27-Hydroxycholesterol induces the transition of MCF7 cells into a mesenchymal phenotype

CRISTIAN G. TORRES[1], MARÍA E. RAMÍREZ[2], PAMELA CRUZ[2], MARÍA J. EPUÑAN[2], LUIS E. VALLADARES[2] and WALTER D. SIERRALTA[2]

[1]Laboratorio de Medicina Experimental, Escuela de Medicina Veterinaria, Universidad Andrés Bello, Santiago 8370251; [2]Laboratorio de Nutrición y Regulación Metabólica, INTA-Universidad de Chile, Santiago 7830489, Chile

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Abstract. A decrease in the expression of E-cadherin and β-catenin, paralleling the loss of adherens junction complex, was observed in MCF7 cells exposed for longer than 48 h to 2 μM 27-hydroxycholesterol (27OHC), indicating an epithelial-mesenchymal transition (EMT). Upon removal of 27OHC from the culture medium, the cells released by the exposure of 72 h to the oxysterol grew as loosely packed cell groups. In these cells, accumulation of E-cadherin and β-catenin in the cytoplasm and the prolonged expression of epidermal growth factor receptor 2 (EGFR2/neu) in the plasma membrane were observed, suggesting that the acquired phenotype was related to the expression of this tyrosine kinase-growth factor receptor. The results presented here are discussed on the basis of the claimed relationship between 27OHC, hypercholesterolemia, macrophage infiltration and therapy-resistant ERα$^+$ breast cancer incidence.

Introduction

Breast cancer remains one of the leading causes of morbidity and mortality in women worldwide; not surprisingly, considerable efforts have been made in the development of additional therapies to fight this disease. Frequently, tumors derived from a healthy mammary gland retain activities which were present in the normal tissue (1); particularly important in this respect is the regulation of cell proliferation by estrogens (2). In responsive mammary cancer cells, a variety of processes are regulated at the genomic level after the binding to the cognate receptors (ERα, ERβ) of estradiol (E2) and other estrogens which are called selective modulators of estrogen receptors (SERMs) (3). In addition, several non-genomic activities are regulated by the association of E2 or a SERM to estrogen receptors present in the cytoplasm or in the plasma membrane of target cells (4,5). The attachment of an estrogen to an ER specifically alters its conformation; this change defines the successive recruitment of one or more discrete co-regulatory molecules, thus controlling particular cell activities (6).

Uptake from the circulation and local steroid production provide target cells with endogenous estrogen for the activation of their ERs (7). Therefore, treatments with estradiol antagonists or with inhibitors of aromatase and other enzymes involved in the local synthesis of estradiol are widely used therapeutic strategies to impede proliferation of ER-positive mammary cells in cancer patients (8). Unfortunately, many ER-positive tumor cells do not respond to these therapies and even acquire resistance during treatment (9); therefore, a further knowledge of the activation of ER signaling pathways by other compounds in cancer cells is required.

Body fluids bathing organs, tissues and cells contain molecules with the potential to operate as SMERs. 27-Hydroxycholesterol (27OHC), a major circulating cholesterol metabolite reported to act as a non-classical SMER in estrogen-target cells is one of these (10). In endothelial cells, 27OHC functions as a SMER antagonist and appears to be responsible for the damage to endothelial and muscle cells bordering atherosclerotic plates (10). 27OHC also behaves as an antagonist in the bone, deteriorating bone homeostasis and promoting osteoporosis (11). In ERα-positive mammary tumor cells, 27OHC acts as a SMER agonist, enhancing proliferation (12). We recently reported on the differential effect of this oxysterol on mammary tumor cell lines and on the inhibition of 27OHC-stimulated cell growth by simvastatin and by the pure estradiol receptor antagonist ICI 182,780 (13).

In this study, we found that MCF7 cells exposed to 27OHC showed a decrease in the expression of E-cadherin and β-catenin, in parallel with the evanescence of adherens junctions, indicating that an epithelial-mesenchymal transition (EMT) (14) was in progress. Once 27OHC was removed from the culture medium, the cells, released by exposure to the oxysterol, were capable of growing as loosely packed cell colonies. In these MCF7 cells a marked immunoreactivity of EGFR2/neu was detected in the plasma membrane, and abundant aggregates of E-cadherin and β-catenin were observed in the cytoplasm.

Correspondence to: Dr Walter D. Sierralta, Laboratorio de Nutrición y Regulación Metabólica, INTA-Universidad de Chile, El Líbano 5524, Santiago 7830489, Chile
E-mail: wsierra@inta.uchile.cl

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The results suggest that the maintenance of a mesenchymal phenotype in 27OHC-released cells is related to the expression of the tyrosine kinase-growth factor receptor EGFR2. The mechanisms underlying this regulation are discussed.

A relationship exists between the plasma levels of cholesterol and 27OHC and, accordingly, the circulating levels of the oxysterol are higher in obese patients than in normal subjects (15). It has been suggested that hypercholesterolemia, macrophage infiltration and therapy-resistant ERα$^+$ breast cancer incidence are interrelated (16). Therefore, we discuss the results presented in this study considering these issues.

Materials and methods

Materials. Tissue culture material was obtained from Nalge Nunc Intl. (Rochester, NY, USA). 27-Hydroxycholesterol (C6570-000) was purchased from Steraloids Inc. (Newport, RI, USA). Dulbecco's phosphate-buffered saline (DPBS) was from Gibco-Invitrogen Corp. (Carlsbad, CA, USA). Pure antiestrogen ICI 182,780 (ICI) was purchased from Tocris Bioscience (Ellisville, MO, USA). The FITC-Annexin V (no. 556419) and FITC BrdU flow kits (no. 559619) were obtained from BD-Pharmingen (San Diego, CA, USA). Most of the chemicals used were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Antibodies. Mouse anti-human E-cadherin (MAB3199Z) and anti-actin (MAB1501) monoclonal antibodies were obtained from Upstate-Chemicon-Millipore (Temecula, CA, USA). Mouse anti-EGFR2/neu (sc-08), anti-β-catenin (sc-65482) and anti-α-tubulin (sc-5286) monoclonal antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). FITC-conjugated goat anti-mouse IgG (F0257) and peroxidase-conjugated goat anti-mouse IgG (A9917) were purchased from Sigma-Aldrich Inc.

Cells. Estradiol-sensitive MCF7 epithelial cells from human metastatic breast cancer tissue (HTB 22; ATCC, USA) were cultured in DMEM/F12 containing 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. In the proliferation studies, the cells were transferred 24 h after seeding to DMEM/F12 containing ITS (insulin, transferrin, selenium), 1% charcoal/dextran twice-treated serum (CDTS), 3% hydroxyethylated starch (HAES), 50 U/ml penicillin and 50 μg/ml streptomycin. Non-tumorigenic E2-insensitive epithelial cells from cell line MCF10 (CRL-10317; ATCC, USA), used for control studies, were cultured under the same conditions as the MCF7 cells. In each of the experiments, the cells were cultured at 37˚C in a humidified incubator in a 5% CO_2 atmosphere.

Proliferation studies. MCF7 or MCF10 cells (approximately 20,000 cells/cm^2) were seeded and incubated for 24 h to allow attachment. Subsequently, non-adherent cells and media were removed. The remaining cells were washed and further incubated for various time periods with low-serum culture medium containing either 2 nM E2, or different micromolar concentrations of 27OHC, in the absence or presence of 100 nM ICI. At the completion of the incubation period, cells were washed with DPBS, detached (0.25% trypsin in 0.2 mM EDTA), resuspended in DPBS, counted and assessed for viability (trypan blue). Each experiment was performed at least three times in triplicate.

Immunofluorescence studies. Cells exposed to different experimental conditions were grown on sterile coverslips and then fixed (absolute methanol at -20˚C), rinsed with DPBS and blocked for 30 min with 2% BSA in DPBS. The samples were then incubated with the primary antibodies for 1 h at RT. After extensive washes (DPBS containing 2% BSA), samples were incubated with the appropriate secondary antibody for 1 h and washed. The samples were mounted with Biomeda gel/mount (Foster City, CA, USA) and inspected with a Zeiss Axiophot epifluorescence microscope fitted with a color CCD camera (Kappa GmbH, Goettingen, Germany). In each experiment, the images were obtained under fixed settings of illumination, exposure times and camera gain.

Cell cycle analyses. Approximately 8x10^5 MCF7 cells/well were seeded in 6-well plates and treated as described for the respective experiments. The analyses were carried out using the FITC BrdU flow kit following the instructions of the manufacturer. Briefly, after exposure to different stimuli, cells were incubated for 240 min with 10 μM BrdU and were detached using trypsin-EDTA before fixation in BD Cytofix/Cytoperm buffer. After DNase treatment to obtain better exposure of the epitopes, the incorporated BrdU was detected with the FIT-conjugated anti-BrdU-antibody. DNA was counterstained with 7-aminoactinomycin D for 30 min. The samples were finally resuspended in staining buffer and analyzed within 1 h. Before any treatment, the MCF7 cells were synchronized by 24 h serum deprivation, and then incubated for 24-48 h with vehicle, 2 nM E2 or different concentrations of 27-OHC, in the absence or presence of 100 nM ICI.

Annexin V/PI labeling. The analyses were carried out using the BD FITC-Annexin flow kit, following the instructions of the manufacturer. In brief, cells were grown in P60 dishes and incubated in medium alone or stimulated for different periods of time with E2 or 27OHC. After completion of the selected times, the supernatants and trypsin-EDTA-detached cells from each dish were combined and centrifuged after addition of 10% serum to stop the protease activity. The cell sediments were carefully washed and resuspended at a concentration of 1x10^6 cells/ml in BD-Annexin V binding buffer. The suspension (100 μl) was mixed with 5 μl of FITC-Annexin V and 10 μl PI. The cells were gently stirred with a vortex and incubated for 15 min at RT (25˚C) in the dark. After addition of 400 μl of binding buffer to each tube, the cell populations were analyzed within 30 min by flow cytometry using a BD-FACSCalibur cytometer.

Fate of cells released during exposure to 27OHC. During the exposure to 27OHC a number of living cells was released. To evaluate survival, the MCF7 cells set free during the 72-h incubation with 27OHC were collected by centrifugation, washed with DPBS and suspended in medium with 10% fetal bovine serum. After obtaining an aliquot for counting, the cell suspension was plated on coverslips for fluorescence studies or

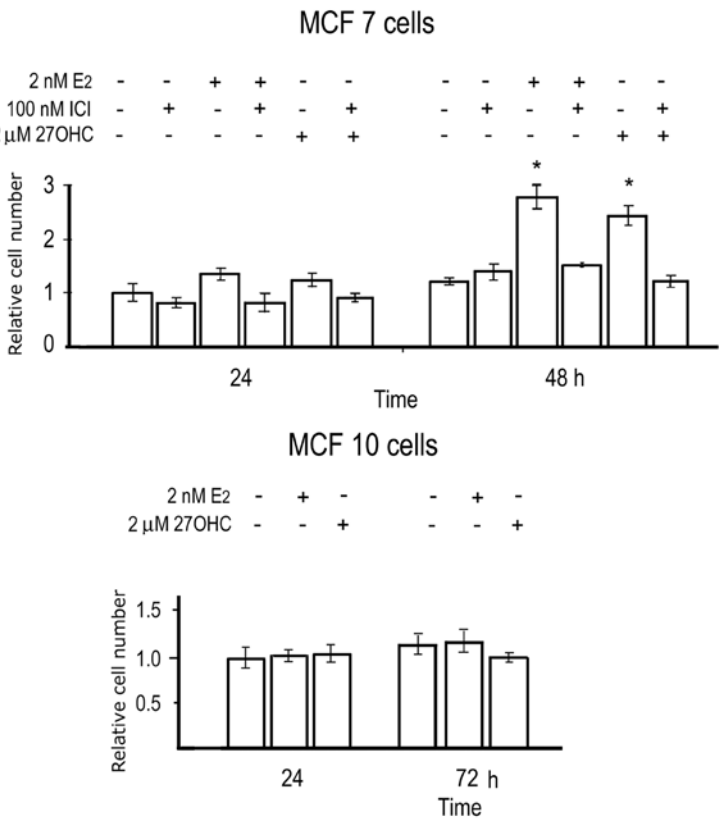

Figure 1. Effect of estradiol and 27-hydroxycholesterol on the proliferation of MCF7 and MCF10 cells in culture. MCF7 or MCF10 cells were incubated for the indicated times in a medium containing 1% charcoal-treated serum and 3% HAES, with 2 nM E2 or 2 μM 27OHC in the absence or presence of 100 nM ICI 182780. The proliferation rate was expressed as a relative cell number calculated in relation to the cell number of control cells at 24 h after seeding, which was set to 1. Values are mean ± SD of 3 individual experiments carried out in triplicate. *p<0.05 compared with the non-stimulated control cells.

directly placed in the wells of P24 plates for counting purposes. After 16 h, the number of adherent cells was determined. When the total incubation time of 72 h was completed, the supernatant was discarded, and the adherent cells were washed with DPBS and analyzed by immunofluorescence. In parallel, following detachment from the wells with trypsin/EDTA, the cells were centrifuged, resuspended in DPBS, mixed with trypan blue and counted using haemocytometers.

Invasion assays. Invasion assays were carried out using BD-BioCoat™ Matrigel™ invasion chambers (Transwell® 8-μm pore size, 24 wells) (BD Biosciences, Bedford, MA, USA). Cells in medium supplemented with 1% CDTS and 3% HAES and containing or not 2 nM E2 or 2 μM 27OHC were incubated for 24 h against a gradient of 5% FBS. Non-invading cells were wiped from the upper side of the filter, and the nuclei of invading cells were stained with Hoechst 33258. After fixation with cold methanol, the nuclei were inspected by epifluorescence. For each condition, three Transwell units were used in the experiments; five microscopic fields were counted per insert.

Statistical analyses. The Student's t-test was used to evaluate differences between the samples and the respective controls. P<0.05 was considered significant. Data were analyzed with Statistica for Windows Software, release 6 (Statsoft Inc., USA).

Results

As previously demonstrated, 2 μM 27OHC stimulated the proliferation of MCF7 cells in culture medium containing low levels of charcoal-treated calf serum; comparable results were obtained with ER-positive cells from the ZR75-1 cell line (13). The simultaneous addition of the pure anti-estrogen ICI 182,780 blocked the mitogenic effects, meaning that a functional ER was required to augment the proliferative activity (Fig. 1). In the non-tumorigenic MCF10 cell line, neither estradiol nor 27OHC exhibited any effect on cell proliferation (lower panel of Fig. 1).

A 24-h exposure to 2 μM 270HC or 2 nM E2 stimulated similar numbers of adherent MCF7 cells into the S phase as shown by BrdU incorporation studies; the proportion of cells at G2+M phases of the cycle marginally varied between the experimental groups. After 48 h of stimulation with both compounds, similar numbers of cells were distributed in the S and G2M phases. Representative dot plots from these cell cycle experiments are presented in Fig. 2A. This figure also shows that the simultaneous addition of 100 nM ICI effectively blocked the effects of E2 and 27OHCI. Results of the cell cycle analyses are summarized in Fig. 2B.

Marked expression of E-cadherin was observed in both non-stimulated and estradiol-stimulated MCF7 cells, but a progressive decline in E-cadherin expression was detected in cells exposed to 27OHC for 48 h or longer (left panel of

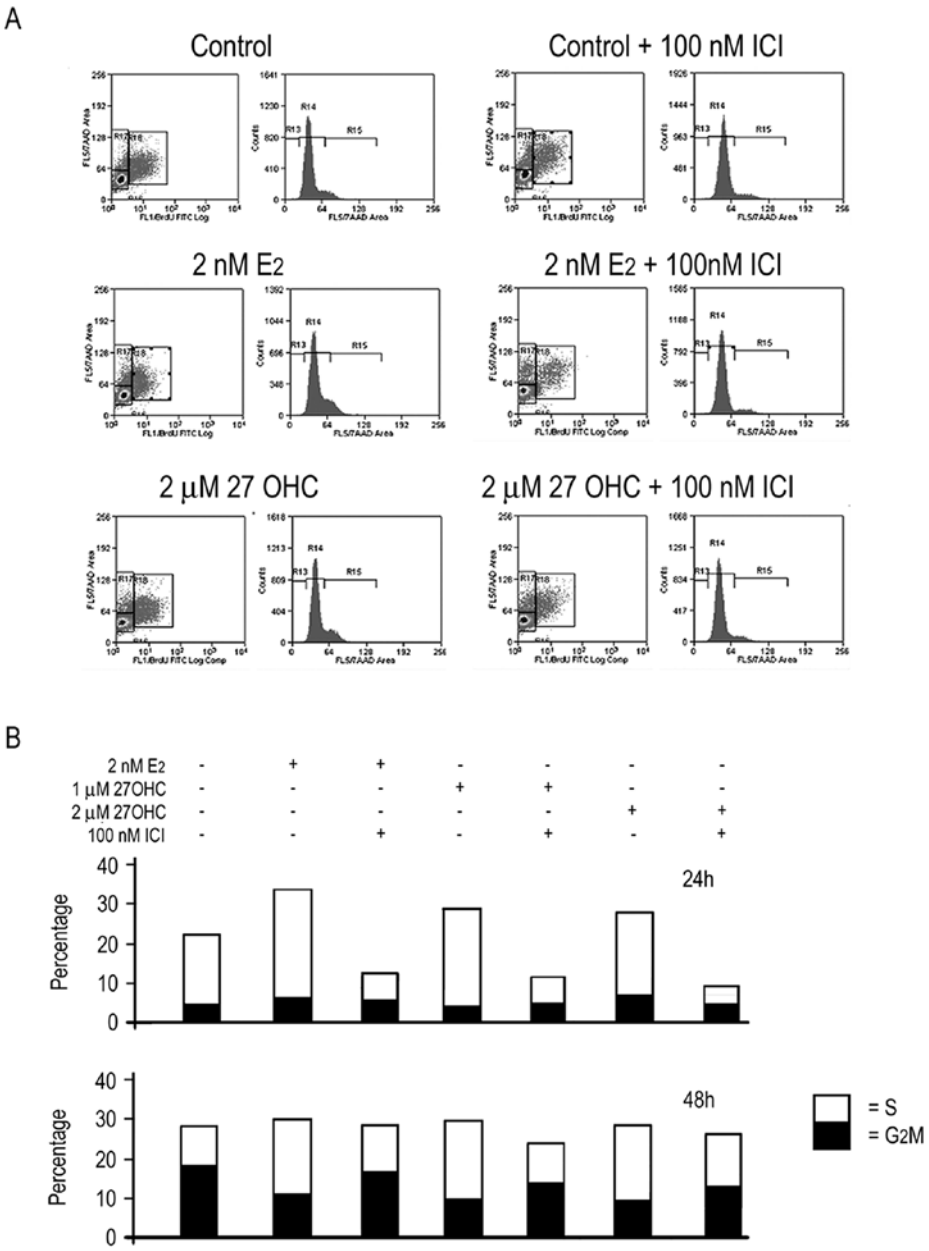

Figure 2. Cell cycle analysis of MCF7 after stimulation with estradiol or 27-hydroxycholesterol. (A) Representative dot-plots in which the labels indicate the percentage of cells found at the S stage after exposure for 24 or 48 h to either vehicle, or 2 nM E2, or 2 μM 27OHC, in the absence or presence of 100 nM ICI. (B) Histogram depicting the percentage of cells found at the S and G2/M phases from two individual experiments.

Fig. 3A). The dissolution of the basolateral cell-cell contacts in epithelium signals the induction of an anchorage-independent cell growth. The expression of β-catenin followed coherent variations (right panel of Fig. 3A). 27OHC induced the transit of β-catenin from the cell margin to the perinuclear space and a decline in expression at 72 h. In the E2-stimulated cells and non-stimulated controls, changes in the localization of β-catenin were not detected. Fig. 3B illustrates the variations in the expression of E-cadherin and β-catenin, analyzed by Western blotting in whole cell extracts.

Exposure to 27OHC for 72 h induced the release of up to 40% of the total MCF7 cells; most of the cells were alive as judged by the trypan blue exclusion test. To determine the fractions of surviving cells and those undergoing different cell death stages, we carried out Annexin V/PI labeling (Fig. 4). 27OHC induced slightly more cells to transgress into early apoptosis, as compared with the non-stimulated control cells. Cell viability was not modified during the first 48 h of exposure to 27OHC but was reduced at 72 h, in comparison to the non-stimulated control cells.

Since the exposure of MCF7 cells to 27OHC induces a release of cells, we performed Transwell invasion assays. As shown in Table I, an induction of invasiveness was noted. This effect was not affected by the presence of 100 nM ICI, suggesting that an active estradiol receptor was not required for this action.

We analyzed the response of the 27OHC-mobilized cells to the removal of 27OHC from the culture medium. The cells released by the 72-h stimulation with 2 μM 27OHC were collected, washed, suspended in complete medium and plated

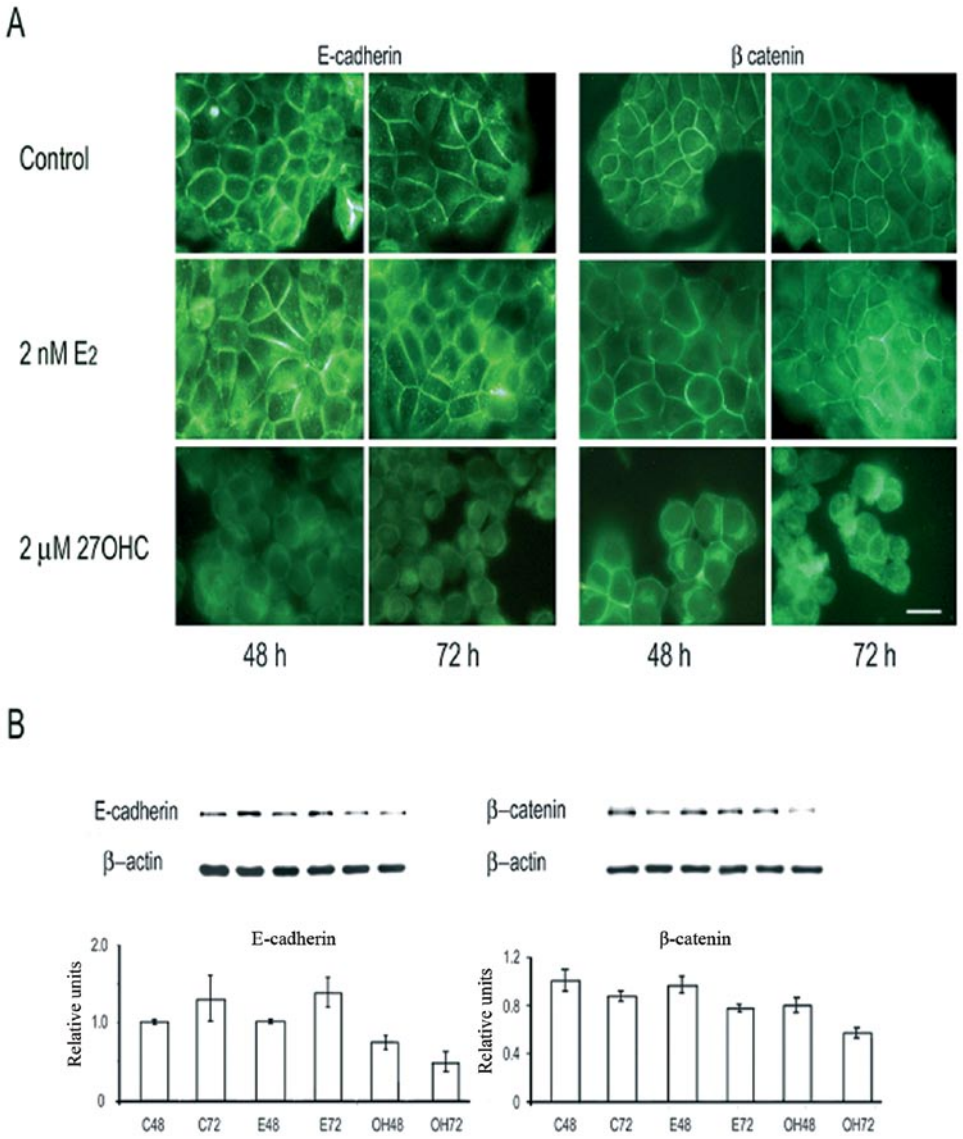

Figure 3. Analyses of E-cadherin and β-catenin expression in MCF7 cells unexposed or exposed to either E2 or 27OHC. (A) Representative fluorescence images of E-cadherin (left) or β-catenin (right) in the MCF7 cells grown for 48 or 72 h in medium containing 1% charcoal-treated serum and 3% HAES and 2 nM E2 alone; or 2 μM 27OHC alone or maintained in medium alone (control). Bar, 20 μm. (B) Representative results from the analysis of cell extracts analyzed by Western blotting. The histogram shows the mean ± SEM from three independent analyses. Extracts from C, control cells; E, estradiol-stimulated cells; OH, 27OHC-stimulated cells at 48 or 72 h.

Table I. Effect of 27-hydroxycholesterol on the invasiveness of MCF7 cells.

Treatment	Cells/field
None	10.13±1.09
2 nM estradiol	11.95±1.06
2 μM 27-hydroxycholesterol	16.00±1.44
2 μM 27-hydroxycholesterol/100 nM ICI	15.46±1.71

MCF7 cells were seeded into Matrigel-coated Transwell inserts and cultured for 24 h in serum-free medium or with 2 nM E2, or 2 μM 27OHC, or 2 μM 27OHC plus 100 nM ICI. Non-invading cells were wiped from the upper side of the filter, and the nuclei of invading cells were stained with Hoechst 33258. Randomly selected fields were inspected using a fluorescence microscope with a x10 Neofluar objective, and the nuclei were counted from digitalized images; means ± SEM from 3 experiments.

in P24 plates. As early as 24 h after seeding, >62% of the previously released cells showed the capacity to adhere. These adherent cells did not organize a regular epithelium but, instead, formed loosely packed clusters with granular cytoplasmic accumulation of E-cadherin and β-catenin with intense EGFR2/neu immunoreactivity at the plasma membrane (Fig. 5).

Discussion

The preservation of many of the characteristics of the normal mammary epithelium supports the use of MCF7 cells as a model to analyze the molecular and cell biological features aspects of human breast cancer. These cells possess functional estradiol receptors α and β, which are required for the preservation of the sensitivity to estrogens and exhibit the tendency to build well-formed epithelia and primitive ducts in culture (17). Based on these and other reasons, MCF7 cells are considered a

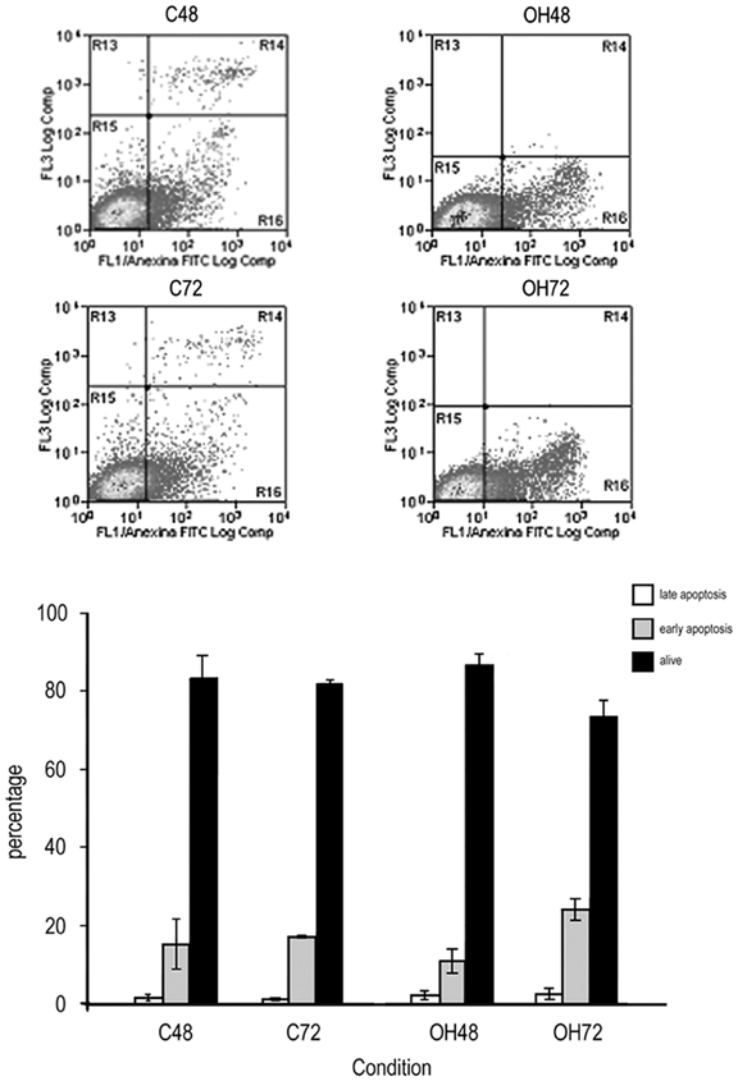

Figure 4. 27-Hydroxycholesterol moderately affects the survival of MCF7 cells. Annexin V/PI staining of MCF7 cells treated with 2 μM 27-hydroxycholesterol for the indicated time periods. The dot plots depict the distribution of the cytometrically analyzed cells according to the four categories (R13, necrotic; R14, late apoptotic; R15, alive; R16, early apoptotic). The histogram in the lower panel summarizes the percentage of alive cells and those in early- and late apoptosis (means ± SEM from three independent assays).

suitable model by which to analyze changes occurring in estradiol-responsive mammary tumors exposed to SERMs (18).

A number of oxidized metabolites of cholesterol are present in the blood of human subjects (19). One of these is 27-hydroxycholesterol, whose plasma concentration is proportional to that of cholesterol (20). This oxysterol participates in cell signaling and vesicle transport (21), acting as an intracrine/paracrine modulator in target tissues (15). In mammary tumor cells containing ERα, oxysterol stimulates proliferation (12,13). Similar to estradiol, 27OHC induces breast tumor cells to accumulate into the S and G2/M phases of the cell cycle, and these effects are neutralized by the pure estrogen-antagonist ICI 182,780, emphasizing the requirement of functionally active ERs for 27OHC action. Neither estradiol nor 27OHC was found to alter the proliferation or modify the cell cycle of ER-negative MCF10 cells (13).

The migration of epithelial cancer cells requires dissolution of the adherent contacts that keep the cells joined together while maintaining their polarity and shape (22). In epithelial cells, E-cadherin is localized to the cell surface and associates with β-catenin at the adherens junctions, contact regions that constitute a marker of the tissue fate (23). Estrogens may regulate the morphology and performance of several ER-positive cellular types, including mammary tumor cells (24). In these cells, exposure to endogenous or exogenous estrogens causes modifications of the cytoskeleton, the cell membrane and/or the junctional complexes, facilitating the migration process which is a prerequisite for the invasion of tissue cells and the establishment of metastases (25,26). In embryonic and cancer tissues, epithelial architecture may be altered by the epithelial mesenchymal transition (EMT), a process involving the disassembly of the junction complexes, the expression of mesenchymal proteins, remodeling of the extracellular matrix and migration of cells (reviewed in ref. 27). It has been demonstrated that β-catenin plays a dual role of mediator of E-cadherin dysfunction and regulator of gene transcription. Its own phosphorylation status determines the capacity of β-catenin to modulate these functions. The phosphorylation of specific serine/threonine residues destabilizes the adhesion complexes of β-catenin with cadherin and allows the

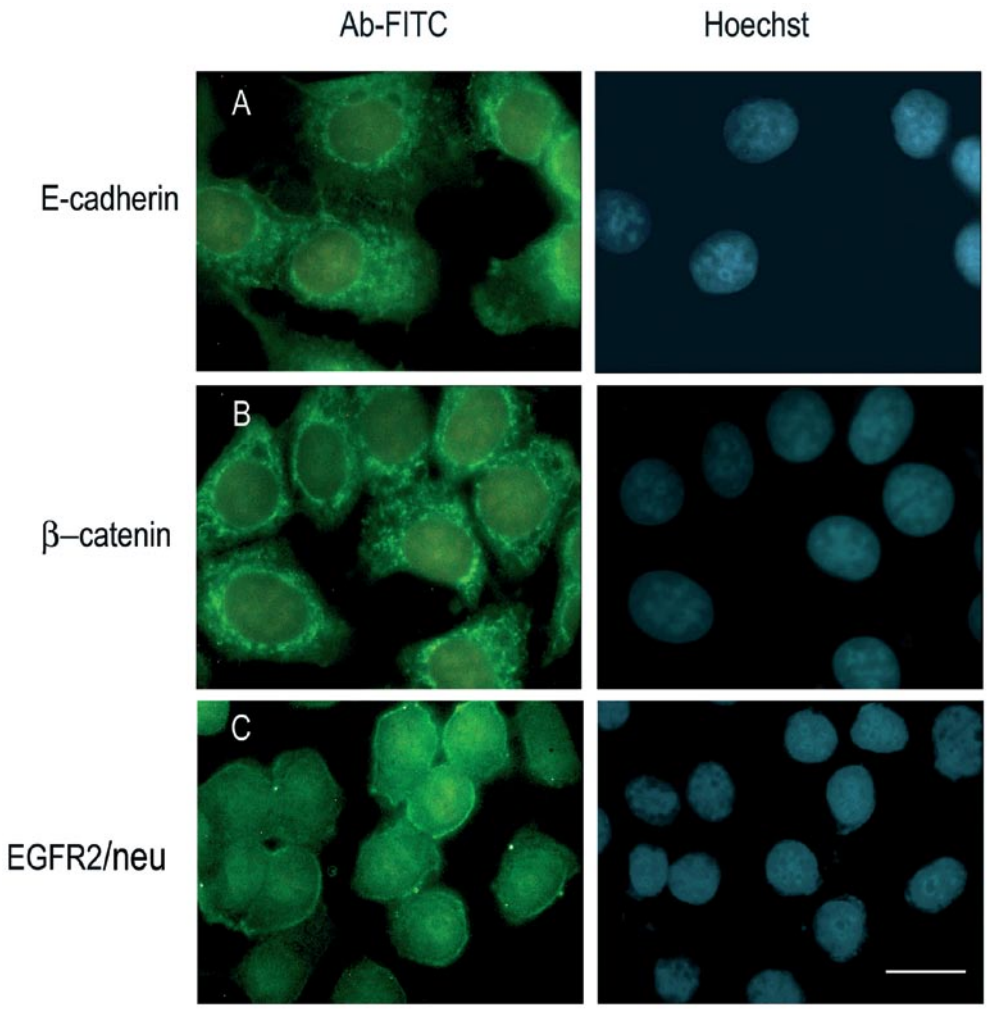

Figure 5. Immunofluorescence of E-cadherin, β-catenin and EGFR2/neu expression in MCF7 cells previously exposed to 27-hydroxycholesterol. Representative fluorescence images of (A) E-cadherin, (B) β-catenin or (C) EGFR2/neu in MCF7 cells released by a 72-h treatment with 2 μM 27OHC, then plated on coverslips and incubated for 1 week in culture medium containing 10% fetal bovine serum; with medium renewed every 48 h. Bar, 20 μm.

translocation of β-catenin into the nucleus, to regulate gene expression (28). In contrast, tyrosine phosphorylation of β-catenin promotes its dissociation from E-cadherin, impairing cell to cell adhesion and stimulating cell spreading (29). It has been shown that in many cell typess, the receptor-tyrosine kinase EGFR2/neu may associate with β-catenin and phosphorylate key tyrosine residues, abolishing the adherens junction (30). The down-regulation of E-cadherin indicates a shift in epithelial cells into a mesenchymal invasive phenotype (31). Together with the results from the Annexin V/PI labeling, our observations indicate that 27OHC modifies cell invasiveness without altering cell viability or increasing cell death. After withdrawal of 27OHC, most of the cells released upon exposure adhered to the plates and formed dispersed small colonies, without the development of cell-cell adherens junctions even two weeks after plating in serum-rich medium. These cells expressed E-cadherin and β-catenin; however, the proteins did not associate by building junctional complexes. Instead, they aggregated in the cytoplasm, suggesting that 27OHC induced a transformation with loss of the capacity to rebuild a normal epithelium. The morphology of the transformed cells resembled the mesenchymal phenotype, displaying increased EGFR2/neu immunoreactivity at the plasma membrane. This suggests that the new phenotype was maintained by tyrosine phosphorylation of β-catenin, driven by an activated EGFR2/neu. In many aspects, the cells altered by the treatment with 27OHC are similar to MCF7 cells made resistant to tamoxifen (TamR cells) (32). In TamR cells, the EMT involves adherens junction degradation, EGFR2/neu activation and tyrosine phosphorylation of β catenin; in addition, β-catenin accumulates in the cytoplasm due to its impaired degradation by the proteasome (32).

The incidence of obesity-related cancers has increased dramatically in the last few years (33), but the mechanisms linking obesity and breast cancer are poorly understood. Many groups have proposed that obesity increases the risk of breast cancer primarily due to the high levels of aromatase present in adipose and/or tumor tissues, which converts androgens into estrogens (34). Therapy of breast cancer with aromatase inhibitors is successful in a number of patients (35); however, other sources of estrogen-like molecules are not eliminated (reviewed in ref. 7).

Tamoxifen is a widely used therapy for breast cancer; however, loss of efficacy develops during treatment, probably

due to a complex molecular cross-talk between the SERM-filled ERs and the receptor-tyrosine kinase EGFR2/neu (36). Remarkably, many therapy-resistant breast cancers do retain ER expression, suggesting a sustained operation of the estrogen signaling pathways. Endogenously produced SERMs, similar to 27OHC, may associate to ERs keeping them activated. Cells expressing high levels of P450 Cyp27 hydroxylase, such as macrophages and fibroblasts, can be adjacent to neoplastic cells and supply these with 27OHC through the metabolism of widely available cholesterol (37). Obesity is associated with elevated cholesterol and 27OHC circulating levels; therefore, in overweight patients a stimulatory effect of this oxysterol on the proliferation of ER-positive cancer cells may be of relevance. This assumption is supported by studies demonstrating that hydrophobic statins may reduce the progression of mammary tumors (38). Our observations convey a warning to obese patients regarding the potential risk of high circulating levels of 27-hydroxycholesterol for the growth of estrogen-sensitive breast tumors. Furthermore, these results raise the question of whether tumor relapses and the incidence of metastases are higher in patients with elevated circulatory and local 27OHC levels. A similar question is pertinent in subjects with infiltrated mammary tumors, where macrophages and fibroblasts with a large capacity to metabolize cholesterol accumulate, synthesizing large amounts of 27OHC locally.

A deeper understanding of how ER cells respond to 27OHC and other SERMs requires further study. In the meantime, a careful characterization of the mechanisms involved in estrogen-stimulated breast cancer growth is needed to improve diagnosis and to predict responses to tumor-specific therapies (39). It is reasonable to state that many more interacting, still unknown, factors are probably involved in the modulation of mammary cancer cell growth.

Acknowledgements

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