Abstract. Expression of the CXC chemokine receptor-4 (CXCR4), a G protein-coupled receptor, and HER2, a receptor tyrosine kinase, strongly correlates with tumor progression and metastatic potential of breast cancer cells. We report the identification of pomolic acid (PA) as a novel regulator of HER2 and CXCR4 expression. We found that PA downregulated the expression of HER2 and CXCR4 in SKBR3 cells in a dose- and time-dependent manner. When investigated for the molecular mechanism(s), it was found that the downregulation of HER2 and CXCR4 was not due to proteolytic degradation but rather to transcriptional regulation as indicated by downregulation of mRNA expression. Moreover, we show that PA inhibits phosphorylation of ERK and reduces NF-κB activation. Suppression of CXCR4 expression by PA correlated with the inhibition of CXCL12-induced invasion of HER2-overexpressing breast cancer cells. Overall, our results demonstrate for the first time that PA is a novel inhibitor of HER2 and CXCR4 expression via kinase pathways and may play a critical role in determining the metastatic potential of breast cancer cells.

Introduction

Metastasis is the main cause of morbidity and fatality in various cancers. Breast cancer is a major public health issue and is the most common malignancy in females (1). Expression of numerous genes has been linked to impart metastatic potential in breast cancer. In the clinic, breast cancer is classified mainly into four molecular subtypes: luminal A/B, human epidermal growth factor receptor type II (HER2) and basal-like. The HER2 subtype is overexpressed in ~25-30% of all breast cancer cases and HER2 overexpression is strongly associated with an aggressive phenotype and poor outcomes (2).

Chemokine receptors belong to G protein-coupled receptor (GPCR) family, which trigger chemotactic and growth signals following reciprocal action with their ligands. CXCR4, the receptor of CXCL12/stromal cell-derived factor-1α (SDF-1α), has recently been shown to play an important role in breast cancer metastasis (3). The CXCR4/CXCL12 axis makes breast cancer cells to leave of the circulation and traffic into specific organs with large amounts of chemokines, thus forming angiogenesis, proliferation and metastatic tumors (4). The RTK HER2 and GPCR CXCR4 are two structurally unrelated receptors, but a recent study indicated that HER2 enhances CXCR4 expression and that CXCR4 is required for HER2-induced breast cancer metastasis (5). In addition, a potential relationship between CXCR4/CXCL12 and HER2 in breast cancer cells is not completely understood, especially on its role in metastasis. Therefore, targeting disease-associated proteins for HER2 and CXCR4 represents a promising alternative therapeutic strategy in breast cancer.

Pomolic acid (PA) is a pentacyclic triterpene isolated from Euscaphis japonica, and is highly effective in inhibiting cell growth (6) and induces apoptosis (7,8). In a previous study, our group showed that PA suppressed CXCR4 expression in breast cancer cells (9), but how CXCR4 is regulated in these cancer cells is not understood. Although it has been established that HER2-mediated upregulation of CXCR4 protein is responsible for the invasiveness of HER2-overexpressing breast cancer cells (5), no information is available regarding the regulation of CXCR4 in HER2-overexpressing breast cancer cells.

Therefore, the aim of this study was to examine the effects of PA on CXCR4 and HER2 regulation in HER2 overexpressing SKBR3 cells. We found that PA inhibited HER2 and CXCR4 expression at both the protein and mRNA levels, with critical involvement of the ERK pathway and suppression of NF-κB activation. Furthermore, we investigated the PA-inhibited metastatic potential of HER2-overexpressing breast cancer cells when the cells were transactivated by CXCL12.

Materials and methods

Cell culture and tumor cell lines. SKBR3, MCF7 and MDA-MB-231 human breast cancer cell lines were obtained.
from the American Type Culture Collection (ATCC; Rockville, MD, USA).

The human HER2-overexpressing breast cancer cell line SKBR3 was cultured in RPMI-1640 containing 25 mM HEPES, 10% fetal bovine serum (FBS) and 1% antibiotics. MCF7 breast cancer cell lines was cultured in RPMI-1640 supplemented with 10% FBS and 1% antibiotics. MDA-MB-231 breast cancer cell lines was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and breast cancer cell lines was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37°C in an atmosphere of 5% CO₂-95% air. All cells were passaged at 80% confluence in 0.25% trypsin-EDTA for 3-5 min. RPMI-1640, DMEM, FBS, antibiotic and trypsin-EDTA were purchased from Gibco (Gibco, Grand Island, NY, USA).

Materials and reagents. Pomolic acid (PA) was received from Dr Ki Yong Lee, a professor of the College of Pharmacy, Korea University (10). Pomolic acid was dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA) as a 10-mM stock solution and stored at 4°C. Further dilution was done in cell culture medium. Lactacystin and chloroquine were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against CXCR4 was obtained from Abcam (Cambridge, MA, USA). HER2, phospho-ERK, ERK, phospho-p38, p38, phospho-AKT, AKT, phospho-JNK, JNK, β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). β-actin was used as a loading control. CXCL12 was purchased from R&D Systems (Minneapolis, MN, USA).

Western blot analysis. SKBR3 cells, grown under our experimental conditions, were lysed for 30 min on ice in radioimmunoprecipitation assay (RIPA) lysis buffer [150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 1% deoxycholate and 5 mM ethylene diamine tetra acetic acid (EDTA)] enriched with a complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined by using bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

Total proteins (25 µg) was loaded onto 10% SDS-polyacrylamide gel, separated, and transferred onto polyvinyl difluoride (PVDF) membrane (Roche, Penzberg, Germany). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tris-buffered saline with Tween-20 (TBST; 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20) and incubated with primary antibody at 4°C. After three washes of 10 min each in TBST, the membranes were incubated with hybridization with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 2 h and subsequently washed again. The transferred proteins were incubated with super-signal pico-chemiluminescent substrate or dura-luminol substrate (Thermo Scientific, Waltham, MA, USA) for 2 min according to the manufacturer's instructions and visualized with ImageQuant™ LAS 4000 (Fujifilm Life Science, Roche Diagnostics).

RNA extraction and PCR analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and 11 µg of total RNA was reverse-transcribed using AccuPower® Rocketscript™ cycle RT premix (Bioneer, Daejeon, Korea). The relative expression of CXCR4 and HER2 was analyzed by quantitative RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The following pairs of forward and reverse primer sets were used: HER2 sense, 5'-AGC CGG AAC CCA AGT-3'; antisense, 5'-TTG GTG GGC AGG TAG GTG AGT T. CXCR4, sense, 5'-CCG TGG CAA ACT GGT ACT TT-3'; antisense, 5'-TTT CAG CCA ACA GCT TCC TT-3'. The RT-PCR reaction mixture contained 2.5 µl of 10X Taq reaction buffer, 0.5 µl of each 10 mM dNTP, 1 µl each of forward and reverse primers, and 2 µl template DNA each of in a final volume of 25 µl. Amplification products were resolved by 1.5% agarose gel electrophoresis stained with safe dye and photographed by Imagequant LAS 4000.

Quantitative real-time PCR. Real-time PCR was performed on the cDNA using the selective primers for HER2 (sense, 5'-AGC CGG CAC CCC CCA AGT-3'; antisense, 5'-TTG GTG GGC AGG TAG GTG AGT T) CXCR4 (5'-CCG TGG CAA ACT GGT ACT TT-3'; antisense, 5'-TTT CAG CCA ACA GCT TCC TT-3') and GAPDH (sense, 5'-CAG CCT CAA GAT CAT CAG CA-3'; antisense, 5'-GTC TTC TGG GTG GCA GTG AT-3'). PCR was performed in a Light Cycler 480 (Roche Diagnostics, Indianapolis, IN, USA) using the Light Cycler DNA Master SYBR Green kit (Roche Diagnostics) following the manufacturer's recommended amplification procedure. Reaction conditions of CXCR4 consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 55°C for 30 sec. Reaction conditions of HER2 consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec. All reactions were triplicate repeated, and relative mRNA expression levels for target genes were determined using the 2-ΔΔCT method with normalization by GAPDH (11).

MAPK inhibitor treatment and siRNA transfection. SKBR3 cells were pre-treated with mitogen-activated protein kinase (MAPK) inhibitors (Calbiochem, CA, USA) such as ERK1/2-specific inhibitor: PD98059 (20 µM), JNK-specific inhibitor: SP600125 (20 µM), p38-specific inhibitor: SB203580 (20 µM) and PI3K-specific inhibitor: LY234002 (20 µM). After 30 min, the cells were treated for 24 h. Cells were transfected with control siRNA and ERK1/2-MAPK siRNA (Cell Signaling Technology) using RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

In vitro invasion assay. In vitro invasion of SKBR3 cells was measured using Bio-Coat Matrigel invasion assay system (BD Biosciences, Lexington, KY, USA) according to the manufacturer's instructions. Cancer cells (5x10⁴/ml) were suspended in medium and seeded into the Matrigel-precoated Transwell chambers with polycarbonate membranes of 8 µm pore size. After preincubation with or without pomolic acid (25 µM), Transwell chambers were placed into 24-well plates, in which was added the basal medium only or basal medium containing 100 ng/ml CXCL12. After incubation (24 h for SKBR3), the upper surface of Transwell chambers was wiped off with a
cotton swab and invading cells were fixed and stained with a Diff-Quick stain. The invading cell numbers were counted in five randomly selected microscope fields (x100). Average cell numbers in each field were used for statistical analyses. Each experiment was performed in triplicate. All experiments were conducted in triplicate and the invasion index was expressed as the percentage of invaded cell number compared with the corresponding control.

Electrophoretic mobility shift assay. A DIG Gel Shift kit (Roche, Mannheim, Germany) was used to detect electrophoretic mobility shift assay (EMSA). The nuclear protein was harvested and NF-κB binding with DNA was detected according to the manufacturer’s instructions. The NF-κB sequence consensus oligonucleotide used in this study was 5'-CTT GAA GGG ATT TCC CTG GCT TGA AGG GAT TTC CCT GG-3' containing the NF-κB binding motif end-
labeled with DIG-ddUTP. For the binding reaction, 10 µg of the sample protein was incubated at room temperature for 30 min with a DIG-labeled probe. For supershift experiments, 4 µg of specific anti-p65 was added to the binding reaction and incubated for 30 min, after which we added the DIG-labeled probe. The DNA-protein complexes were separated by electrophoresis in 6% non-denatured polyacrylamide gels using 0.5X TBE as a running buffer. After electrophoresis, the gels were transferred to nylon membranes and detected chemiluminescently. Signal intensity was quantified by ImageQuant LAS 4000.

Statistical analysis. Experiments were performed at least three times, with consistent results. The results are given as mean ± standard deviation (SD). The P-value was assessed using ANOVA and Student-Newman-Keul tests. Results were considered statistically significant at P<0.05, and P<0.001.

Results

HER2 and CXCR4 expression is downregulated in the breast cancer cells by PA. Recently, several studies have shown that the relationship between HER2 and CXCR4 is involved in the breast cancer metastasis to organs (lung, liver and bone) (3,12). We first investigated the relative expression of CXCR4 and HER2 in three breast cancer cell lines. Expression of CXCR4 was detected in all three cell lines, but constitutive HER2 was found only in SKBR3 cells (Fig. 1B). Hence, we determined whether HER2 overexpression affects CXCR4 expression, and the pathway involved in this regulation. Furthermore, we assessed whether PA can modulate both HER2 and CXCR4 expression in HER2-overexpressing SKBR3 cells. When SKBR3 cells were incubated either with different concentrations of PA for 24 h or with 25 µM PA for different times, CXCR4 and HER2 expression was suppressed in a dose- and time-dependent manner (Fig. 1C and D).

PA-induced downregulation of HER2 and CXCR4 is not mediated through its degradation. Degradation of HER2 leads to binding of Hsp90 and ubiquitination of the receptor that targets the protein for proteasomal degradation (13). Also, CXCR4 undergoes ubiquitination at its lysine residue, followed by degradation (14,15), and so we investigated the possibility that PA enhances the rate of both HER2 and CXCR4 degradation through proteasomal activation. We determined if PA induced the degradation of HER2 and CXCR4, by treating SKBR3 cells with the proteasomal inhibitor lactacystin. SKBR3 cells were pretreated with lactacystin for 1 h before being exposed to PA. As shown in Fig. 2A, lactacystin had no effect on PA-induced downregulation of HER2 and CXCR4.

Several studies have shown that degradation of HER2 is dependent on both lysosomal and proteasomal proteases (16). CXCR4 undergoes ligand-dependent lysosomal degradation (14), so we investigated whether chloroquine, a lysosomal inhibitor, blocks PA-induced degradation of SKBR3. Cells were pretreated with chloroquine 1 h before exposure to PA. The lysosomal inhibitor had no influence on the downregulation of HER2 and CXCR4 (Fig. 2B), indicating that this was not the primary pathway for suppressing HER2 and CXCR4 expression.

PA significantly downregulates both HER2 and CXCR4 at transcriptional level. Because PA did not downregulate HER2 and CXCR4 expression by enhancing its degradation, we investigated whether suppression occurred at transcriptional level using RT-PCR and real-time PCR. Cells were treated with PA for the indicated concentrations and then mRNA levels of HER2 and CXCR4 were examined. As shown in Fig. 3A and B, downregulation of HER2 and CXCR4 mRNA levels was detected in a dose-dependent manner.
PA inhibits constitutive activation of NF-κB in HER2 overexpression breast cancer cells. NF-κB activity is closely related to modulation of critical genes involved in cancer metastasis (17). There is a positive correlation between HER2 overexpression and constitutive activation of NF-κB in breast cancer cells (18,19). Also, the extracellular signal-activated transcription factor NF-κB regulates the expression of the chemokine receptor CXCR4, which has recently been implicated in organ-specific metastasis of breast cancer cells (20). Therefore, we tried to determine whether the PA exerts its effect on CXCR4 by suppressing NF-κB activation. We performed a DNA-binding assay to explore the effect of PA on constitutive NF-κB activation in SKBR3 cells, and demonstrated that PA treatment suppressed NF-κB activation in a dose-dependent manner (Fig. 3C).

PA downregulates the MAPK/ERK pathway. Because PA downregulates both HER2 and CXCR4, we focused on further signaling pathways related to these receptors. Various signaling pathways have been implicated in CXCR4 and HER2, particularly MAPKs (21-24). Therefore, we identified the MAPK signaling pathway involved in HER2 and CXCR4, utilizing specific kinase inhibitors for ERK (PD98059), JNK (SP600125), p38 (SB203580) and PI3K (LY294002). Fig. 4A shows that PD98059, SP600125, LY294002 and PA strongly inhibited the expression of HER2 and CXCR4 in SKBR3 cells. Moreover, PA inhibited the phosphorylation of ERK1/2 in a dose-dependent manner (Fig. 4B).

We confirmed upregulation of ERK-MAPK-dependent HER2 and CXCR4 by treating SKBR3 cells with ERK1/2 siRNA. After transfection with ERK1/2 siRNA for 24 h, the
expression of ERK1/2 and p-ERK1/2 was abolished (Fig. 4C), and cells transfected with ERK1/2 siRNA did not show a change in HER2 and CXCR4 expression levels. Together, these data suggest that the underlying mechanism by which PA suppresses HER2 and CXCR4 in SKBR3 breast cancer cells is inhibition of the ERK-MAPK pathway.
PA suppresses CXCL12-induced invasion by HER2-positive SKBR3 breast cancer cells. The CXCL12/CXCR4 biological axis also modulates the chemotactic motility of cancer cells. We next evaluated whether PA inhibited this process. As shown in Fig. 5A, CXCL12 increased significantly the number of invasive cells. On the contrary, PA abrogated the invasiveness of SKBR3 cells induced by CXCL12.

Discussion

The mortality rate of metastatic breast cancer is higher than that of the primary tumor. Recently several clinical studies have demonstrated that overexpression of HER2 is associated with increased proliferation, invasiveness and a poor prognosis (2,25,26). Further, CXCR4 expression is strongly correlated...
with the degree of metastasis to various organs in breast cancers (27,28). A previous study reported that expression of CXCR4 was associated with invasiveness and migration, as well as HER2 overexpression (29). Other researchers also suggested that HER2 and CXCR4 expression was related to a poor survival rate in primary breast tumor tissues (5). Furthermore, regulation of CXCR4 by estrogens acting through ER may play an important role in determining the metastasis of breast cancer cells.

Our previous study reported that PA inhibited CXCR4 via the NF-κB pathway in breast cancer cells (9). However, the signaling pathway involved in CXCR4 inhibition was not fully investigated, neither the relationship between HER2 and CXCR4. Therefore we explored the mechanism by which PA suppresses metastasis of breast cancer cells expressing both HER2 and CXCR4. We found that PA not only showed a significant inhibitory effect on HER2 and CXCR4 expression, but also regulated ERK and NF-κB activation in SKBR3 cell lines.

Recent reports have shown that degradation of CXCR4 involves atrophin-interacting protein (AIP)-4 mediated ubiquitination and degradation (15). Moreover, degradation of HER2 is due to ubiquitination of the receptor that targets the protein for proteasomal and lysosomal degradation (16). However, our data indicate that PA does not downregulate HER2 and CXCR4 through these mechanisms. This suggests that PA downregulates the expression of HER2 and CXCR4 at the transcriptional level.

Nonetheless, the exact mechanism of HER2 and CXCR4 by PA is not fully understood. The ERK signaling pathway has a central action in the regulation of various biological process, such as proliferation, survival and metastasis (30). Pharmacological inhibition of ERK signaling has been demonstrated to reduce tumor growth in various human cancers (31,32). One study suggested that multiple signaling pathways, including ERK, act as downstream effectors to promote the invasive potential of breast cancer cells (33). Also, activation of CXCR4 leads to activation of multiple signaling pathways, including ERK, in several cell types (34). Therefore, it is possible that SKBR3 cells acquire invasive capacity through induction of HER2 and CXCR4 by constitutive activation of the ERK signaling pathway.

Clinically, NF-κB expression is strongly correlated with expression of HER2 and CXCR4 in metastatic breast cancer cells (20,35). Recent studies have shown that expression of CXCR4 in cancer cells is dependent on the MEK/ERK signaling cascade and NF-κB activation (36). Also, NF-κB is constitutively active in breast cancer cells, and this is correlated with increased expression of HER2 (37). This study supports these findings in terms of showing that PA inhibits the ERK signaling pathway and reduces NF-κB activation.

Previous studies showed that CXCL12, the ligand for the CXCR4 receptor, is upregulated by estrogen in parental breast cancer cells (38). Estrogen upregulates the ligand in HER2 expressing breast cancer cells and increases cell migration (28). Moreover, the effect of estrogen in high HER2 expressing, estrogen receptor (ER)-positive cells suggests that ER and HER2 affect CXCR4 levels and upregulate CXCL12, which is critical for activating the pathway associated with invasiveness (39). Hence, we determined whether PA suppressed the invasion of HER2-positive breast cancer cells induced by CXCL12. Our result showed that PA suppressed CXCL12-induced breast cancer cell invasion.

In addition, PA induced apoptosis in cells from patients with chronic myeloid leukemia exhibiting different drug resistance profile (8). Also, PA may overcome multi-drug resistance mediated by overexpression of anti-apoptotic bcI-2 proteins (40). It has been reported that bioactive natural products such as resveratrol, quercetin and catechin will become major sources of therapeutic agents with breast cancer combination therapy (41). Therefore, PA may be used as a potential anticancer drug to facilitate earlier treatment in patients with breast cancers.

In conclusion, our data demonstrated that PA suppressed HER2 and CXCR4 expression in HER2-positive breast cancer cells. This result was correlated with inactivation of the ERK pathway and suppression of constitutive NF-κB activation.

Further studies with in vivo model are needed to manifest the relevance of these results to cancer treatment.

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References


