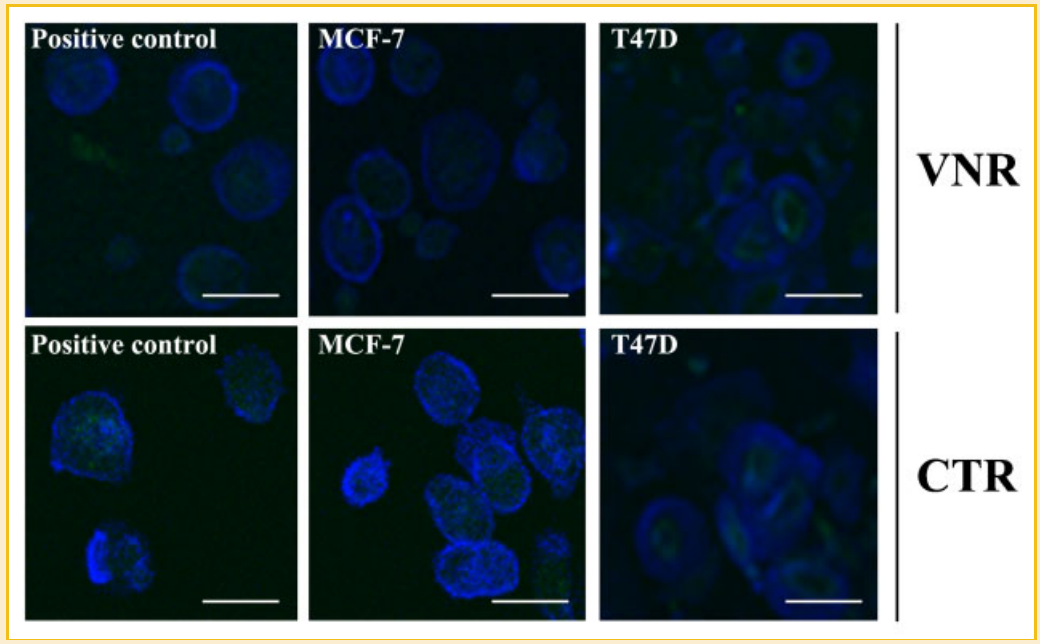


Fig. 4. CLSM visualization of PBMC cultures performed in the presence of recombinant M-CSF and RANKL (positive control) or 10% conditioned medium (CM), from MCF-7 cell line (7 days of culture) or T47D cell line (48 h of culture). Cells are stained blue for F-actin and green for vitronectin receptors (VNR) or calcitonin receptors (CTR). White bars represent 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Calcium phosphate resorbing ability. The ability of PBMC cultures to resorb calcium phosphate layers is shown in Figure 5. In the absence of M-CSF and RANKL or any CM, just few isolated lacunae were found, with a low value for the total resorbed area. The presence of both M-CSF and RANKL significantly increased the response by about 100%. Compared to positive control, the presence of the CM from the breast cancer cell lines sharply increased the total resorbed area (2.2–3.7 times). CM from T47D cell line elicited the highest resorbing ability, followed by SK-BR-3 and Hs-578T (with a similar response), and MCF-7.

Gene expression of osteoclast-related proteins. PBMC cultures performed in the absence or presence of M-CSF and RANKL expressed the osteoclast-related genes c-myc, c-src, TRAP, CATK, and CA2, with a significantly higher expression in the cultures supplemented with the growth factors (31–102%; Fig. 6). Compared to the positive control, CM from the breast cancer cell lines elicited an increased expression of these genes (12–51%). CM from MCF-7 cell line caused a slightly lower response compared to the CM from the other cell lines, nevertheless similar to the positive control.

Osteoclastogenic signaling pathways. Figure 7 shows the TRAP activity of PBMC cultures treated with U0126 and PDTC (inhibitors of MEK and NF-κB signaling pathways, respectively) and indomethacin, a PGE2 synthesis blocker. In the absence of growth factors or CM (negative control), the presence of U0126 or PDTC significantly abolished TRAP activity to values barely detectable or even undetectable, whereas indomethacin did not affect the cell response. On PBMC cultures treated with M-CSF and RANKL, the lower concentration of U0126 decreased by half the values of TRAP activity, while the higher concentration completely abolished enzyme production, at day 21. PDTC also impaired TRAP activity, while indomethacin had no significant effect. On PBMC cultures supplemented with CM from SK-BR-3 or MCF-7 cell lines, U0126 and PDTC revealed a very strong inhibitory effect, completely abolishing TRAP activity, even at lower concentrations. Indomethacin also decreased the enzyme activity, approximately 33% at day 21. CM from Hs-578T and T47D cell lines elicited a response that was only partially inhibited by low concentrations of U0126 (~38% and 77%, respectively). However, higher concentrations of U0126, or the presence of PDTC, completely blocked TRAP activity. Indomethacin also promoted a decrease of about 50% and 70% in TRAP activity of cultures supplemented with CM from Hs-578T and T47D cell lines, respectively.

A similar profile was observed for the number of TRAP-positive multinucleated cells observed in each experimental condition (data not shown).

DISCUSSION

Bone metastasis is a frequent complication of breast cancer [Chambers et al., 2002; Mundy, 2002; Kakonen and Mundy, 2003]. Although it is recognized that breast cancer cells have the ability to affect osteoclastogenesis through the secretion of soluble molecules that act as paracrine modulators [Chambers et al., 2002; Mundy, 2002; Kakonen and Mundy, 2003], information on this issue is still incomplete and sometimes controversial. This study compared the paracrine cellular and molecular effects of four human breast cancer cell lines on human osteoclastogenesis. The tested cell lines were selected by their different ability to express estrogen receptors, a factor known to be related with the bone

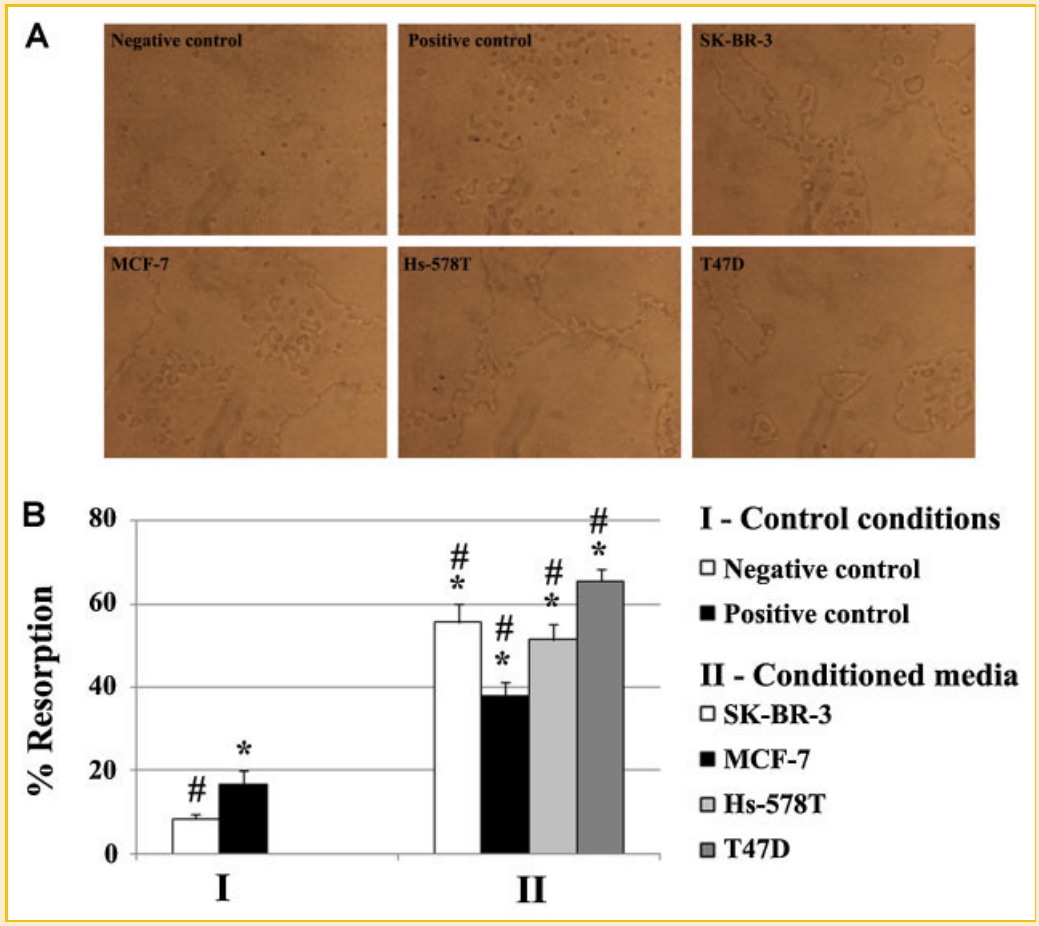
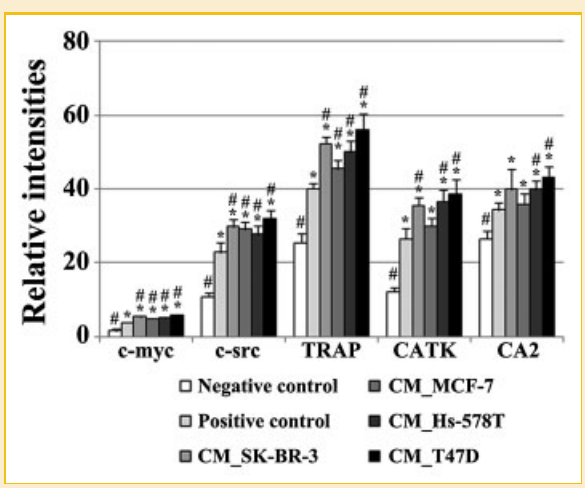


Fig. 5. Calcium phosphate resorption activity of PBMC cultures treated with 10% conditioned medium (CM) from breast cancer cell lines. CM from SK-BR-3, MCF-7, and Hs-578T was collected after 7 days of culture and CM from T47D was collected after 48 h of culture. After 21-day culture on calcium phosphate layers, resorption lacunae were identified **(A)** and total resorbed area was quantified **(B)**. Negative and positive controls represent PBMC cultures maintained in the absence or presence of both M-CSF and RANKL, respectively. *,#Significantly different from negative and positive control, respectively. Results are the mean of three independent experiments from five different donors. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]



metastatic behavior of breast cancer cells [Koenders et al., 1991; Solomayer et al., 2000; James et al., 2003]. PBMC cultures were supplemented with CM from the breast cancer cell lines and characterized for the osteoclastogenic response.

Cultures of the cancer cell lines used as a source of the CM were assessed for the expression of M-CSF and RANKL, known to have a central role on osteoclast development [Zhao et al., 2007]. The four cell lines expressed M-CSF, although at different degrees, which is in line with previous reports, showing the expression of this gene by

Fig. 6. RT-PCR analysis of PBMC cultures treated with 10% conditioned medium (CM) from breast cancer cell lines. CM from SK-BR-3, MCF-7, and Hs-578T was collected after 7 days of culture and CM from T47D was collected after 48 h of culture. Cultures performed in the absence or presence of recombinant M-CSF and RANKL were used as negative and positive control, respectively. Cell layers were assessed for the expression of GAPDH, c-myc, c-src, TRAP, CATK, and CA2. RT-PCR products were subjected to a densitometric analysis and were normalized to the corresponding GAPDH value. *,#Significantly different from negative and positive control, respectively. Results are the mean of three independent experiments from five different donors.

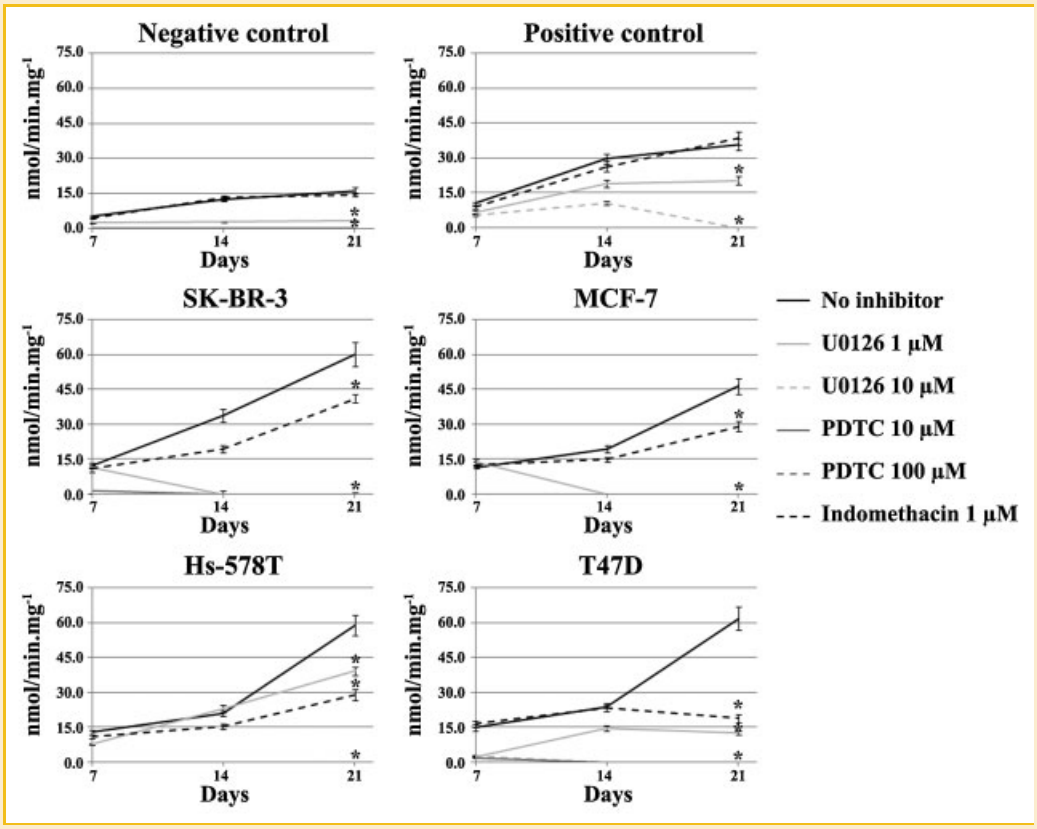


Fig. 7. TRAP activity of PBMC cultures performed in the absence or presence of both M-CSF and RANKL (negative and positive control, respectively) or supplemented with 10% conditioned medium (CM) from breast cancer cell lines. CM from SK-BR-3, MCF-7, and Hs-578T was collected after 7 days of culture and CM from T47D was collected after 48 h of culture. PBMC cultures were treated with 1 or 10 μM U0126 (MEK signaling pathway inhibitor), 10 or 100 μM PDTC (NF-κB signaling pathway inhibitor) and 1 μM indomethacin (PGE2 synthesis blocker). *Significantly different from the results obtained in the absence of any inhibitor at day 21. Results are the mean of three independent experiments from five different donors.

different cell lines, including MCF-7 [Pederson et al., 1999; Hunt et al., 2001; Morgan et al., 2004; Bendre et al., 2005]. On the other hand, RANKL expression was observed in three breast cancer cell lines, with the one exception being MCF-7. Some authors have claimed that breast cancer cells express RANKL, including MCF-7 cell line [Van Poznak et al., 2006; Nicolin et al., 2008], while other reports (that also included results for T47D and MCF-7 cell lines) revealed no expression of this gene [Thomas et al., 1999; Bendre et al., 2005]. Considering the low levels of RANKL expression found in breast cancer cells, other soluble molecules might play a role. Thus, the expression of other important osteoclastogenic genes (TNF-α, GM-CSF, IL-1β, IL-6, and IL-17) by breast cancer cells was also addressed in the present work. IL-1β was produced by all cell lines (at levels similar to the ones obtained for RANKL), while IL-6 expression was only not observed on Hs-578T and IL-17 on SK-BR-3. The expression levels varied among the different cell lines. The ability of breast cancer cells to express some of those genes was also described in previous reports [Pederson et al., 1999; Hunt et al., 2001; Lau et al., 2007]. Furthermore, SK-BR-3 and T47D were able to express TNF-α, which is in line with previous findings [Mrusek et al., 2005; Stavik et al., 2010].

The present work showed that the tested breast cancer cell lines have the ability to stimulate osteoclast differentiation through paracrine mechanisms, at different degrees. In selected conditions, the response was higher than that achieved in PBMC cultures supplemented with recombinant M-CSF and RANKL. The osteoclastogenic response was particularly significant regarding the calcium phosphate resorbing activity, and the tested breast cancer cell lines were able to induce it at a degree that was more than the double of that seen in PBMC cultures supplemented with the growth factors. Results showed differences on the osteoclastogenic potential of the cell lines, considering the profile of the cell response through the culture period and the characteristics of the CM that elicited the maximum response. T47D cell line exhibited the higher osteoclastogenic potential and MCF-7 cell line showed the lower response. In line with the present work, previous studies involving these and other cell lines also reported a significant variability on the induced osteoclastogenic response. It was shown that CM from 4-day cultures of MCF-7 cells induced an incomplete osteoclast differentiation of RAW 264.7 cell line [Nicolin et al., 2008]. However, other authors have observed osteoclastogenesis induced by CM of MCF-7 and MDA-MB-231 in PBMC cultures [Lau et al., 2007]. It was also reported that CM from MCF-7 and MDA-MB 435 cell lines were able to elicit a high degree of osteoclast development on co-cultures of UMR106 and PBMC, or on PBMC cultures [Hunt et al., 2001]. In a study conducted with rabbit osteoclasts, 3-day CM

from SK-BR-3 cells seemed to be more osteoclastogenic than that from MCF-7 [Gallet et al., 2006]. Finally, in another report, CM from T47D, MCF-7, MDA-MB-231, and MDA-MB-435 cell lines stimulated chicken osteoclasts, at different degrees, being CM from T47D cells the one that elicited the highest response [Pederson et al., 1999], which is in line with the highest osteoclastogenic potential observed in the present study. Regarding cell lines not tested in the present work, it was shown that CM from 48-h cultures of the cell lines MDA-MB-231, MDA-MET, and A549 CM were able to elicit osteoclast differentiation on PBMC cultures [Bendre et al., 2005]. Also, 24-h CM from MDA-231 cell line induced osteoclast differentiation in human bone marrow cells [Grano et al., 2000]. In another report, CM from the mouse mammary carcinoma cell line (BALB/c-MC) did not induce osteoclast formation on mouse bone marrow cells [Ono et al., 1998]. It is worth to note that there are no reported studies on the osteoclastogenic potential of Hs-578T cell line.

Globally, the higher tested concentration of the CM (particularly those conditioned for longer periods) induced a low osteoclastogenic response. This situation could be related to a low abundance of the osteoclastogenic cytokines in these CM. Nevertheless, as observed in Figure 1B, in the CM from 7-day cultures, some of the cytokines were detected. Moreover, the values obtained with CM from 14-day cultures were similar for IL-1β and IL-6, and slightly higher (particularly in T47D) for TNF-α (data not shown). Thus, it seems that the observed inhibitory effects of 20% CM from higher conditioned periods might be related to toxic effects, probably caused by the accumulation of cellular wastes and metabolites from breast cancer cells, rather than a direct decrease in their osteoclastogenic potential.

The present results showed differences regarding the relative relevance of the tested signaling pathways in the osteoclastogenic response induced by the breast cancer cell lines. However, MEK and NF-κB pathways seemed to have an important contribution, with PGE2 production also playing a role. MEK and NF-κB pathways are activated by several factors, including M-CSF and RANKL [Zhao et al., 2007; Bradley et al., 2008]. The importance of NF-κB signaling for breast cancer induced osteolysis was also suggested before [Park et al., 2007]. Regarding this, anti-RANKL therapeutics showed efficacy in patients with breast cancer-related bone metastases [Lipton et al., 2007] and also inhibited osteolysis in a mouse model of experimental bone metastasis involving MDA-MB-231 cell line [Morony et al., 2001], suggesting that RANKL-mediated osteoclastogenesis appears to be involved in osteoclast activation. As mentioned above, the four cell lines presented significant differences regarding the expression profile of M-CSF and RANKL. The cell lines SK-BR-3 and Hs-578T expressed high levels of M-CSF and also expressed RANKL, and these molecules might play a role on the induced osteoclastogenic response. On the other hand, MCF-7, which showed a low expression of M-CSF and did not express RANKL, presented a lower osteoclastogenic potential compared to the other cell lines, nevertheless similar to that seen in the positive control. However, the cell line T47D revealed low expression of both M-CSF and RANKL and showed the highest osteoclastogenic potential. These observations, together with the fact that MEK and NF-κB signaling pathways can be synergistically activated by different factors [Watanabe et al., 2004; Otero et al., 2007], suggest the presence of other soluble molecules that may also have an important role in the osteoclastogenesis induced by the breast cell lines. Regarding this, T47D cells exhibited the highest expression levels of the pro-osteoclastogenic genes IL-1β, IL-6, and TNF-α, which might help to explain the paracrine-induced osteoclast differentiation induced by this cell line. In addition, there are also other osteoclastogenic molecules that are known to be produced by breast cancer cells, like, for example, IL-3, IL-8, IL-11, TGF-β1, TGF-β2, and PTHrP [Pederson et al., 1999; Hunt et al., 2001; Benoy et al., 2004; Morgan et al., 2004; Bendre et al., 2005; Lau et al., 2007], and might be involved in the observed response. Thus, the communication between breast cancer cells and osteoclasts (or their precursors) appears as a very complex network of crosstalks, that usually drives bone metabolism towards bone destruction. The observed effects are most probably the result of the contribution of many molecules, whose expression varies among different breast cancer cell lines. Interestingly, it seems that at least partially, the different combinations of osteoclastogenic molecules might converge in the intracellular mechanisms affected, where MEK and NF-κB signaling pathways might be central players of the process.

CONCLUSION

The breast cell lines SK-BR-3, Hs-578T, MCF-7, and T47D were able to induce human osteoclastogenesis by paracrine-mediated mechanisms. Differences were noted regarding the time-dependent profile of the cell response and the experimental conditions that elicited the maximum osteoclastogenic behavior. Also, some differences were observed in the underlying intracellular mechanisms. MEK and NF-κB pathways seemed to play an important role, and the contribution of other soluble molecules in addition to M-CSF and RANKL is suggested. Information regarding the osteoclastogenic potential of breast cancer cells, their variability, and the contributing mechanisms are of crucial importance for the development of new therapeutic strategies for breast cancer-related bone metastasis and subsequent skeletal complications.

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