RANKL-induced migration of MDA-MB-231 human breast cancer cells via Src and MAPK activation

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Abstract. Accumulating studies have shown that the receptor activator of nuclear factor-κB ligand (RANKL)/RANK pathway plays an important role in tumor metastasis. However, the involvement of the RANKL/RANK signal transduction pathway in breast cancer metastasis remains unclear. The present study, therefore, investigated the role of downstream molecules of RANKL/RANK signaling in breast cancer cells using Transwell chemotaxis assays. RANKL was shown to direct the migration of MDA-MB-231 breast cancer cells. Osteoprotegerin (OPG; soluble decoy receptor of RANKL) inhibited RANKL-induced migration. RANKL activated Src kinase in MDA-MB-231 cells, as shown by Western blotting, and pretreatment with a Src inhibitor abrogated RANKL-induced cell migration, in a similar manner to OPG. Short-hairpin RNA against RANK, delivered via a lentiviral vector, significantly abolished the expression of phosphorylated Src. Stimulation by RANKL induced the phosphorylation of mitogen-activated protein kinases (MAPKs) (ERK, p38, JNK), and specific inhibitors of MAPKs blocked RANKL-induced cell migration. Furthermore, the expression of phosphorylated MAPKs could be blocked by a Src inhibitor and by small interfering RNA against Src. These findings suggest that Src and MAPK pathways may be involved in RANKL-induced MDA-MB-231 breast cancer cell migration.

Introduction

The outcomes of breast cancer patients depend largely on the development of distant metastases. Bone is the preferred target site for breast cancer metastasis, and bone metastasis occurs in up to 70% of patients with advanced breast cancer (1). A number of treatments are currently available for patients with bone metastases, including conventional radiation, chemotherapy and bisphosphonates. Although these strategies can provide palliative relief, none of them is curative (2). The mechanisms responsible for determining the directional migration of tumor cells are still poorly understood, and investigation of these biological mechanisms should lead to the development of potential new remedies.

Breast cancer cells commonly cause osteolytic metastases, a process that is at least partly dependent on osteoclast-mediated bone resorption (1). Osteoclasts and osteoclast precursor cells express the receptor activator of nuclear factor-κB (RANK), while osteoblasts and stromal cells express RANK ligand (RANKL). The binding of RANKL to RANK can lead osteoclast precursor cells to differentiate into active, fully mature osteoclasts. Osteoprotegerin (OPG) acts as a soluble decoy receptor by binding to RANKL and reducing its ability to interact with RANK (3). The activity of the RANKL/RANK signaling pathway during osteoclastogenesis has been well-established. RANKL interacts with RANK, inducing trimerization between RANK and tumor necrosis factor receptor-associated factor 6, leading to the activation of nuclear factor-κB (NF-κB), Src kinase and mitogen-activated protein kinases (MAPKs) in osteoclasts (4).

Interestingly, recent studies found that functional RANK was expressed in some primary and some secondary bone tumors, including osteosarcoma (5,6), chondrosarcoma (7), breast cancer (8), prostate cancer (9,10), renal cell cancer (11) and malignant melanoma (8). RANKL can promote cancer cell migration and invasion in in vitro experiments, and induce osteoclast chemotaxis (7,10,12). Inhibition of RANKL/RANK signaling in several murine bone metastasis models effectively reduced tumor burden in the skeleton (8,10,13). Several studies have reported roles for some signaling molecules in the RANKL/RANK pathway in breast cancer cells. Jones et al (8) found that RANKL could stimulate AKT/PKB and ERK1/2 phosphorylation in T47D human breast cancer cells, and could induce ERK1/2 phosphorylation in MDA-MB-231 human breast cancer cells, while Schramek et al (14) reported that RANKL could induce ERK and p38 activation in SKBR3 human breast cancer cells. However, the role of the RANKL/RANK signal transduction pathways in inducing the migration of human breast cancer cells is still not well understood.

Recent evidence supports the idea that cancer cells possess the ability to acquire a bone cell phenotype, called osteomimicry, which facilitates their homing to and colonization in...
the bone (15). Some studies found that the signaling critical for bone cell development could also be important for cancer cell metastasis and growth within the bone (16,17). Previous studies have reported that RANK, which has been well studied in osteoclasts, was also expressed in MDA-MB-231 breast cancer cells (18). In this study, we therefore determined if MDA-MB-231 breast cancer cells shared similar downstream signaling pathways with osteoclasts, thus facilitating their metastasis.

Materials and methods

Cell culture and reagents. The human breast cancer cell line MDA-MB-231 was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). Recombinant human RANKL and human OPG were purchased from Peprotech Inc. (Rocky Hill, NJ, USA). The ERK inhibitor PD98059, the p38 MAPK inhibitor SB203580 and Cell Counting Kit-8 were acquired from the Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). The Src kinase inhibitor PP2 was obtained from Invitrogen Corp. (Carlsbad, CA, USA) and the JNK inhibitor SP600125 was obtained from Enzo Life Sciences (Lausen, Switzerland). Antibodies against RANK, phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK, JNK, Src and GAPDH were purchased from Santa Cruz Biotechnology (Beverly, MA, USA).

Wound healing assay. MDA-MB-231 cells were seeded into a 24-well plate and allowed to reach 90% confluency in complete medium. Then the cell monolayer was wounded horizontally by a sterile 10-µl plastic tip. After wounding, cultures were washed gently several times with PBS to remove the detached cells. Subsequently, cells were cultured with or without human recombinant RANKL in DMEM plus 0.1% bovine serum albumin (BSA). The cells were allowed to migrate into the scratched area for 12 h and were photo-micrographed at 0 and 12 h with a magnification of x100. The wound area between the two edges of the scratch was measured and counted as change of wound closure relative to 0 h using Image Pro Plus (version 6.0) software (Media Cybernetics, Bethesda, MD, USA).

Transwell chemotaxis assay. The chemotaxis assay was performed using Millicell inserts (Millipore, Billerica, MA, USA) with 8 µm pore size in 24-well dishes. Briefly, 2x10^4 cells in 200 µl of serum-free DMEM/0.1% BSA medium were placed into the upper chamber. The same medium which contained RANKL was placed into the lower chamber. In order to inhibit RANKL-induced migration, RANKL was incubated with OPG for 1 h in the lower chamber before the upper chamber was inserted into the plate. If other inhibitors were used, cells were pretreated for 1 h with PP2 (Src inhibitor), PD98059 (ERK inhibitor), SB203580 (p38 MAPK inhibitor), and SP600125 (JNK inhibitor), respectively. The plate was incubated at 37°C, 5% CO2 for 12 h. Cells on the upper side of the filters were removed with a cotton tip. Then cells that had migrated to the lower surface of the filters were fixed with 4% paraformaldehyde for 20 min and stained with crystal violet for 15 min. Cells on the underside of the filters were counted under a microscope in five randomly different fields (x100) per membrane. The number of cells in each image were counted using the Image Pro Plus (version 6.0) software.

RNA interference. The lentiviral vectors were constructed and packaged according as previously described and produced by Telebio Biomedical Company (Shanghai, China) (19). Briefly, the effectively selected RANK short-hairpin RNA (shRNA) and negative control oligonucleotide sequences were as follows: RANK-targeted shRNA, sense: 5'-TCCACAGAAGATGAAATTCTTCATTTGTTGTGGTTT TTTC-3'; negative control shRNA sense: 5'-TTTCTCCGAA CGTGTACGTATTATTCAAGAAGTTACGACACG TCCGGAGAATTTTTTT-3'. The produced lentiviruses containing RNA interference (RNAi) sequences and negative control sequences were named as I002 and SKF, respectively. The cells which were transfected by virus with RANK-targeted shRNA were thus named MDA-MB-231-I002 cells, and the cells which were transfected by control virus were named MDA-MB-231-SKF cells. The MDA-MB-231 cells were infected at a multiplicity of infection 15 by purified lentivirus (final titer = 3x10^4 T/U/ml) with 8 µg/ml polybrene. Transduction efficiency was estimated by fluorescence microscopy after 96 h. To silence Src protein expression in MDA-MB-231 cells, we used small interfering RNA (siRNA) approach. Cells were transfected with siRNA against human Src or control siRNA (100 nM; Santa Cruz) using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions as previously described (20). The efficiency of the knockdown was determined by Western blot analysis.

Cell viability assay. Cell viability was analyzed using a WST-8 Cell Counting Kit-8. In brief, MDA-MB-231 cells (1x10^4 cells per well) were plated into 96-well plates at 37°C, 5% CO2 in 100 µl of DMEM containing 0.1% BSA. Cells were incubated in the absence or presence of RANKL, OPG, PP2, PD98059, SB203580 and SP600125 for 12 h to parallel the conditions of the migration assays. WST-8 solution (10 µl) was added to each well and the cultures were incubated for 2 h at 37°C. Then, the OD was determined at 450 nm using an immunoreader.

Western blot analysis. Cells were washed with ice-cold PBS and scraped in RIPA lysis buffer including protease inhibitors. After SDS-PAGE electrophoresis, the proteins were electrophoretically transferred to a polyvinylidine difluoride membranes. Membranes were blocked in TBS-T containing 6% non-fat dry milk for 2 h at room temperature, and then incubated overnight with the primary antibodies. Antibodies directed against RANK, phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK, JNK and GAPDH were all at a dilution of 1:1000. The efficiency of the knockdown was determined by Western blot analysis.

Statistical analysis. The SPSS 13.0 software (IBM, Somers, NY, USA) was applied to complete data processing. All results...
are expressed as the mean ± SEM. The data were analyzed using one-way factorial analysis of variance (ANOVA) with the Student-Newman-Keuls (SNK) tests for comparisons between the means. The Student’s t-test was conducted when two groups were compared. P<0.05 is considered as a significant difference.

**Results**

**RANKL induces MDA-MB-231 cell migration in a dose-dependent manner.** We investigated the biological effects of RANKL in breast cancer cells using a wound-healing assay to examine the motility of MDA-MB-231 cells. As shown in Fig. 1A, RANKL (25, 50 and 100 ng/ml) significantly increased the migration of MDA-MB-231 cells into the slit area over 12 h, compared with the untreated group (P<0.05). In the Transwell assay, the migration rates of MDA-MB-231 cells mediated by RANKL (25, 50 and 100 ng/ml) increased by 0.78-, 1.8- and 3.9-fold, respectively (P<0.05) (Fig. 1B). The RANKL-induced cell migration was inhibited by OPG in a dose-dependent manner. There was no significant difference in the migration between the OPG/RANKL (500:100 ng/ml) and blank groups (P>0.05) (Fig. 1C). Our results also show that OPG alone did...
Figure 2. Down-regulation of RANK expression inhibits RANKL-induced migration of MDA-MB-231 cells. (A) MDA-MB-231 cells transduced with lentivirus-mediated RANK-targeted shRNA (MDA-MB-231-I002) or control-negative shRNA (MDA-MB-231-SKF) were harvested. Western blotting was used to analyze the expression levels of RANK protein. *P<0.05 versus the MDA-MB-231 and MDA-MB-231-SKF groups. (B) MDA-MB-231-SKF, MDA-MB-231-I002 and untransfected MDA-MB-231 cells were put into the Transwell chambers and migration towards RANKL (100 ng/ml) was measured after 12 h. Results are the means ± SEM of three experiments. The MDA-MB-231 cells without RANKL treatment served as the control. *P<0.05 versus the control. #P<0.05 versus the MDA-MB-231 and MDA-MB-231-SKF groups which were treated with RANKL.

Figure 3. RANKL activates Src kinase in MDA-MB-231 cells. (A) MDA-MB-231 cells were incubated with RANKL (100 ng/ml) for 0-60 min, and levels of activated Src were detected by Western blot analysis. (B) MDA-MB-231 cells were incubated for 60 min with RANKL (0-100 ng/ml) or with RANKL/OPG (100:500 ng/ml), and levels of Src activation were detected by Western blot analysis. (C) MDA-MB-231-SKF, MDA-MB-231-I002 and untransfected MDA-MB-231 cells were incubated for 60 min with RANKL (100 ng/ml), and Src phosphorylation was examined by Western blot analysis. *P<0.05 versus the MDA-MB-231 and MDA-MB-231-SKF groups. (D) MDA-MB-231 cells were pretreated with vehicle (0.1% DMSO) or PP2 (2.5, 5 and 10 µM) for 60 min respectively, followed by treatment with 100 ng/ml RANKL. Src phosphorylation was examined by Western blot analysis. Results are the means ± SEM of three experiments. *P<0.05 versus no PP2 treatment.
not increase cell migration (Fig. 1C). In addition, treatment of MDA-MB-231 cells with RANKL (100 ng/ml) and OPG (500 ng/ml) for 12 h had no significant effect on cell viability (P>0.05) (Fig. 1D).

**shRNA-induced RANK silencing inhibits the migration of MDA-MB-231 cells.** The silencing effect of lentivirus-mediated RANK-targeted shRNA was confirmed using Western blot assays to determine the expression of RANK protein in transfected MDA-MB-231 cells. As shown in Fig. 2A, the expression levels of RANK protein in MDA-MB-231-I002 cells transduced with lentivirus-mediated RANK-targeted shRNA were decreased by ~59.1% compared with those in the untransduced MDA-MB-231 cells (P<0.05). There was no obvious difference between the levels in MDA-MB-231-SKF cells transduced with the lentivirus-mediated negative control shRNA and untransduced MDA-MB-231 cells (P>0.05) (Fig. 2A). Fewer MDA-MB-231-I002 cells migrated towards RANKL, compared with MDA-MB-231 cells (Fig. 2B).

**Src kinase is involved in the RANKL-induced migration of MDA-MB-231 cells.** Western blot analysis was used to investigate the effects of RANKL on Src kinase. Levels of phosphorylated Src began to increase within 5 min after treatment with RANKL (100 ng/ml) (Fig. 3A), in a concentration-dependent manner. The expression of phosphorylated Src was markedly decreased when MDA-MB-231 cells were co-incubated with OPG/RANKL (500:100 ng/ml), compared with the RANKL (100 ng/ml) alone group (Fig. 3B). RANKL-induced expression of phosphorylated Src was significantly lower in MDA-MB-231-I002 cells transduced with lentivirus-mediated RANK-targeted shRNA than in MDA-MB-231 cells (Fig. 3C). To confirm the involvement of Src kinases in RANKL-induced MDA-MB-231 cell migration, MDA-MB-231 cells were pretreated with the specific Src inhibitor PP2 to inhibit the activation of Src. Src phosphorylation induced by RANKL was markedly suppressed by the addition of 10 µM PP2, as detected by Western blotting (Fig. 3D). Pre-incubation with increasing concentrations of PP2 decreased RANKL-induced chemotaxis of MDA-MB-231 cells in a dose-dependent manner (Fig. 4B). Transfection of cells with Src siRNA reduced Src protein expression (Fig. 4A) and antagonized RANKL-induced migration (Fig. 4B).

**MAPK kinases are involved in the RANKL-induced migration of MDA-MB-231 cells.** Western blot analysis was performed to investigate the effects of RANKL on MAPKs. RANKL markedly increased phosphorylation of ERK1/2, JNK and p38 within 30 min (Fig. 5A). The phosphorylation of ERK1/2, JNK and p38 were induced by RANKL in a dose-dependent manner (Fig. 5B). The phosphorylation levels of ERK1/2, JNK and p38 were all inhibited by OPG (Fig. 5B). PD98059 (ERK inhibitor; 30 µM), SB203580 (p38 inhibitor; 20 µM), SP600125 (JNK inhibitor; 20 µM) inhibited ERK, p38 and JNK phosphorylation, respectively (Fig. 5B). Moreover, the Src inhibitor PP2 (10 µM) also inhibited the RANKL-induced phosphorylation of ERK1/2, JNK and p38 (Fig. 5B). The phosphorylation levels of ERK1/2, JNK and p38 were all decreased in cells transfected with Src siRNA, compared with MDA-MB-231 cells, after treatment with RANKL (Fig. 5C). Cell migration was also effectively decreased by treatment with PD98059 (30 µM), SB203580 (20 µM), SP600125 (20 µM) (Fig. 6A). Cell viability assays showed that PP2 (10 µM), PD98059 (30 µM), SB203580 (20 µM) and SP600125 (20 µM) had no effects on the proliferation of MDA-MB-231 cells after treatment for 12 h (Fig. 6B).
Discussion

Like other metastatic processes, bone metastasis of breast cancer is the result of several sequential steps, and depends on interactions between tumor cells and the host environment. In order to develop metastatic lesions, cancer cells must possess or acquire the ability to disrupt and hijack the intricate signals that exist in the bone cells and the surrounding stroma. These
signals usually govern the physiologic bone remodeling that facilitates normal bone turnover (2). The dramatic roles of RANK, RANKL and OPG in bone turnover have been well explored since their discoveries in the late 1990s. Many studies also have demonstrated an essential role for Src in the process of osteoclast-mediated bone resorption (21). In the current study, we confirmed that RANKL increased MDA-MB-231 migration, and that Src kinase and MAPK pathways were involved in the RANKL-directed migration of these breast cancer cells. To the best of our knowledge, this provides the first evidence of a role for the RANKL/RANK/Src signaling pathway in the regulation of MDA-MB-231 breast cancer cell migration.

Src kinase is a membrane-associated non-receptor tyrosine kinase that belongs to the Src-family kinase group. As a pleiotropic activator, Src kinase also regulates numerous cell signaling pathways important for survival, proliferation, invasion, migration and angiogenesis of cancer cells (22). Previous breast cancer studies have found that Src can act as a downstream signal of receptor tyrosine kinases, such as epidermal growth factor receptor and human epidermal growth factor receptor 2, as well as G-protein-coupled receptors (2). In the current study, we demonstrated that Src phosphorylation could be promoted by RANKL in MDA-MB-231 breast cancer cells, while silencing of RANK expression, and OPG, could abrogate the Src phosphorylation mediated by RANKL. The results suggest that Src acts as a downstream signal of RANKL/RANK in MDA-MB-231 cells. Src activation is known to correlate with poor prognosis in breast cancer, and Zhang et al (23) have reported that Src activity was tightly associated with the development of bone metastases and late-onset bone relapse. Although a previous study also found that inhibition of Src expression or activity in MDA-MB-231 breast cancer cells specifically reduced the formation of bone metastases in a nude mouse model (24), the mechanism of Src in mediating breast cancer bone metastasis remains unclear. The results of the current study showed that pretreatment with a Src inhibitor (PP2) or Src siRNA antagonized the cell migration induced by RANKL, which is abundant in the bone environment (4). Sabbota et al (17) also recently identified Src as a key downstream mediator of RANKL-induced migration of prostate cancer cells. Overall, these results suggest that Src kinase is involved in mediating the effects of RANKL on cell motility, and may thus play a role in metastasis.

Although previous studies reported that RANKL could induce ERK1/2 and p38 phosphorylation in breast cancer cell lines (8,14), we found that RANKL could also activate JNK, and that treatment of MDA-MB-231 cells with OPG could inhibit JNK, ERK1/2 and p38 activation. These results indicate that MAPKs also act as downstream signals of RANKL/RANK in MDA-MB-231 cells. MAPK pathways are important signaling pathways for cell migration. Several cytoskeleton-associated proteins and signaling molecules, as well as adaptor proteins, have been identified as MAPK substrates. MAPK kinases can induce the phosphorylation of paxillin, calpain, DCX, MAP1B, MAP2, MAPKAPK 2/3 and MLCK, and are thus involved in regulating the dynamics of focal adhesions and the reorganization of filamentous actin (25). In this study, we found that PD98059 (an ERK inhibitor), SB203580 (a p38 inhibitor) and SP600125 (a JNK inhibitor) were all able to suppress RANKL-induced cell migration. Overall, the results suggest that RANKL mediates MDA-MB-231 cell migration via MAPK signaling pathways.

Further evidence indicates that Src and MAPK pathways may cooperate to promote cell motility. Activated Src kinase can form complexes at the cell periphery with cytoplasmic proteins such as FAK. The active FAK-Src complex can recruit ERK/MAPK, and then induce proteolytic cleavage and disruption of adhesions to increase motility (26). Li et al (27) recently demonstrated that Src kinase mediated the phosphorylation of ERK, PI3K and JNK induced by estradiol, to facilitate cell migration. In the present study, the Src inhibitor (PP2) and knockdown of Src with siRNA blocked the phosphorylation of ERK1/2, JNK and p38 induced by RANKL. These data suggest that Src kinase may play a role in the RANKL-induced activation of MAPKs. Sabbota et al (17), however, found no effect of PP2 on RANKL-induced activation of MAPKs in prostate cancer cells. These inconsistent observations may be attributable to the different cancer cell types used. Further studies are needed to explore the precise relationship between Src and MAPK pathways in the RANKL-induced migration of breast cancer cells.

Although the osteomimicry theory was initially presented as cancer cells acquiring an osteoblast-like phenotype, such as bone-matrix proteins and Wnt signaling (15,28), many studies have also found that breast cancer cells can acquire normal regulatory pathways for osteoclasts to facilitate bone metastasis (29,30). The RANKL/RANK and Src pathways are well known to play important roles in the functions of both osteoclasts and tumor cells. Several Src inhibitors and a human monoclonal RANKL antibody have undergone clinical trials in patients with bone metastasis (2). The current study, as well as that of Sabbota et al (17), demonstrated that the RANKL/RANK/Src axis was a key pathway in the RANKL-induced migration of prostate cancer and breast cancer cells. The results suggest that the use of Src inhibitors, in combination with RANKL inhibitors, could provide a new therapeutic strategy for the treatment of bone metastasis. It is therefore crucial to understand the molecular mechanisms underlying the osteoclast-like properties of cancer cells, and to identify new therapeutic targets for patients with bone metastases.

In conclusion, the results of this study confirmed that RANKL can induce the migration of MDA-MB-231 breast cancer cells, and that this migration involves Src kinase and MAPK pathways. Further investigations into the RANKL/RANK-mediated signaling pathway in breast cancer cells may increase our understanding of the mechanisms of metastasis, and thus lead to the development of more effective therapies.

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References


