Lipids isolated from bone induce the migration of human breast cancer cells

Jeane Silva,* Somsankar Dasgupta,* Guanghu Wang,* Kannan Krishnamurthy,* Edmond Ritter,† and Erhard Bieberich[1,*]

Institute of Molecular Medicine and Genetics,* and Department of Surgery,† School of Medicine, Medical College of Georgia, Augusta, GA 30912

Abstract **Bone is the most common site to which breast cancer cells metastasize. We found that osteoblast-like MG63 cells and human bone tissue contain the bile acid salt sodium deoxycholate (DC). MG63 cells take up and accumulate DC from the medium, suggesting that the bone-derived DC originates from serum. DC released from MG63 cells or bone tissue promotes cell survival and induces the migration of metastatic human breast cancer MDA-MB-231 cells. The bile acid receptor farnesoid X receptor (FXR) antagonist Z-guggulsterone prevents the migration of these cells and induces apoptosis. DC increases the gene expression of FXR and induces its translocation to the nucleus of MDA-MB-231 cells. Nuclear translocation of FXR is concurrent with the increase of urokinase-type plasminogen activator (uPA) and the formation of F-actin, two factors critical for the migration of breast cancer cells. Our results suggest a novel mechanism by which DC-induced increase of uPA and binding to the uPA receptor of the same breast cancer cell self-propel its migration and metastasis to the bone.**—Silva, J., S. Dasgupta, G. Wang, K. Krishnamurthy, E. Ritter, and E. Bieberich. **Lipids isolated from bone induce the migration of human breast cancer cells.** ***J. Lipid Res.*** **2006.** 47: **724–733.**

Supplementary key words bile acids • deoxycholate • farnesoid X receptor • urokinase-type plasminogen activator

The migration of cancer cells is a key factor in metastasis, which is a multistep event that involves the interaction of host and tumor tissue. Bone is the most common site to which breast cancer cells metastasize (1, 2). The mechanism underlying this specific metastatic behavior is not completely understood. Recently, we reported that oxygenated derivatives of cholesterol (oxysterols) synthesized and released by human osteoblast-like MG63 cells induce the migration of nonmetastatic human breast cancer MCF-7 cells (3). Bile acids, another species of oxygenated cholesterol, have long been implicated in the tumorigenesis of colorectal cancer (4, 5). Recent studies have shown that an increased concentration of bile acids in breast cyst fluid is indicative of a higher risk of developing breast cancer (6, 7). It remained to be elucidated, however, which cell signaling pathways for tumorigenesis and breast cancer cell migration are triggered by bile acids.

The secondary bile acid deoxycholic acid or its salt deoxycholate (DC) is synthesized by intestinal bacteria and transported to the liver and other tissues by the serum (8–11). The specific enrichment of deoxycholic acid and other bile acids in breast cyst fluid shows that tissues other than liver can take up and accumulate bile acids from the serum (7, 12, 13). Bile acids bind to the nuclear receptor farnesoid X receptor (FXR) and activate a variety of genes involved in cholesterol metabolism and transport (14–17). FXR is expressed in colorectal tumor cells; however, it is not clear how bile acid-induced activation of FXR triggers cancerogenesis (18). In this study, we show for the first time that bone tissue and MG63 cells contain DC. We provide evidence that DC is taken up from the medium and can be released at a concentration that induces the migration of MDA-MB-231 cells. We also show for the first time that MDA-MB-231 cells express FXR, which allows for DC-induced receptor activation. Our results suggest that the release of DC from bone as well as the DC-induced migration of human breast cancer cells may contribute to the migration and metastasis of breast cancer cells to bone or other tissues that contain or release bile acids.

EXPERIMENTAL PROCEDURES

Materials

MDA-MB-231 human breast cancer cells (ATCC HTB-26) were a generous gift from Dr. Yoshihiko Takeda (Medical College of

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Abbreviations: CDC, chenodeoxycholate; DC, deoxycholate; FXR, farnesoid X receptor; HPTLC, high-performance thin-layer chromatography; MG63-CM, MG63-conditioned medium; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.

[1] To whom correspondence should be addressed.
e-mail: ebieberich@mail.mcg.edu

Georgia). Human osteoblast-like MG63 (ATCC CRL-1427) cells were purchased from the American Type Culture Collection (Manassas, VA). DMEM, 0.25% trypsin-ETDA, and antibiotic-antimycotic solution was from Cellgro (Herndon, VA), and FBS was from Atlanta Biologicals, Inc. (Lawrenceville, GA). Vybrant-CM diI cell-labeling solution, Alexa Fluor 647 phalloidin, and Alexa Fluor 594 wheat germ agglutinin were purchased from Molecular Probes (Eugene, OR). Polyclonal rabbit IgG against FXR (sc-13063) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-urokinase-type plasminogen activator (uPA) monoclonal mouse IgG (clone 4-19) was from Lab Vision (Fremont, CA). The Boyden chamber (Transwell) plates were from Corning, Inc. (Corning, NY). All cholesterol derivatives used as standards or for the incubation of cells were obtained from Steraloids, Inc. (Newport, RI). Preparative and high-performance silica gel 60 thin-layer chromatography [preparative TLC and high-performance thin-layer chromatography (HPTLC)] plates were from Merck (Darmstadt, Germany). Silicic acid and sodium DC were from Sigma (St. Louis, MO). All reagents or organic solvents were of the highest analytical grade available.

Cultivation and treatment of breast cancer cells

MDA-MB-231 or MG63 cells were maintained in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution at 37°C in a humidified atmosphere containing 5% CO_2. Control cells were treated with the same volume of serum-containing medium (positive control) or serum-free medium (negative control). Cells were harvested by trypsinization or scraping and used for Transwell migration assays, protein determination, and lipid isolation. Supplementation of isolated lipids to serum-free medium was performed as described previously (3).

Migration assay

The migration of MDA-MB-231 cells was monitored using the agarose drop migration assay (3) or Transwell cell culture Boyden chambers with 6.5 mm diameter polycarbonate membrane filters perforated with 8 μm pores. The filter membranes were coated with 200 μl of 50 μg/ml collagen. Aliquots of 2.5×10^4 cells in 200 μl of fresh serum-free medium labeled with Vybrant-CM diI were seeded into the upper wells of the chamber inserts. Serum-containing control medium or serum-free DMEM with 0.1% albumin was supplemented with 5 μM of the cholesterol derivatives. After 24 h of incubation, the migrated cells were detached from the bottom side of the Transwell membrane using 400 μl of 0.25% trypsin-EDTA solution. The cells were washed with medium, and the cell number of Vybrant-CM diI-positive cells was counted using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Extraction and quantitation of lipids from bone tissue and MG63-conditioned medium

Cancellous human bone was collected according to the guidelines of and with approval from the Ethics Committee of the Medical College of Georgia. Total lipids were extracted from crushed bone samples (wet weight, 10 g) or MG63-conditioned medium (MG63-CM) as described previously (3). Briefly, crushed bone was washed with cold PBS and dissected into small pieces, and the bone fragments were homogenized in 4–6 ml of methanol and 8–12 ml of chloroform (chloroform-methanol, 2:1, v/v). The lipid was extracted by ultrasound (sonication) treatment using a Sonic Dismembrator (Fisher Scientific) for 5–10 min at room temperature. The homogenate was centrifuged at 10,000 *g* for 10 min, and the supernatant was combined with that of two additional extracts obtained from the pellet. The organic solvent was removed from the extract by vacuum evaporation. The dried residue was dissolved in 20 ml of $CHCl_3/CH_3OH$ (2:1, v/v), 5 ml of water was added, and the reaction mixture was stirred for 1 h at room temperature. After phase separation, the upper phase was discarded and the lower phase was evaporated to dryness in a stream of nitrogen. The extract was dissolved in 100–500 μl of hexane and applied to a silicic acid column (40 × 100 mm). After washing with 15 ml of hexane, lipid fractions were successively eluted with 15 ml of hexane/$CHCl_3$ mixtures at proportions of 1:1, 1:5, 1:7, and 1:9 (all v/v), followed by elution with 15 ml of $CHCl_3/CH_3OH$ (2:1 and 1:2, v/v). Individual lipid bands in each eluate were separated by analytical TLC using running solvents composed of $CHCl_3/CH_3OH$ (95:5, v/v) or $CHCl_3/CH_3OH/CH_3COOH$ (90:5:5, v/v) and visualized with cupric acetate reagent as described previously (3). The amount of individual lipids was quantified by scanning densitometry using standard lipids of known concentration for comparison. The lipid fractions collected from the silicic acid column were further purified by preparative TLC (200 × 200 mm plate) using $CHCl_3/CH_3OH/CH_3COOH$ (90:5:5, v/v) as running solvent, and the lipid bands were identified by iodine absorption. Bands comigrating with the standards were carefully scraped off, and the lipid was recovered from the gel using 15 ml of $CHCl_3/CH_3OH$ (2:1 and 1:2, v/v).

Analysis of lipid composition

To identify the lipid species, the lipid fractions (20–50 μg) obtained from preparative TLC were dried under nitrogen and placed in a vacuum desiccator overnight. The trimethylsilyl derivative was prepared by adding 0.1 ml of Sylon BTZ (Supelco, Bellefonte, PA), followed by heating at 60°C for 2 h. The reaction mixture was separated from the product by adding 0.5 ml of deionized water, followed by phase partitioning with 1 ml of petroleum ether. The ether layer was carefully removed and dried under a stream of nitrogen. The residue was dissolved in 50 μl of $CHCl_3$, and an aliquot of 1 μl was injected into a Hewlett-Packard 5980 series II plus gas chromatograph equipped with a 5972 mass selective detector. The lipid species were separated on a DB-1 column using a temperature gradient program of 50–250°C, with increasing temperature at a rate of 10°C/min. The individual peaks were identified by their retention times compared with those of reference standards and characterized by mass ions (*m/z*).

Immunocytochemistry

Cells were fixed with 4% *p*-formaldehyde in PBS and then permeabilized by incubation with 0.2% Triton X-100 in PBS for 5 min at room temperature. Nonspecific binding sites were saturated using a blocking solution of 3% ovalbumin and 2% donkey serum in PBS. The incubation with antibodies was performed at concentrations of 5 μg/ml of the primary antibodies or 10 μg/ml of the secondary antibodies in 0.1% ovalbumin in PBS. Epifluorescence microscopy was performed with an Axiophot microscope (Carl Zeiss MicroImaging, Inc.) and equipped with a Spot II charge-coupled device camera. Confocal fluorescence microscopy was performed using a Zeiss LSM confocal laser scanning microscope equipped with a two-photon argon laser at 488 nm (Cy2), 543 nm (Cy3; Alexa Fluor 546), or 633 nm (Cy5; Alexa Fluor 647).

RT-PCR

Total RNA was prepared from breast cancer cells using Trizol® (Life Systems) according to the manufacturer's protocol. The RNA was treated with DNase I (Invitrogen) to remove residual genomic DNA. First-strand cDNA was synthesized using the Omniscript® RT kit (Qiagen) according to the manufacturer's

protocol. The amount of template from each sample was adjusted until PCR yielded equal intensities of amplification for GAPDH. PCR was performed for 35 cycles with the following primer combinations and annealing temperatures: FXRsense, 5′-tgctgaaagggtctgcggttg-3′, and FXRantisense, 5′-cacgtcccagatttcacagag-3′ (56°C); uPAsense, 5′-actactacggctctgaagtcacca-3′, and uPAantisense, 5′-gaagtgtgagactctcgtgtagac-3′, (56°C); and uPARsense, 5′-ctggagcttgaaaatctgccg-3′, and uPARantisense, 5′-ggtttttcggttcgtgagtgc-3′ (56°C).

Statistical analysis and miscellaneous

Data are expressed as means ± SEM. Statistical analysis was performed using ANOVA or Student's *t*-test when appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Cholesterol derivatives from human bone tissue and MG63-CM induce the migration of MDA-MB-231 cells

Recently, we reported that 25-hydroxycholesterol (oxysterol) induces the migration of nonmetastatic human breast cancer (MCF-7) cells (3). We analyzed the migration-inducing effect of oxysterol on other human breast cancer cell lines and characterized the migration-inducing factors in human lumbar vertebral cancellous bone freshly obtained from patients undergoing spinal decompression. In contrast to MCF-7 cells, migration of metastatic MDA-MB-231 cells was not induced by oxysterol (results not shown). However, using the agarose drop coculture migration assay that was developed in our laboratory (3), we observed that MDA-MB-231 cells were attracted to fragments of living human bone tissue placed into the tissue culture dish with Vybrant-CM diI-labeled breast cancer cells embedded in agarose (**Fig. 1A**). Lipid extraction of the bone tissue with organic solvent but not protein denaturation by heating in a microwave oven eliminated the migration-inducing effect. Staining of living cells in bone using Hoechst dye (DNA staining) showed that MDA-MB-231 cells migrated to living cells in the bone tissue (Fig. 1B, C). Induction of migration resulted in the attachment of MDA-MBA-231 cells to cells within the bone tissue (arrows in Fig. 1A–C), suggesting that a migration-inducing lipid contributed to the homing of metastatic breast cancer cells into the bone.

We supplemented serum-free medium with the total lipid fraction from human bone and quantified its migration-inducing effect on MDA-MB-231 cells using a Boyden chamber assay. Migrating cells were released by trypsinization from the bottom side of the Transwell membrane and counted by flow cytometry. Figure 1D shows that the total lipid fraction from human bone increased the migration of MDA-MB-231 cells by 6-fold compared with serum-free medium. We then determined the migration of MDA-MB-231 cells induced by medium that was conditioned by cultivated bone tissue and human osteoblast-like MG63 cells. MG63-CM induced the migration of MDA-MB-231 cells by almost 20-fold (Fig. 1E). Medium from dextran-charcoal-treated (delipidated) MG63-CM, however, did not induce breast cancer cell migration. In summary, our results showed that human bone cells in culture or in bone tissue released a lipid to the medium that induced the migration of human breast cancer cells.

DC is a migration-inducing factor that is taken up by and released from MG63 cells and human bone tissue

To identify the lipid species that induced the migration of MDA-MB-231 cells, we developed a chromatographic purification procedure for different lipid fractions from MG63 cells and human bone tissue. The composition of the purified lipid fractions was determined by GC-MS. The lipid extracts were first purified by silicic acid column chromatography, followed by preparative TLC (Fig. 1F, G). A lipid compound comigrating with the bile salt sodium DC was identified to be the migration-inducing factor in the human bone-derived lipid extract. The purified lipid was further characterized as a trimethylsilyl derivative (retention time, 10.3 min) and identified by its mass fragmentation products (*m/z* 281, 256, 213, 199, 157, and 143) compared with standard DC (**Fig. 2**). Quantitative HPTLC showed a concentration of 2.5 μg DC/g bone tissue (wet weight) and 2.0 μg DC/ml (4.8 μM) MG63-CM. Using several bile acid and oxysterol derivatives, we found that the migration-inducing effect was specific for DC (59 ± 7% of control with serum-supplemented medium), whereas 5-androsten-3β-ol-7,17-dione, 5-androsten-3β,7α-diol-17-one, and cholic acid (5 μM each) did not induce migration compared with serum-free medium (19 ± 2% of control with serum-supplemented medium). The highly oxygenated cholestan-3β,5α,6β-triol (5 μM) even suppressed the migration of MDA-MB-231 cells to 15% of that observed with serum-free medium.

It is well known that secondary bile acids such as deoxycholic acid are not synthesized in mammalian cells but in intestinal bacteria. We tested the capacity of MG63 cells to take up DC from the medium and to release it afterward. Cells were cultivated for 3 days in serum-free medium and then incubated for another 3 days with 10 μM DC. The DC was removed, and cells were incubated overnight with fresh, serum-free medium. DC released to the medium was extracted with organic solvents and quantified using HPTLC. Figure 1H shows that MG63 cells (10^6 cells) released DC to serum-free medium (2 ml) at a concentration of 4 μM. In summary, these results suggested that DC is a lipid species that induces the migration of breast cancer cells to the bone. Because secondary bile acids have not been shown to be synthesized in osteoblasts, it is likely that DC is first enriched from serum and then released from MG63 cells or bone tissue to the medium.

DC promotes cell survival and induces the nuclear translocation of FXR and increase of uPA

DC has been shown to bind to the bile acid receptor FXR in vitro and in vivo (15–17). The expression of FXR in human breast cancer cells, however, has not been reported yet. In our attempt to test the effect of DC and chenodeoxycholate (CDC) on the expression and subcellular distribution of FXR, we noticed a surprising response of MDA-MB-231 cells with respect to DC- and CDC-inducible

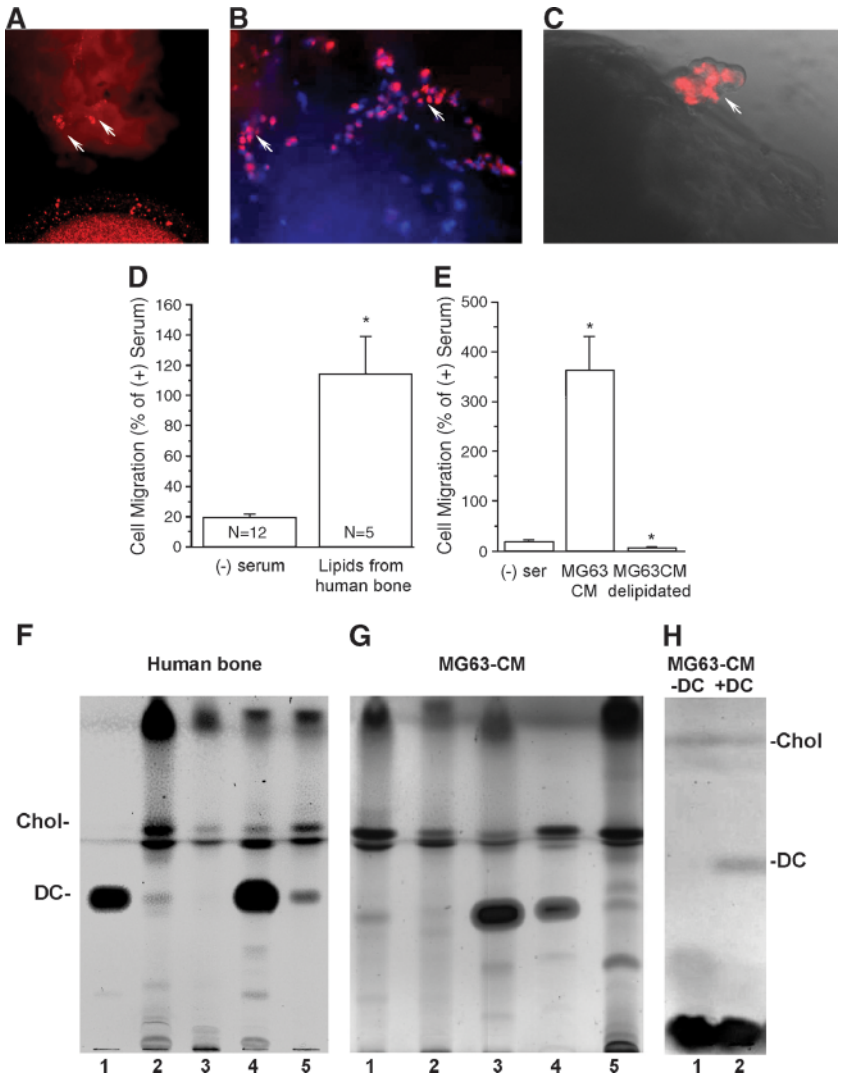

Fig. 1. Deoxycholate (DC) from human bone tissue or MG63 cells induces breast cancer cell migration. A–C: MDA-MB-231 cells were labeled with Vybrant-CM diI, embedded in a soft agarose drop as described (3), and incubated overnight next to a fragment of human cancellous bone freshly obtained from plastic surgery. The breast cancer cells settle with living cells in the bone tissue, as visualized by staining of nuclei with Hoechst dye (B). Micrometastasis attached to bone surface is indicated by arrows. D: Total lipids (30–150 μg) from human cancellous bone were added to 400 μl of serum-free medium in the bottom chamber of a Transwell chamber that was seeded with 2.5×10^5 Vybrant-CM diI-labeled MDA-MB-231 cells onto the gelatin-coated membrane of the top chamber. After overnight incubation, MDA-MB-231 cells on the bottom side of the Transwell membrane were detached by trypsinization and counted using flow cytometry. E: MG63-conditioned (serum-free) medium was used to induce the migration of MDA-MB-231 cells. For D and E, results are means ± SEM of at least five independent experiments (* $P < 0.05$). The number of cells migrating after incubation with serum-supplemented medium is the positive control, set to 100%. The number of cells migrating after incubation with serum-free medium is the negative control. F, G: Thin-layer chromatography of lipids isolated from human cancellous bone or MG63-conditioned medium (MG63-CM) and enriched by silicic acid chromatography. The high-performance thin-layer chromatography plate was developed in $CHCl_3/CH_3OH/CH_3COOH$ (90:5:5, v/v), and the lipid species were visualized by staining with cupric acetate reagent. F: Human bone. Lane 1, standard DC; lane 2, $CHCl_3/CH_3OH$ (2:1) extract; lane 3, $CHCl_3/CH_3OH$ (1:2) extract; lane 4, $CHCl_3/CH_3OH$ (2:1) extract of DC standard; lane 5, $CHCl_3/CH_3OH$ (1:2) extract of DC standard. G: MG63 cells. Lane 1, $CHCl_3$ extract; lane 2, $CHCl_3/CH_3OH$ (2:1) extract; lane 3, $CHCl_3/CH_3OH$ (2:1) extract; lane 4, $CHCl_3/CH_3OH$ (1:2) extract of DC standard; lane 5, standards cholesterol and DC. H: Uptake and release of DC. MG63 cells were cultivated for 3 days in serum-free medium and then exposed for 72 h to 10 μM DC. After washing, cells were incubated for another 24 h in serum-free medium without DC, and the DC released to the medium was isolated and determined by organic solvent extraction and quantitative TLC. Lane 1, exposure without DC; lane 2, exposure with DC for 24 h followed by 24 h in serum-free medium.

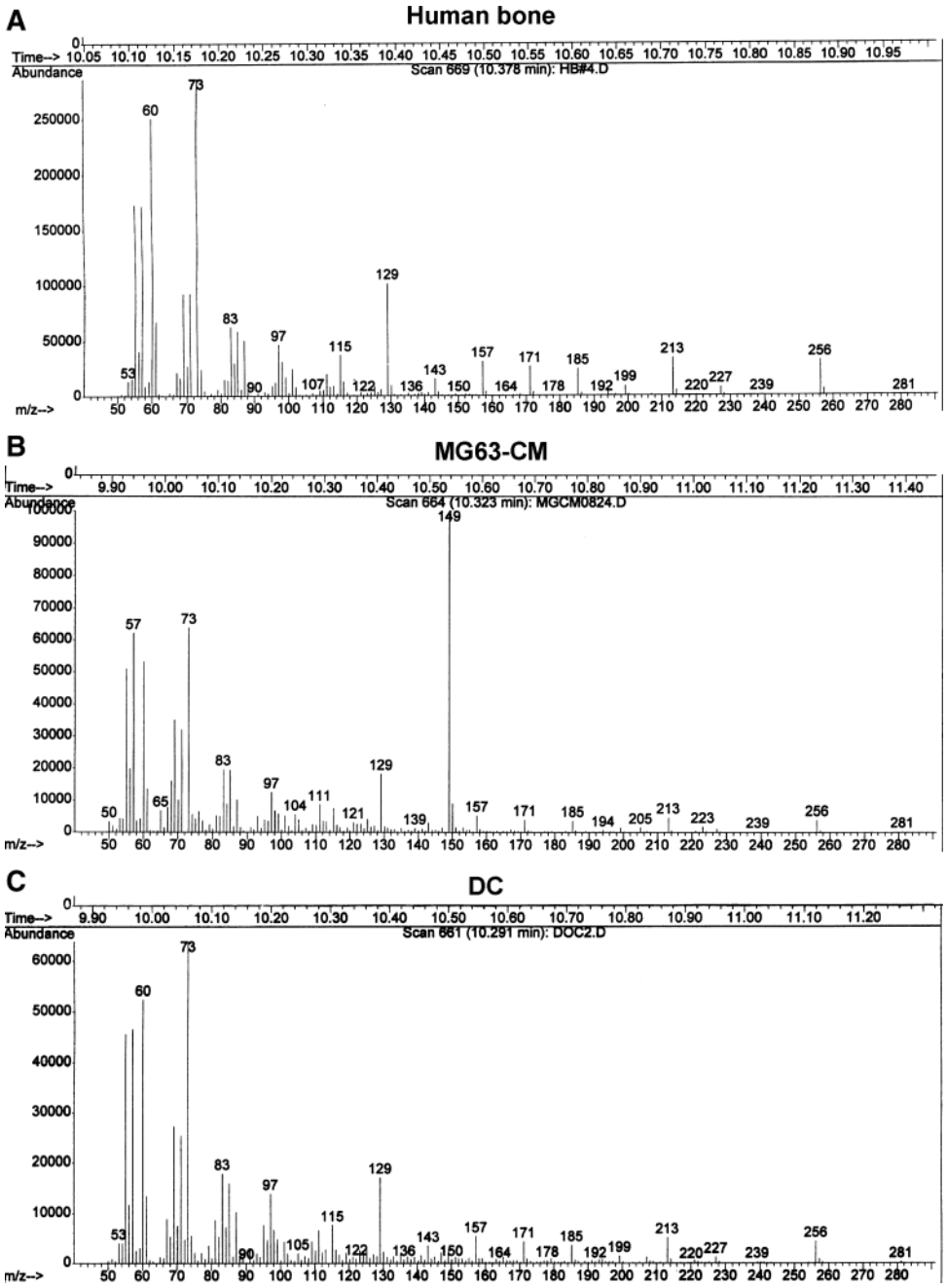

Fig. 2. GC-MS of the purified lipid from human cancellous bone and MG63-CM. Trimethylsilyl derivatives of the lipids purified from human bone (A), MG63-CM (B), and standard DC (C) were analyzed by GC-MS. The peaks of DC were identified by the retention time (10.3 min) compared with that of the analytical DC standard. The mass peak at *m/z* 281 indicates the presence of DC. Mass peaks at *m/z* 256, 213, 199, 171, 157, 143, and 129 are fragmentation products of DC.

apoptosis. **Figure 3A, C** shows that at low concentration (10 μM), DC or CDC reduced cell death observed after prolonged serum deprivation (>72 h). This result was in contrast to the effect of DC at higher concentrations (100–150 μM), which was consistent with the apoptotic response of cancer cells to bile acids reported in the literature (results not shown) (19–21). MDA-MB-231 cells not only survived better at lower concentrations of DC or CDC, but they adopted a morphology that was less clustered and more of an epithelial phenotype (Fig. 3A). Incubation of MDA-MB-231 cells with the FXR antagonist Z-guggulsterone induced apoptosis, as shown in Fig. 3B, C. Supplementation of Z-guggulsterone with DC reduced apoptosis, again suggesting that DC antagonized the proapoptotic effect of Z-guggulsterone by activation of FXR.

We determined FXR expression and subcellular distribution by immunochemistry and RT-PCR using RNA from MDA-MB-231 cells with or without prior treatment with DC. **Figure 4A** shows that the number of cells with nuclear translocation of FXR increased by 5-fold when cells were

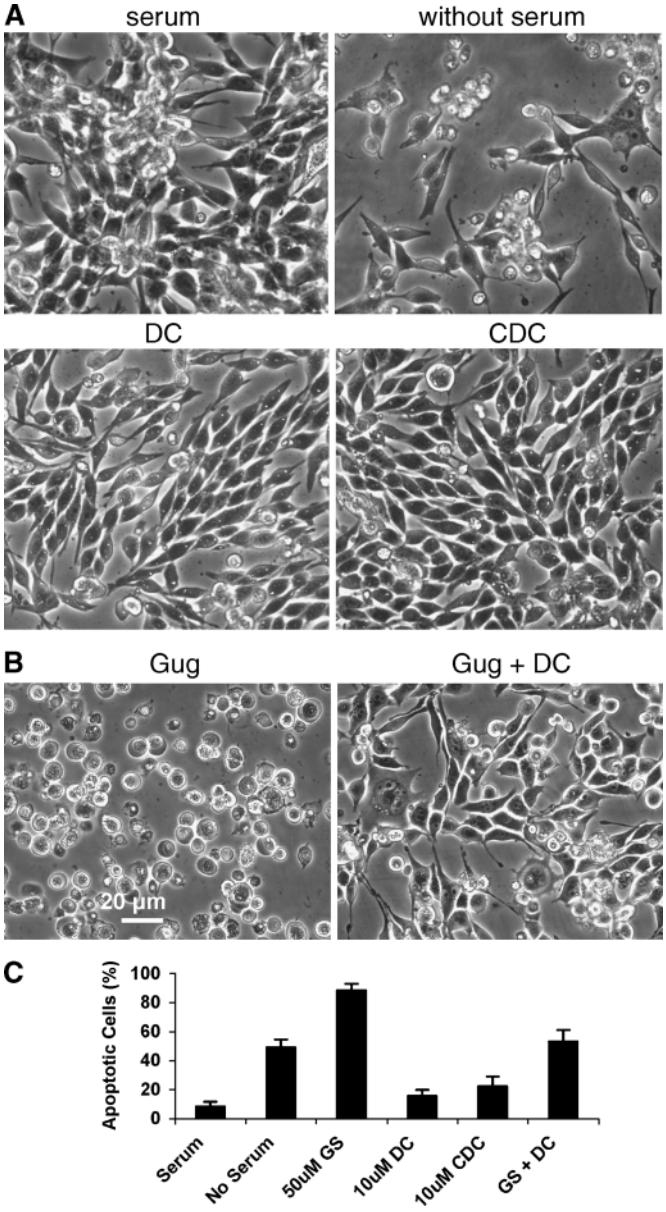

Fig. 3. DC and chenodeoxycholate (CDC) prevent apoptosis induced by serum deprivation or guggulsterone (Gug). A: MDA-MB-231 cells were incubated for 72 h with or without serum and with or without 10 μM sodium DC or CDC. B: MDA-MB-231 cells were incubated for 48 h in serum-free medium supplemented with 50 μM Z-guggulsterone with or without 10 μM DC. C: Plot for data presented in A and B. Note that guggulsterone increases the degree of apoptosis, whereas DC is antiapoptotic. Results are means ± SEM.

incubated overnight with 5–10 μM DC (1% in serum-free control vs. 5% in DC-treated cells). This number correlated well with the degree of increased migration (3-fold compared with serum-free medium) or reduction of apoptosis (one-fourth compared with serum-free medium). In the majority of MDA-MB-231 cells, nonnuclear FXR was distributed to a distinct perinuclear compartment (Fig. 4A). The perinuclear distribution of FXR is not commonly observed, although it has been shown to occur during the differentiation of germ cells in the marbled newt (22). Staining for the Golgi apparatus using fluorescently labeled wheat germ agglutinin suggested that FXR was codistributed with a Golgi compartment unless translocation to the nucleus was induced by incubation with DC (Fig. 4B).

Figure 4A, D shows that in cells with nuclear translocation of FXR, the expression level and the membrane translocation of uPA were higher than those of control cells cultivated in serum-free medium (Fig. 4C shows a negative control without primary antibodies). uPA is a protease that promotes cell survival and migration of MDA-MB-231 cells (23–25). Using RT-PCR, we determined the level of gene expression for FXR, uPA, and the urokinase-type plasminogen activator receptor (uPAR). Figure 4E shows that serum-free MDA-MB-231 cells (lane 1) expressed FXR-specific mRNA in trace amounts. Treatment with serum or 5 μM DC, however, significantly induced the gene expression of FXR. Positive or negative feedback regulation is a common feature of nuclear receptors, including FXR. This indicates activation of the receptor by binding of its ligand (14). RT-PCR for the gene expression level of uPA and uPAR showed a significant increase when cells were incubated with serum or DC (Fig. 4B). In cells with increased expression and membrane translocation of uPA, the polymerization of actin was enhanced, as indicated by stronger immunofluorescence staining for F-actin (**Fig. 5A, B**). Together, these results suggested that DC or CDC at physiological concentrations induced the nuclear translocation of FXR and enhanced the gene expression of uPA and uPAR as well as F-actin formation in human breast cancer cells.

DISCUSSION

In previous studies, we and others have shown that human osteoblast-like MG63 cells synthesize cholesterol derivatives such as estrogen and oxysterol (3, 26). We have also reported that MG63-derived oxysterol induces the migration of nonmetastatic human breast cancer MCF-7 cells (3). These observations prompted us to investigate the significance of bone-derived cholesterol derivatives for the induction of breast cancer cell migration. We found that MG63 cells and human bone released the bile acid DC to the tissue culture medium. Conditioned medium as well as DC itself induced the migration of metastatic MDA-MB-231 cells but not of MCF-7 cells. We were puzzled by these results for two reasons. *i*) DC is not synthesized by mammalian cells but by intestinal bacteria. Hence, bone or MG63 cell-derived DC was most likely taken up from serum and then released to the medium. The accumulation of bile acids from serum by >30-fold has been reported for breast cyst fluid and is discussed as a potential risk factor in breast cancer (6, 7, 12, 13). We have shown that MG63 cells also take up and accumulate DC from medium, suggesting that bone tissue has similar capabilities for DC enrichment from serum. Uptake of bile acids by tissues other than liver has been described previously and is most likely attributable to (organic) anion trans-

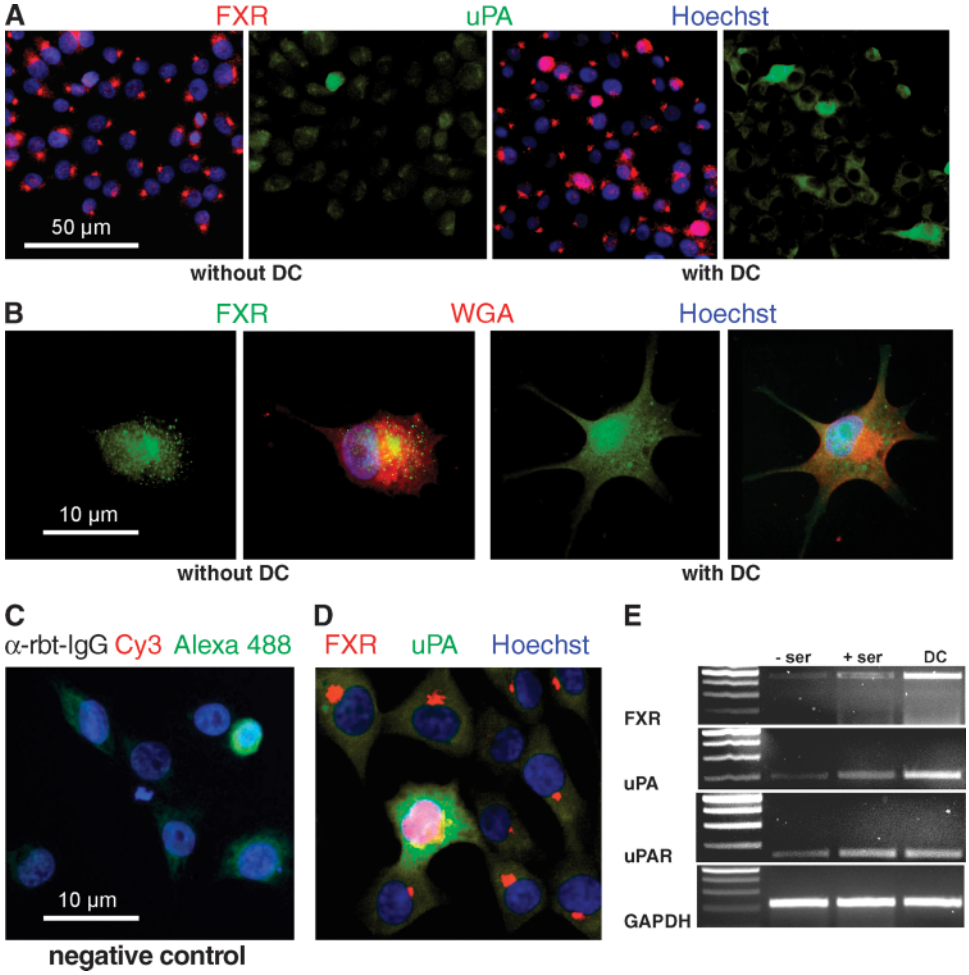

Fig. 4. DC induces the expression and nuclear translocation of farnesoid X receptor (FXR) concomitant with urokinase-type plasminogen activator (uPA) increase. A: MDA-MB-231 cells were incubated for 24 h with or without 5 μM DC in serum-free medium. Immunocytochemistry was performed using antibodies against FXR (Cy3, red) and uPA (Cy2, green). Nuclei were stained with Hoechst dye. B: MDA-MB-231 cells were incubated with or without DC, and immunocytochemistry was performed as described for A using an antibody against FXR (Alexa 488, green) and Alexa Fluor 594-conjugated wheat germ agglutinin (WGA, red) for staining of the Golgi apparatus. C: Negative control for immunocytochemistry performed in A and B. Primary antibodies were omitted, and two secondary antibodies were used as indicated. D: Immunocytochemistry as in A at higher magnification. E: RT-PCR using RNA prepared from MDA-MB-231 cells with or without prior incubation with DC for 24 h. Lane 1, serum-free; lane 2, serum-treated; lane 3, serum-free with DC. The intense band at the top of the 100 bp marker ladder represents a 500 bp band. uPAR, urokinase-type plasminogen activator receptor.

port, which is consistent with the high anion intake of bone-derived cells (27–30). *ii*) DC has been reported to induce apoptosis in multiple cancer cell types, whereas in our study, DC promoted cell survival and the migration of breast cancer cells (19–21, 31, 32). The previous observations were most likely attributable to the use of fairly high concentrations (>100 μM) of the bile acid for incubation of the cancer cells. In our study, we incubated cells at a physiologically significant concentration that is one magnitude less (5–10 μM) than that used in previous studies. At this low concentration, DC may activate cell survival and migration-inducing signaling pathways that are different from the proapoptotic pathways triggered at higher concentrations.

In colon cancer cells, DC has been found to elicit a variety of effects that promote cancer spread and metastasis (21, 31, 33). We show that in MDA-MB-231 cells, low concentrations of DC induce the uPA cell signaling pathway, whose activation is commonly linked to metastasis formation (24, 34, 35). uPA is a protease that degrades the extracellular matrix and promotes the invasive migration of breast cancer cells (23, 36, 37). uPA may bind to the uPAR on the cell surface of the uPA-releasing cancer cells, thereby self-propelling migration. In our study, DC upregulated the gene expression of uPA and induced the formation of F-actin, a prerequisite for cancer cell migration (38). At present, it is not clear whether uPA also contributes to the DC-mediated protection against apoptosis

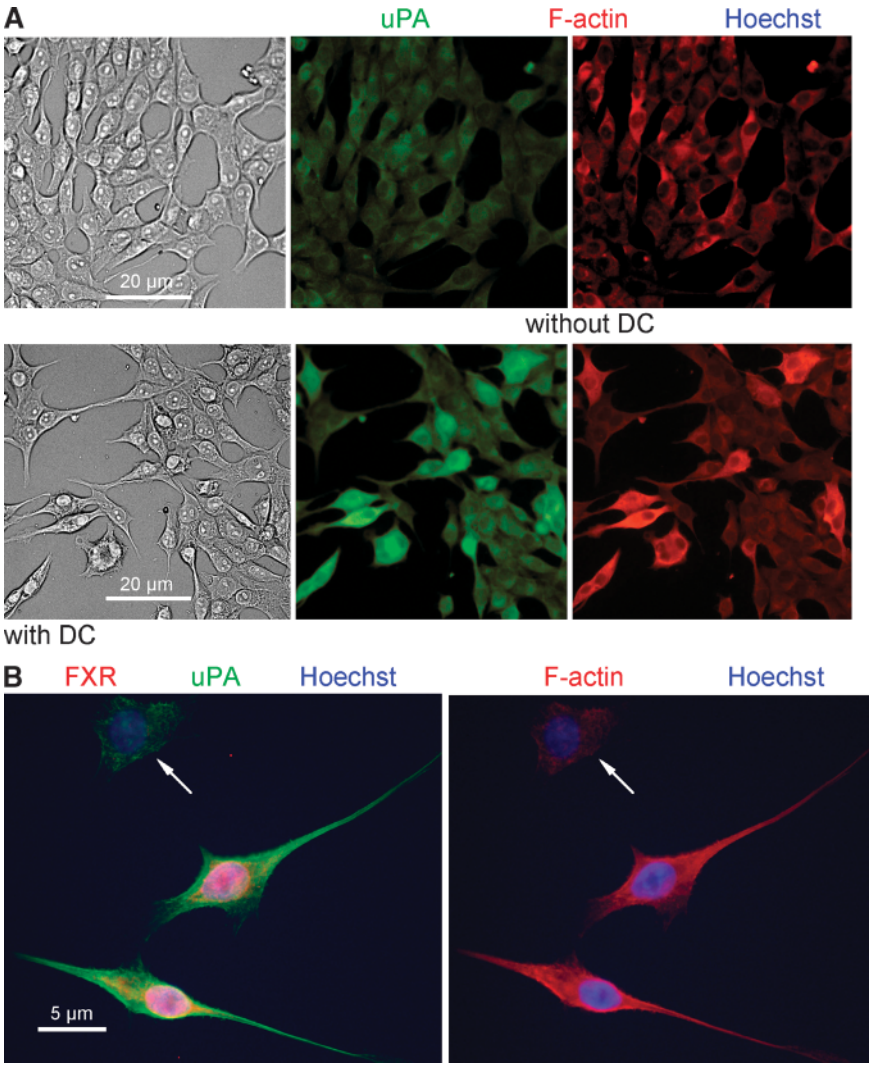

Fig. 5. Increase of uPA is concurrent with the formation of F-actin. A: MDA-MB-231 cells were incubated for 24 h with or without 5 μM DC in serum-free medium. Immunocytochemistry was performed using antibodies against uPA (Cy2, green). Actin was stained with Alexa Fluor 647 phalloidin (pseudocolored red), and nuclei were stained with Hoechst dye. B: As in A but also stained for FXR (Cy3, red). The arrow indicates a cell without nuclear translocation of FXR and, hence, no increase of uPA or enhanced formation of F-actin.

induced by serum deprivation. Previous studies have demonstrated that the increase of endogenous uPA expression prevents the apoptosis of MDA-MB-231 cells (25). Therefore, it is likely that DC-induced uPA expression promotes both cell survival and the migration of breast cancer cells.

It is known that secondary bile acids bind to the nuclear FXR, thereby activating gene expression for various enzymes in cholesterol and lipoprotein metabolism of liver cells (14, 18). The role of FXR expression in cancer cells, however, is still not clear. In contrast to liver or colon cancer cells, breast cancer cells have not been described to express FXR. Our results using RT-PCR show that only low levels of FXR gene expression are detectable unless MDA-MB-231 cells are incubated with DC. Autoactivation of FXR gene expression by DC has been described for HepG2 cells and is an autoregulatory loop that has been observed for a variety of nuclear receptors (14). It is not known, however, how the DC-induced activation of FXR upregulates the expression or protein level of FXR. It also remains to be elucidated how FXR activation stimulates the expression of uPA. Recent studies have shown that DC increases the expression of the cyclooxygenase 2 product prostaglandin E_2 and that cyclooxygenase 2 activation enhances the gene expression of uPA (39–43). We are currently investigating the DC-induced activation of cyclooxygenase 2 as a potential upstream regulator of uPA expression in MDA-MB-231 cells. The significance of FXR activation for DC-induced cell survival and migration is supported by the observation that the FXR antagonist Z-guggulsterone induces apoptosis, which can be counteracted by DC. The concentration of Z-guggulsterone required to induce

apoptosis is significantly higher than that predicted by its affinity to FXR. This suggests that Z-guggulsterone triggers apoptosis by other cell-signaling pathways in addition to antagonizing the activation of FXR (44). The antiapoptotic effect of DC, however, is consistent with the concentration range used to determine the antagonism of DC and Z-guggulsterone for binding to FXR (15). This indicates that DC promotes cell survival by counteracting Z-guggulsterone as a result of the activation of FXR. Together, our results suggest that DC first binds to FXR, which triggers its own gene expression and that of uPA. Secreted uPA translocates to the cell surface by binding to uPAR in lipid membrane domains, which stimulates F-actin formation and cancer cell migration.

Our results predict that breast cancer metastasis will be promoted by increased bile acid levels in tumor and bone. Indeed, recent epidemiological studies found that postmenopausal women with newly diagnosed breast cancer show increased levels of DC in plasma and breast cyst fluid (7). Our data provide a plausible explanation for these epidemiological data and show for the first time that bile acids, particularly DC, are released from bone tissue and induce the migration of metastatic breast cancer cells. Nutritional strategies that decrease the systemic level of DC, or natural FXR antagonists such as guggulsterone, may be useful for new adjuvant cancer treatments. Intriguingly, in traditional Ajurvedic medicine, guggul leaf (*Commiphora mukul*) extract is not only used to decrease cholesterol levels but also has been used to treat cancer (45, 46). Moreover, recent epidemiological studies suggest that breast cancer rates are lower in regions with high consumption of low-fat fermented milk or soy products (47, 48). Results of other studies suggest that this may be attributable to the effect of *Lactobacillus* processing bile acids (49, 50). This opens up the intriguing possibility that nutritional supplements may contribute to reduce the risk of breast cancer linked to serum-derived bile acids. In future studies, we will investigate the mechanism(s) for the uptake of serum-derived DC and its release from bone tissue and further determine the cell signaling pathways mediating the DC-induced migration of breast cancer cells.

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