Osteoblast-derived Oxysterol Is a Migration-inducing Factor for Human Breast Cancer Cells*

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Bone metastasis is the major reason for death caused by breast cancer. We used human breast cancer (MCF-7) cells that are poorly metastatic but show highly inducible migration to determine bone-derived factors that induce migration of initially non-disseminating breast cancer cells. We have found that a lipid fraction from human osteoblast-like MG63 cell-conditioned medium (MG63CM) contains a migration-inducing factor for MCF-7 cells. In this fraction, we have identified oxysterol (OS) as a lipid mediator for tumor cell migration. In MCF-7 cells, insulin-like growth factor 1 elevates the expression of OS-binding protein-related protein 7. Binding of OS to OS-binding protein or OS-binding protein-related protein is known to trigger elevation of sphingomyelin, a sphingolipid that organizes lipid microdomains in the cell membrane. In MCF-7 cells, OS increases the intracellular concentration of sphingomyelin and other phospholipids and induces the translocation of the small GTPase p21Ras to GM1- and cholesterol-rich membrane areas. The induction of migration by MG63CM is prevented by incubation of MG63 cells with mevinolin, a statin-type cholesterol biosynthesis inhibitor that depletes the conditioned medium of OS. Osteoblast-derived OS may, thus, be a yet unrecognized lipid mediator for bone metastasis of breast cancer and a new target for anti-metastasis chemotherapy with statins.

The analysis of cell signaling for bone metastasis of breast cancer cells is crucial for the development of novel approaches for treatment of cancer. A great body of work has been done on the identification of proteinogenic growth and migration-inducing factors (1, 2). Bone-derived IGF-1 and chemokines play central roles as trophic factors that attract breast cancer cells to bone tissue (3–5). Only little is known, however, about the significance of lipid mediators in this process. Numerous studies show that the response of cancer cells to growth or migration-inducing factors critically depends on the lipid microenvironment of their receptors (6, 7). For example, incubation with cholesterol has been found to significantly enhance IGF-1 and chemokine-dependent migration of human breast cancer MCF-7 cells toward the source of the growth or migration-inducing factor (8). These studies have also shown that the use of MCF-7 cells with a low degree of intrinsically active cell migration allows for the sensitive determination of exogenous factors that induce migration by alteration of the membrane lipid composition. The potential of these factors to induce migration in poorly metastatic MCF-7 cells indicates that they are likely to turn a non-disseminating into a disseminating tumor.

Lipids of the plasma membrane, in particular cholesterol and sphingolipids (i.e. sphingomyelin, GM1) are clustered in lipid microdomains that are known to associate with growth factor or chemokine receptors (9–11). The tight packing between cholesterol and sphingolipids has been shown to organize the formation of membrane lipid microdomains (12). This observation indicates that up-regulation of intracellular cholesterol and sphingomyelin may enhance lipid microdomain formation and in turn assist or amplify growth factor or chemokine-dependent cell signaling for breast cancer cell migration (6, 8). It has also been shown that lipid domains in the plasma membrane recruit small GTP-binding proteins (e.g. Ras, Rho) and growth factor or chemokine receptors to the leading edge of migrating cancer cells (8, 13, 14). The association of these proteins in cholesterol/sphingolipid-rich membrane domains may, thus, form a cell signaling platform or module that triggers or controls the induction of tumor cell migration (3, 6, 8, 15).

The cholesterol and sphingomyelin biosynthesis is tightly regulated by soluble lipids, in particular steroids. We have identified hydroxylated cholesterol or oxysterol (OS) in medium that has been conditioned by osteoblast-like MG63 cells. OS is a soluble and cell-permeable form of cholesterol that has been reported to modulate both the intracellular cholesterol and sphingomyelin concentration in a variety of cell species (16–21). To our knowledge, however, this is the first study that reports OS-induced migration of tumor cells. We have developed an improved drop agarose migration assay that allows us to determine the expression of OS-binding proteins and microdomain-associated lipids specifically in migrating tumor cells. This assay has been used to visualize the effect of OS on lamellipodia formation at the leading edge of MCF-7 cells. Our analyses show that osteoblast-derived OS may be an important lipid mediator for the navigation of migrating breast cancer cells to bone tissue. Furthermore, we discuss results that suggest a therapeutic potential of cholesterol biosynthesis inhibitors for anti-metastasis therapy.

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‡ The abbreviations used are: IGF-1, insulin-like growth factor 1; IGF-1R, IGF-1 receptor; EMEM, minimum essential Eagle’s medium with Earle’s salts; HPTLC, high performance thin layer chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; MAPK, mitogen-activated protein kinase; OS, 25-hydroxycholesterol; OSBP, oxysterol-binding protein; ORP, oxysterol-binding protein-related protein; Rock, Rho-associated protein kinase; HPS, heat shock 70-kDa protein 8 isoform 1; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; RT, reverse transcription; CM, conditioned medium; PBS, phosphate-buffered saline; GM1, Galβ1,3GalNAcβ1,4NeuAcα2,3Galβ1,4Glcφβ1,1-ceramide.

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EXPERIMENTAL PROCEDURES

Materials—Human breast cancer MCF-7 (ATCC HTB-22) and human osteosarcoma MG63 (ATCC CRL-1427) cells were purchased from the American Type Culture Collection (Manassas, VA). EMEM medium was from Invitrogen. Dextran-coated charcoal, LongR3-IGF-1 peptide analog, low melt agarose, wortmannin, arachidonic acid, acetylsalicylic acid, filipin, sphingomyelin, cholesterol, and 25-hydroxycholesterol were from Sigma. Polyclonal rabbit IgG against IGF-1 receptor β-subunit and monoclonal mouse IgG against RhoA IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal IgG against anti-phospho-Thr-202/Tyr-204-p42/44-MAPK and rabbit polyclonal IgG against p21Ras was from Cell Signaling (Beverly, MA). Y-27632, PD98059, prostatlandins, and rabbit polyclonal IgG against p21Ras was from Calbiochem. Alexa 488-labeled goat anti-rabbit IgG and Alexa 594-labeled cholera toxin B were from Molecular Probes (Eugene, OR). HPTLC plates were obtained from Merck. All other chemicals were analytical grade or higher, and organic solvents were freshly re-distilled before use.

Tumor Cell Culture and Medium Conditioning—MCF-7 and MG63 cells were propagated in a humidified incubator at 5% CO2 using EMEM medium with or without phenol red or fetal bovine serum. Medium was conditioned by incubation of MG63 or MCF-7 cells with serum-free EMEM for 48 h followed by filtration of the conditioned medium through a 0.2-μm filter membrane. To remove lipids, the filtered medium was incubated with dextran-coated charcoal for 2 h, and the supernatant was used for incubation of MCF-7 cells as described (22). In one series of experiments, the MG63-conditioned medium was supplemented with 1 μg/ml anti-IGF-1R antibody to prevent signaling via the IGF-1-induced pathway following a previously published procedure (23).

Migration Assays—The soft agarose drop migration assay was performed following the procedure introduced by Varani et al. (24). MCF-7 cells (10^4–10^5 cells) in 10 μl of serum-free medium were mixed with 10 μl of 0.6% low melting point agarose liquefied in serum-free medium and then dropped onto the pre-cooled surface of a 6-well dish. The agarose was allowed to solidify for 15 min at 4 °C and then covered with 0.5 ml of serum-free medium. After incubation at 37 °C for 5 h, growth or migration factors were added, and the assay mixture was incubated for another 24 h at 37 °C. Cells that migrated out of the rim of the drop were counted for quantitative determination of migrating cells. A modified drop agarose migration assay (co-culture migration assay) was designed to determine the trophic effect of growth or migration factors on MCF-7 cells that were released from a local source over a period of time. A dialysis tube containing MG63 cells was placed into a 60-mm dish before adding the agarose drop with MCF-7 cells to the dish. Alternatively, the dialysis tube was filled with medium that has been supplemented with migration or growth factors. After incubation of the cells, the agarose was removed, and the tumor cells were fixed and analyzed by immunocytochemistry. Statistically significant differences in data sets (p < 0.05) were assessed from at least 10 independent experiments using one way analysis of variance.

Lipid Preparation and Analysis—Lipids were extracted from conditioned medium or agarose-embedded cells in 500 μl of deionized water by thoroughly mixing with an equal volume of CHCl3/CH3OH (1:1, by volume). The lower (organic) phase was evaporated to dryness with a stream of nitrogen, and the residue was dissolved in CHCl3/CH3OH (1:1, by volume) for further analysis by HPTLC and followed by mass spectrometry or preparative chromatography of the migration-inducing lipid fraction. HPTLC of cholesterol, ceramide, and prostatlandins was performed in the chloroform/methanol/formic acid (50:50:1, by volume) solvent system and visualized by staining with 3% cupric acetate in 8% phosphoric acid and compared to standard lipids as described (25). Phospholipids, including sphingomyelin, were resolved by HPTLC in CHCl3/EtOH/H2O/triethylamine (35:30:7.5:3.5, by volume) and specifically stained using a modified Dittmer’s (CuSO4/HSO4/molybdate) reagent (26). Cholesterol and oxysterol were separated using HPTLC in CHCl3/CH3OH (95:5, by volume) and visualized by staining with 5% ammonium molybdate in 10% sulfuric acid as described (27). For preparative HPTLC, individual lipid fractions that co-migrated with cupric acetate-stained bands were scraped from the silica plate and extracted with CHCl3/CH3OH (1:1, by volume). After removal of the silica particles by centrifugation, the supernatant was dried, and the residue was redissolved in serum-free EMEM medium or organic solvent for incubation of MCF-7 cells or analysis by mass spectrometry, respectively. Lipid extracts (in CHCl3/CH3OH, 1:1 by volume) and matrix (2,5-dihydroxybenzoic acid in H2O/ EtOH, 9:1 by volume) were co-crystallized on the target in a dried-droplet application. MALDI-TOF mass spectra were acquired in positive ion mode on a Voyager DE STR (Applied Biosystems, Foster City, CA) equipped with a UV laser (λ = 337 nm). The assignment of mass peaks to lipid species and their fragments followed previously published procedures (28).

SDS-PAGE and Proteomics Analysis—Protein was extracted from free or agarose-embedded MCF-7 cells using SDS-sample buffer according to Laemmli (29) for SDS-PAGE or sample buffer for isoelectric focusing (9 M urea, 4% CHAPS, 10 mM dithiothreitol, 0.2% (w/v) Bio-Lytes 3/10). Two-dimensional gel electrophoresis was performed with pre-cast immobilized pH gradient gels following the procedures given by the manufacturer (Bio-Rad). The SDS gels were stained with Coomassie, and stained bands/proteins were cut out for tryptic digestion and proteomics analysis. Proteins were digested (trypsin, sequencing grade, Promega, Madison, WI). The tryptic digests were extracted from the gel, dried, and dissolved in 50% acetonitrile in trifluoroacetic acid (0.1%). An aliquot was mixed on target with matrix (o-cyano-4-hydroxycinnamic acid in 50% acetonitrile in trifluoroacetic acid (0.1%)). MALDI-TOF mass spectra were acquired as described for the analysis of lipids, and potential peptide sequences were identified using the PROFOUND and MASCOT programs for mass fingerprinting.

RT-PCR—Total RNA was prepared from free or agarose-embedded MCF-7 cells using the Trizol method according to the manufacturer’s (Invitrogen) protocol. An aliquot (0.6–1.0 μg of RNA) was used for RT-PCR with the ThermoScript™RT-PCR system following the supplier’s (Invitrogen) instructions. PCR was carried out by applying 35 cycles with various amounts of first strand cDNA template (equivalent to 0.05–0.2 μg of RNA) and 20 pmol of OSEB-related protein 7 (ORP7)-specific sense (ORP7a 5'-agctgtgtagctagactcgtg-3') and antisense (ORP7a 5'-catgtcctagctagctcgtg-3') oligonucleotide primers.

Immunofluorescence Microscopy—The agarose drop migration assay was performed on cover slips, and the drop was removed after incubation with migration-inducing factors. Migrating cells attached to the cover slips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Unspecific binding sites were saturated by incubation with 3% ovalbumin in PBS for 1 h at 37 °C. The cover slips were then incubated with 5 μg/ml primary antibody in 0.1% ovalbumin, PBS followed by incubation with the appropriate fluorescence-labeled secondary antibody for 2 h at 37 °C. After staining, the antibody-bound GM1 was stained by incubation with 1 μg/ml Alexa 594-labeled cholera toxin subunit B in 0.1% ovalbumin, PBS for 1 h at 37 °C. Cholesterol was stained by incubation with filipin at a concentration of 50 μg/ml in 0.1% ovalbumin, PBS for 1 h at room temperature as described previously (30, 31).

RESULTS

MG63-conditioned Medium Induces Migration of MCF-7 Cells—The observation that 50% of primary breast tumors metastasize to bone suggests the activity of bone-derived growth factors on the migration of breast cancer cells. We assayed the degree of inducible migration of human breast cancer (MCF-7) cells using a soft agarose drop migration assay (Fig. 1A). Medium was conditioned by incubating human osteoblast-like MG63 cells with serum-free EMEM. The conditioned medium (MG63CM) was added to MCF-7 cells that had been embedded into agarose drops, and migration was determined after incubation for 24 h. As shown in Fig. 1A, cells migrating out of the agarose drop surrounded its edge and were also attached to the dish surface beneath the drop (Fig. 1B). The surrounding cells were counted for quantitative determination of migration (Fig. 2). The results were consistent with the number of migrating cells that stayed attached to the dish surface after the drop was removed (Fig. 1B).

The effects of serum or IGF-1, a well known migration-inducing factor for breast cancer cells (3–5), on MCF-7 cell migration were determined as positive controls. Incubation of the agarose drop in serum-free medium resulted in 30% of the migration observed with serum-containing medium (Fig. 2). Supplementation of serum-free medium with LongR3-IGF-1 peptide analog (100 ng/ml) restored migration to 55% of the serum value. MG63CM also restored migration of MCF-7 cells to 60% of the serum value (Fig. 2). This effect was specific for MG63 cells since medium that was self-conditioned by MCF-7 cells only slightly induced migration. We tested whether IGF-1
is a migration-inducing factor in MG63CM since it has been reported that osteoblasts secrete IGF-1 to the medium (32). An antibody against IGF-1R, however, did not suppress the migration-inducing effect of MG63CM, whereas the effect of LongR3-IGF-1 was completely inhibited (Fig. 2). These results indicated that MG63CM contained a migration-inducing factor that was different from IGF-1. Migration was also not affected by the presence of phenol red in the conditioned medium or by the addition of estrogen (not shown), indicating that the migration-inducing effect of MG63CM is independent of estrogen.

**Oxysterol in MG63-conditioned Medium Is a Migration-inducing Factor**—MG63-conditioned medium was treated with charcoal to determine whether the migration-inducing factor was a lipid or protein. As shown in Fig. 3, charcoal treatment of MG63CM suppressed MCF-7 migration to the value obtained with serum-free medium, which suggested that the MG63-derived migration-inducing factor was a lipid. We isolated four different lipid fractions from MG63CM using organic extraction and preparative HPTLC (Fig. 4A). The migration-inducing effect of each lipid fraction was determined after back-addition to serum-free or charcoal-treated MG63CM in the agarose drop assay. Only the lipid fraction termed L1 (Fig. 3 and lane 11, Fig. 4A) restored migration of MCF-7 cells to 85% of the value obtained with MG63CM.

A mass spectrometric (MALDI-TOF) analysis of L1 revealed mass peaks that corresponded to the sodium salts of prostaglandins and cholesterol derivatives (Fig. 5A). The respective mass peaks were also found with prostaglandin, cholesterol, or OS standards, suggesting that these compounds were present in L1. The biologically active prostaglandins PGE\(_2\) (0.5 \(\mu\)M), PGB\(_2\) (0.5 \(\mu\)M), and PGD\(_2\) (0.5 \(\mu\)M, not shown) or their precursor arachidonic acid (30 \(\mu\)M), however, did not induce migration of MCF-7 cells (Fig. 6). These results were consistent with the observation that incubation of MG63 or MCF-7 cells with 200 \(\mu\)M cyclooxygenase inhibitor acetylsalicylic acid did not reduce migration that was induced by MG63CM (Fig. 6).

A mass peak at 425 indicated the presence of the sodium salt of OS in L1. The concentration of OS extracted from MG63CM was quantified by comparison to the mass peak (not shown) or staining intensity of 25-hydroxycholesterol, a biologically active OS species that was added to serum-free medium at different concentrations and then re-extracted with organic solvent for mass spectrometry and quantitative HPTLC (Fig. 4B). The OS concentration in MG63CM was 0.8 \(\pm\) 0.2 \(\mu\)M. The exact structure of the hydroxylated cholesterol was not determined yet. A migration assay, however, showed that 1–10 \(\mu\)M 25-hydroxycholesterol in serum-free medium restored migration that was induced by MG63CM (Fig. 6).

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experiments with standard variances indicated as were determined as the averages from counts in at least 10 independent rum-incubated cells as described in the legend for Fig. 2. All values were incubated with or without serum or MG63CM or lipid fractions induced migration of MCF-7 cells.

Agarose-embedded MCF-7 cells (not shown). We did not induce migration of MCF-7 cells (Fig. 3). We also tested for the migration-inducing effect of cholesterol following a procedure that has previously been reported to induce lamellipodia protrusion and migration in MCF-7 cells (8). However, we were not able to detect a direct effect of cholesterol added to serum-free medium on cell migration of agarose-embedded MCF-7 cells (not shown).

**IGF-1 Elevates the Expression of ORP7 in MCF-7 Cells**—To identify a potential alteration of proteins by incubation with OS we determined the protein expression patterns in migrating MCF-7 cells that were incubated with IGF-1, serum, or MG63CM. As shown in Fig. 7, protein was extracted from cells that migrated out of the agarose drop and analyzed by two-dimensional gel electrophoresis. Coomassie-stained protein fractions between 40 and 90 kDa were cut out of the gel and digested with trypsin, and the tryptic fragments were analyzed by mass spectrometry. As shown in Table I, of 17 tryptic fragments from a 85-kDa protein fraction 8 could be assigned to the fingerprint that was only found when MCF-7 cells were incubated with IGF-1, serum, or MG63CM. As shown in Fig. 7, non-reducing and reducing electrophoretic analysis of the OS lipid fraction L1 from mevinolin-treated MG63 cells as compared with MG63CM L1 from untreated cells.

**IGF-1 and OS Induce Migration via Different Cell Signaling Pathways**—Cell signaling for the migration of breast cancer cells relies critically on signal transduction via the small GTPase families Ras and Rho (14, 35). Membrane recruitment and activation of Ras is required for the induction of the IGF-1R-to-MAPK signaling pathway (35). Rho activates the Rho-associated protein kinase (Rock) pathway for rearrangement of actin and myosin filaments (35–37). As shown in Fig. 8A, inhibition of MAPK phosphorylation by the MAPK kinase inhibitor PD98059 or the phosphatidylinositol 3-kinase inhibitor wortmannin prevented IGF-1-induced migration of MCF-7 cells. In contrast to IGF-1, the migration-inducing effect of MG63CM was not significantly affected by PD98059 or wortmannin. These results indicated that IGF-1 and OS induced migration by two independent cell signaling pathways. The IGF-1 signal was transduced by the IGF-1R-to-MAPK and phosphatidylinositol 3-kinase pathway, whereas OS appeared to activate a different pathway. This assumption was corroborated by the observation that serum and IGF-1, but not MG63CM, elevated the degree of p42/44-MAPK phosphorylation in MCF-7 cells (Fig. 8B). Migration induced by IGF-1 as well as MG63CM and OS, however, was completely inhibited by Y-27632, a specific inhibitor of p160Rock and Rock II (Fig. 8C).
FIG. 5. MALDI-TOF mass spectrometric analysis of lipid fraction L1 from MG63-conditioned medium. Lipid fraction L1 was isolated by HPTLC from MG63CM as shown in Fig. 4. Lipids were co-crystallized with 2,5-dihydroxybenzoic acid, used as a matrix, and MALDI-TOF mass spectra were acquired in positive ion mode. Labeled peaks correspond to the masses of sodium salts of the lipids indicated in the figure. A, without mevinolin treatment of MG63 cells. B, with mevinolin treatment of MG63 cells. PGE$_2$/D$_2$, prostaglandins E$_2$/D$_2$. 
Fig. 6. Effects of prostaglandins on migration of MCF-7 cells. Agarose-embedded MCF-7 cells were incubated with or without prostaglandin PGB$_2$, PGE$_2$, arachidonic acid, or acetyl salicylic acid. 100% migration has been calculated from serum-incubated cells as described in the legend for Fig. 2. All values were determined as the averages from counts in at least 5 independent experiments with standard variations indicated as line bars. PGB$_2$/PGE$_2$, prostaglandins B$_2$/E$_2$.

8A). This result suggested that IGF-1 as well as OS triggered RhoA-to-Rock signaling. Fig. 8B verifies that p21Ras as well as RhoA were expressed in MCF-7.

OS Alters the Sphingomyelin and Cholesterol Composition in MCF-7 Cells—OS has been reported to regulate the intracellular concentration of cholesterol and sphingomyelin (16–21), two membrane lipids that critically affect the formation of lipid microdomains on the cell surface (9–11). The effect of serum, IGF-1, MG63CM, or OS on the lipid composition and of MCF-7 cells was determined by organic extraction and quantitative HPTLC. As shown in Fig. 9A, extraction and analysis of intracellular lipids from cells embedded in agarose drops revealed that after incubation with serum (lane 5), MG63CM (lane 7), or OS (lane 8), the level of MCF-7-bound sphingomyelin was almost 2-fold higher than with serum-free medium (lane 4). Elevation of sphingomyelin was less when MCF-7 cells were incubated with IGF-1 (lane 6). As shown in Table II, however, these effects were not specific for sphingomyelin since the concentration of sphingomyelin relative to that of other phospholipids, in particular phosphatidylcholine, was not altered by incubation with serum, MG63CM, or OS.

We then determined the effect of serum, IGF-1, MG63CM, or OS on the concentration of cholesterol in agarose-embedded MCF-7 cells using quantitative HPTLC. The cholesterol concentration was elevated from 5 ± 1 nmol/10$^6$ cells in serum-free medium (lane 2 in Fig. 9B) to 22 ± 4 nmol/10$^6$ cells in cells incubated with serum (lane 3). The elevation of cholesterol was lower in OS-incubated cells (11 ± 2 nmol/10$^6$ cells, lane 6), and it was only slightly elevated when cells were incubated with IGF-1 (7 ± 2 nmol/10$^6$ cells, lane 4). These results suggested that incubation with serum, conditioned medium, or OS elevated sphingomyelin and/or cholesterol, whereas IGF-1 did not significantly affect the lipid composition of MCF-7 cells. Alteration of the lipid composition, however, was not specific for sphingomyelin or cholesterol but involved a variety of phospholipids as well.

OS Attracts MCF-7 Cells and Promotes Membrane Translocation of p21Ras—The OS-induced elevation of intracellular cholesterol and sphingomyelin prompted us to investigate the formation of lipid-enriched domains on the surface of migrating MCF-7 cells. Cholesterol, sphingomyelin, and GM1 are known to cluster in microdomains or lipid rafts and have been shown to co-localize with IGF-1R, other growth factor and chemokine receptors, and small GTPases (8–10). We analyzed the formation of GM1/cholesterol-enriched membrane areas and their co-localization with p21Ras after OS incubation using fluorescence microscopy. We used a co-culture migration assay with MG63 and MCF-7 cells to determine the relative position of the leading edge to the source of the migration factor (Fig. 10A). This assay was also used to determine the effect of a localized OS source on the formation of lipid-enriched membrane domains and their co-localization with p21Ras. Hoechst staining of migrating MCF-7 cells surrounding the agarose drop revealed that the number of cells on the side of the local OS source was higher than on the opposite side. In these cells binding of fluorescence-labeled cholera toxin B subunit to GM1 was used for the identification of GM1-rich membrane domains (8). Fig. 10B shows that in cells incubated with serum-free medium, GM1 was predominantly localized in vesicles throughout the cytosol or in the perinuclear region. Membrane protrusions or lamellipodia in migrating cells, however, did not show enhanced staining for GM1. In contrast, overnight incubation with a local bolus of OS resulted in distinct staining of GM1-rich areas in the plasma membrane at the leading edge of migrating tumor cells. We determined whether OS also induced the enrichment of cholesterol or p21Ras in or at the membrane. Indirect immunofluorescence microscopy showed that after incubation with OS a portion of p21Ras was distributed to the leading edge of the cell that also stained for GM1.
TABLE I
MALDI-TOF analysis of tryptic fragments from ORP7 and heat shock 70-kDa protein 8

Protein from agarose-embedded and IGF-1-treated MCF-7 cells was separated by two-dimensional gel electrophoresis as shown in Fig. 7B. Coomassie-stained protein spots were subjected to tryptic digestion, and the peptide fragments were analyzed by MALDI-TOF mass spectrometry. The table shows the assignment of the tryptic fragments of the ORP7 HSP8 spot (Fig. 7) to known peptide sequences in protein databases using the MASCOT and PROFOUND programs for fingerprint analyses.

| Amino acid position | Mass observed | Sequence |
|---------------------|---------------|----------|
| ORP7                |               |          |
| 628–636             | 1179.61       | FGDHF EWNK |
| 793–804             | 1439.82       | VMEENNI VHQA R |
| 829–842             | 1479.80       | AEPG YNMDA VLYW |
| 146–159             | 1537.75       | LDMPRGSFLSTAH R |
| 792–804             | 1567.75       | KVMENNIVHQA R |
| 448–463             | 1639.95       | CQQKGCVPGRPMGFPR |
| 310–325             | 1716.90       | VHISSLSVLA ALTMER (Ox) |
| 816–828             | 1823.89       | EWWYTNNTQWHLR |
| HSP8                |               |          |
| 459–469             | 1197.67       | FELTGFAPP R |
| 26–6                | 1228.63       | VEHIANQGNR |
| 237–246             | 1235.62       | MVNHF1AEFK |
| 300–311             | 1480.78       | ARPEELNADLFR |
| 37–49               | 1487.71       | TTPSYVAFTDTER |
| 221–236             | 1691.74       | STAGDTHLGGEDFDNR |
| 570–583             | 1716.86       | IILRCCNEIINWLDK |
| 328–342             | 1837.97       | LDEKQHHIVDVOSGTR |
| 310–155             | 1982.02       | TIVTAVVIPAYFNDSQR |

(with cholera toxin subunit B) and cholesterol (with filipin, Fig. 10B). The membrane translocation of GM1 or p21Ras was not found when MCF-7 cells were treated with mevinolin (not shown). Taken together, these results suggest that the chemotaxis induced by OS is concurrent with the translocation of p21Ras to cholesterol/sphingolipid-rich membrane areas at the leading edge of migrating tumor cells.

DISCUSSION

The analysis of trophic signals that control bone metastasis of breast cancer is crucial for the identification of new molecular targets for anti-metastasis therapy. We provide evidence for the first time that osteoblast-derived OS may play an important role for the migration of breast cancer cells toward bone tissue. The significance of OS for the regulation of sphingolipid and cholesterol metabolism has long been investigated and pharmacologically applied (16). The importance of this regulation for the induction of migration in cancer cells, however, has not been studied yet. Our results suggest that IGF-1 and OS may interact by promoting migration in tumor cells in a two-step process. In Step 1, systemic and bone-derived IGF-1 mobilizes the tumor cells and sustains the expression of OSBP or ORP in migrating cancer cells. This assumption is supported by the observation that IGF-1 elevates the ORP7 expression in migrating MCF-7 cells. Interestingly, a recent study shows that an OS-binding protein homologue (ORP4) is specifically up-regulated in metastasizing tumor cells (38, 39). In Step 2, an activated complex of OS and OSBP or ORP modulates lipid biosynthesis in the migrating tumor cells, which induces or enhances the formation of cholesterol/sphingolipid-rich membrane areas. These membrane domains may trigger or amplify the Ras-dependent cell signaling pathways for tumor cell migration toward bone tissue.

Our results indicate that this two-step signaling for tumor cell migration involves the IGF-1-dependent activation of MAPK and phosphatidylinositol 3-kinase. In Step 1, activation of MAPK may trigger or sustain the elevation of ORP7 or other OSBP or ORP. It is likely that in mammalian cells, various OSBPs or ORPs are elevated, which depends on the cell type and the binding specificity for particular OS isomers (39). This assumption is supported by studies showing that in yeast the expression of ORP homologues is up-regulated by MAPK and as part of the cell stress response (39). In MCF-7 cells, cell stress signals during agarose-embedding may trigger ORP7 elevation that is sustained in migrating cells by incubation with IGF-1. This suggestion is consistent with the observation that the expression of heat shock proteins, in particular HSP8, is also up-regulated in migrating cells.

Phosphatidylinositol 3-kinase, on the other hand, may par...
FIG. 9. Analysis of lipids from MCF-7 cells. Agarose-embedded MCF-7 cells were incubated with or without serum or MG63CM or OS. Cells remaining in the agarose drops were isolated by extraction with organic solvent, analyzed by HPTLC, and stained for phospholipids (A, lipid amount equivalent to 100,000 cells) or cholesterol (B, lipid amount equivalent to 20,000 cells). A, phospholipids. Lane 1, standard phospholipids group I (PLI, from bottom phosphatidylserine (PS), phosphatidylethanolamine (PE)); lane 2, standard phospholipids group II (PLII, from bottom to top, sphingomyelin (SM), phosphatidycholine (PC), phosphatidylinositol (PI), and cardiolipin (CL)); lane 3, standard sphingomyelin (SM); lane 4, phospholipids from agarose-embedded MCF-7 cells incubated without serum; lane 5, with serum; lane 6, with IGF-1; lane 7, with MG63CM; lane 8, with OS, B, cholesterol. Lane 1, standard cholesterol; lane 2, cholesterol from agarose-embedded MCF-7 cells incubated without serum; lane 3, with serum; lane 4, with IGF-1; lane 5, with MG63CM; lane 6, with OS.

TABLE II
Relative concentration of phospholipids in MCF-7 cells
Agarose-embedded MCF-7 cells were incubated with or without serum, IGF-1, MG63CM, or OS, and lipids were isolated, separated by HPTLC, and stained for phospholipids as described in the legend for Fig. 9. The relative concentrations of different phospholipid species was calculated from a densitometric analysis of the visualized lipid bands. The data were obtained from three independent experiments. SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; CL, cardiolipin.

|            | -serum | +serum | +IGF-1 | MG63CM | OS  |
|------------|--------|--------|--------|--------|-----|
| SM         | 18 ± 3 | 17 ± 3 | 16 ± 3 | 15 ± 3 | 21 ± 4 |
| PC         | 51 ± 10| 48 ± 10| 46 ± 9 | 54 ± 11| 57 ± 12|
| PI         | 10 ± 2 | 11 ± 2 | 7 ± 2  | 7 ± 2  | 4 ± 1 |
| PE         | 17 ± 3 | 19 ± 4 | 29 ± 6 | 18 ± 4 | 11 ± 2|
| CL         | 4 ± 1  | 5 ± 1  | 2 ± 1  | 6 ± 1  | 7 ± 2 |

The direct effect of OS on ORPs is consistent with the observation that OS-induced migration is not suppressed by MAPK kinase or phosphatidylinositol 3-kinase inhibitors. Binding of OS to ORPs has been shown to participate in the Golgi transport/anchoring and activation of ORPs (42–45). It has also been found that an activated, Golgi-localized OS-ORP complex modulates the biosynthesis or transport of sphingomyelin and cholesterol (16–21, 42–45). Our results are consistent with these reports in that OS elevates sphingomyelin. It should be noted, however, that in contrast to the previous reports our analyses revealed an OS-dependent elevation of cholesterol and a variety of phospholipids. This result indicates that the effect of OS on the sphingomyelin and cholesterol concentration may be cell-specific. In MCF-7 cells, OS appears to elevate phospholipids and cholesterol with less specificity for sphingomyelin than reported for other cell types (42–45). The effect of OS on cholesterol was verified by filipin staining of OS- incubated MCF-7 cells, which showed accumulation of cholesterol in lamellipodia or at the membranes of adjacent cells (not shown).

Hydroxylated cholesterols, in particular 25-hydroxycholesterol, have been described to trigger the biosynthesis of other lipid second messengers that may act as migration-inducing factors on breast cancer cells. In rat kidney and coronary artery endothelial cells, OS enhances eicosanoid production and prostaglandin synthesis (46–48). Interestingly, prostaglandins are known to be secreted by osteoblasts and have been discussed to promote breast cancer metastasis (49). The most prominent prostaglandins (PGD2, PGF2α, and PGE2), however, did not induce migration of MCF-7 cells. We also investigated the possibility of OS-induced migration due to enhanced prostaglandin biosynthesis in MCF-7 cells. Incubation of neither MG63 nor MCF-7 cells with the cyclooxygenase inhibitor aces- tylic acid prevented MG63CM from inducing migration. These results suggest that the migration-inducing effect of MG63CM or its active ingredient, OS, is not due to altered eicosanoid/prostaglandin biosynthesis but may result from altered membrane lipid composition in MCF-7 cells.

In MCF-7 cells OS-induced or enhanced formation of cholesteryl/sphingolipid-rich membrane areas may amplify the small GTPase-dependent activation of the Rho-associated kinase (Rok) cell signaling pathway for actin re-arrangement and lamellipodia protrusion at the leading edge of migrating tumor cells. This assumption is supported by the observation that the Rock inhibitor Y-27632 prevents OS-dependent lamellipodia formation and tumor cell migration. It has not been thoroughly investigated yet how the metabolic regulation of cholesterol or sphingolipids modulates the formation of membrane domains that facilitate lamellipodia protrusion or migration of tumor cells. It is also not clear how the membrane lipid metabolism may affect the activity of other Rho-related GTPases, e.g., Rac1, that are essential for membrane protrusion (50–52). MCF-7 cells have been shown to respond to exogenous cholesterol by formation of lamellipodia at the leading edge of the cell (8). It is, thus, likely that elevation of intracellular sphingomyelin and cholesterol by incubation with OS will also result in enhanced protrusion of lamellipodia. Induction of migration may rely on the increased translocation of GTPases to GM1 and cholesterol-rich membrane areas at the leading edge of the cell. This assumption is supported by the observation that serum-starved MCF-7 cells, although phenotypically similar to those incubated with MG63CM or OS, do not show increased translocation of p21Ras to GM1 and cholesterol-rich membrane areas.

We have shown that the migration-inducing effect of MG63CM is obliterated by treatment of MG63 cells with
mevinolin, an inhibitor of hydroxymethylglutaryl-CoA reductase. Mevinolin treatment results in the disappearance of mass peaks for cholesterol and OS in the migration-inducing lipid fraction of MG63CM. These results strongly suggest that mevinolin or other statins may be pharmacologically useful to inhibit the cholesterol or OS biosynthesis and in turn may reduce OS-induced tumor cell migration or metastasis. This suggestion is consistent with a recent study showing that cerivastatin inhibits Rho-mediated migration of highly metastatic breast cancer cells (53). The authors discuss that this effect is caused by the inhibition of Rho farnesylation/prenylation, which is necessary for Rho translocation from the cytosol to the plasma membrane. Alternatively, statins may reduce tumor cell migration by the inhibition of cholesterol/sphingolipid-dependent membrane formation, which will also affect the translocation and activity of small GTPases. In future studies, we will further characterize OS-induced membrane domains and determine whether they are equivalent to detergent-insoluble membrane fractions that are known as lipid rafts. We will also determine the exact structure of migration-inducing, bone-derived OS-isomers, analyze the gene expression of various OSBP/ORP species in breast cancer cells, and investigate the significance of statins for the inhibition of OS-induced migration and metastasis in animal studies. Taken together, our results strongly suggest that OS is a novel migration-inducing factor and that an understanding of the mechanism for OS-induced breast cancer cell migration will be of significance for the development of new anti-cancer drugs.

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