

Proliferation of human mammary cancer cells exposed to 27-hydroxycholesterol

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Abstract. The aim of the present study was to identify the possible mechanisms by which certain estradiol receptor (ER)-positive mammary tumor cells remain resistant to treatment with anti-estrogens or inhibitors of local estradiol (E₂) production. To this end, we compared the proliferative effects on mammary cancer cells of the novel selective ER modulator 27-hydroxycholesterol (27OHC) to those of E₂, and evaluated their inhibition by ICI 182,780 (ICI). Analysis of the effects on the cell cycle of 27OHC and E₂ in the absence or presence of ICI was conducted. In ER-positive mammary tumor cells, we detected the blocking of 27OHC proliferation-stimulatory activity by simvastatin, as well as the inhibition of E₂-stimulated proliferation by an α -fetoprotein-derived cyclic nonapeptide. The effects reported herein may be extrapolated to infiltrating mammary cancer, where the activity of local macrophages may stimulate tumor growth. We suggest that increased breast cancer growth in obese patients may be related to increased 27OHC circulatory levels.

Introduction

Breast cancer is the leading cause of morbidity and mortality in women worldwide. Some types of breast cancer are under estrogen control (1). By associating with nuclear estrogen receptors (ERs), estradiol and selective modulators of estrogen receptors (SMER) affect the growth of mammary gland neoplasias through the regulation of genomic processes (2). In addition, estradiol and certain SMER associate with cognate receptors in the cell membrane or the cytoplasm, and control non-genomic effects (3,4).

The concentration of estrogens in breast tissue depends on the uptake from circulation and local production; the latter seems to be precisely controlled by the activity of a variety of nuclear receptors (5). Local levels of estradiol maintain the operation of numerous biological functions in normal and neoplastic breast tissue. Of particular importance among these is cell proliferation. For this reason, the blockade of ER activation by anti-estrogens and the reduction in estrogen availability through the use of inhibitors of aromatase and other enzymes involved in the local synthesis of the steroid are important therapeutic strategies used to hinder the progress of ER-positive mammary cancer (6). However, not all ER-positive breast cancers respond to these treatments, and many tumors eventually acquire resistance during therapy, probably due to the sustained functioning of ER-related pathways (7). Therefore, a better understanding of the mechanisms that maintain the activation of the ER signaling pathways in cancer cell progression is required for the development of new therapeutic approaches.

The association of a particular SMER to an ER has an effect on the overall conformation of the protein. The specific conformation of the complex determines the differential recruitment of co-activators or co-repressors as well as the biochemical and physiological activities to be expressed (7-9). Serum and the fluids bathing cells, organs and tissues contain compounds with the potential to imitate estrogens. In this regard, it has been reported that 27-hydroxycholesterol (27OHC), an oxysterol produced by the intramitochondrial oxidation of cholesterol, may act as a non-conventional SMER in estradiol-target cells (10,11). In normal subjects, plasma 27OHC concentration is in the same order of magnitude as the dissociation constant of the complex between ER and 27OHC, thus assuring their stable association. In endothelial cells, 27OHC functions as an estradiol antagonist, and some authors assume that its increased release by atheroma cells is responsible for damage to vascular endothelial and muscular cells in the vicinity of the atherosclerotic plaques (10,11). However, in ER-positive mammary tumor cells 27OHC works as an estradiol agonist, promoting cell proliferation (11).

In the present study, we compared the activity of 27OHC and estradiol on the proliferation of mammary cancer cells in culture, and determined their inhibition by the specific antagonist ICI 182,780. Analysis of the effects of 27OHC

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on the cell cycle was also conducted. Additionally, we evaluated the effects of simvastatin, a specific inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), on the proliferative activity of 27OHC. Lastly, we studied the effects of an α -fetoprotein (AFP)-derived cyclopeptide on tumor cell proliferation under estradiol and 27OHC stimulation.

Materials and methods

Tissue culture materials were obtained from NalgeNunc (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 majority of the other reagents were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

The AFP-derived nonapeptide [AFPep, cyclo(EKTOV-NOGN), where O is hydroxyproline] was generously supplied by Professor H.J. Jacobson and colleagues of the Albany Medical College, NY, USA.

Rabbit anti-c-erbB2 polyclonal antibody (ab2428) was obtained from Abcam plc (Cambridge, UK). Alexa Fluor 488 conjugated goat anti-rabbit IgG (A11008) was purchased from Molecular Probes-Invitrogen Corp. (Carlsbad, CA, USA).

E_2 -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 μ g/ml streptomycin. In the proliferation studies, the cells were transferred 24 h after seeding to DMEM/F12 containing ITS (insulin, transferrin and selenium), 1% charcoal/dextran-treated serum (CDTS), 3% hydroxyethylated starch (HAES), 50 U/ml penicillin and 50 μ g/ml streptomycin. The non-tumorigenic E_2 -insensitive epithelial cell line MCF10 (CRL-10317; ATCC, USA) was used for control studies, and was cultured under the same conditions as MCF7. In each of the experiments, the cells were incubated at 37°C in a humidified incubator under a 5% CO₂ atmosphere.

Proliferation studies. As indicated above, MCF7 or MCF10 (~20,000 cells/cm²), were seeded and incubated for 24 h to allow attachment, then non-adherent cells and media were removed. The remaining cells were washed and further incubated for various periods with low-serum culture medium containing either 2 nM E_2 or different concentrations of 27OHC or cholesterol, in the absence or presence of either 2 μ g/ml AFPep, 1 μ M simvastatin or 100 nM ICI. Upon 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 using the trypan blue assay. Each experiment was performed at least three times in triplicate.

Immunofluorescence studies. Cells exposed to different experimental conditions were grown on a sterile coverglass and then fixed (absolute methanol at -20°C), rinsed with DPBS and blocked for 30 min with 2% BSA in DPBS. Subsequently, the samples were incubated with the primary antibodies for 1 h at room temperature. After extensive washes in DPBS

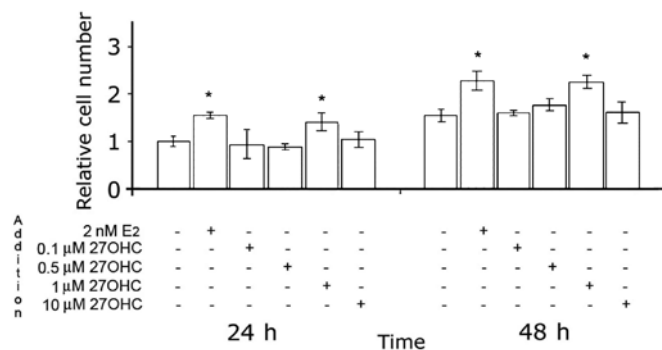


Figure 1. Effect of estradiol and 27OHC on the proliferation of MCF7 cells in culture. MCF7 cells were incubated for the indicated times in medium containing 1% charcoal-treated serum and 3% HAES in the absence or presence of 2 nM E_2 or with increasing concentrations of 27OHC. The proliferation rate was expressed as the relative cell number calculated in relation to the number of control cells at 24 h after seeding, which was set to 1. Values are the mean \pm SD of 3 individual experiments performed in triplicate. * $P < 0.05$ compared to non-stimulated control cells.

containing 2% BSA, the samples were incubated with the appropriate secondary antibody for 1 h and washed. Nuclei were counterstained with 7-aminoactinomycin D (BD Pharmingen, San Diego, CA, USA). After a final washing, 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 time and camera gain.

Cell cycle analysis. Approximately 8×10^5 MCF7 cells/well were seeded in 6-well plates and treated as described for the respective experiments. Analysis was performed using the FITC BrdU Flow kit (BD Pharmingen) following the manufacturer's instructions. After the different stimuli, cells were incubated for 240 min with 10 μ M BrdU and detached using trypsin-EDTA before fixation in BD Cytotfix/Cytoperm buffer. After DNase treatment to obtain a 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, then 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 E_2 or different concentrations of 27OHC in the absence or presence of 100 nM ICI. Flow analysis was performed with a BD-FACSCalibur cytometer.

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

Results

The effects of 27OHC on the proliferation of MCF7 cells in media containing low levels of serum were first analyzed. The results are depicted in Fig. 1, and indicate that 27OHC stimulated mammary tumor cell proliferation. The maximal

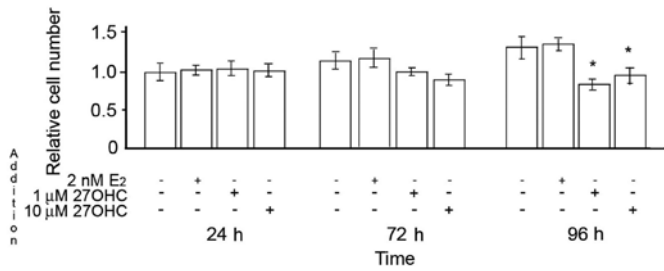


Figure 2. Proliferation of MCF10 cells cultured in the absence or presence of 2 nM estradiol or of two concentrations of 27OHC. The proliferation of MCF10 cells exposed or not to either estradiol or 27OHC is shown. The proliferation rate was expressed as the relative cell number calculated in relation to the number of control cells at 24 h after seeding, which was set to 1. Values are the mean \pm SD of 3 experiments performed in triplicate. Non-significant differences relative to the control cells were noted for cells exposed to 2 nM E₂. *Significant difference (P<0.05) relative to the non-stimulated control cells at 96 h.

stimulatory effect was obtained with 1-2 μ M 27OHC, and was in the same order of magnitude as that obtained with 2 nM E₂. Similar effects of E₂ and 27OHC, albeit with slower proliferation rates, were observed in ER-positive ZR75 cells (data not shown).

In contrast to MCF7 and ZR75 cells, when MCF10 cells were incubated under the same conditions, neither estradiol nor 27OHC had an effect on cell proliferation during the first 24 h. Later during the exposure, 2 nM E₂ still exhibited no effects, while 1-10 μ M 27OHC induced a reduction in the number of MCF10 cells in comparison to non-treated cells (Fig. 2).

The effects of 27OHC and estradiol on the proliferation of MCF7 cells, compared using BrdU pulses to stain proliferating cells in the S-phase of the cell cycle, were completely abolished by 100 nM ICI, as shown in Fig. 3.

Fig. 4 depicts a representative cycle analysis of cells stimulated for 48 h with 2 nM E₂ or with 1-2 μ M 27OHC, and the respective effects of 100 nM ICI. Stimulation with E₂ or 27OHC brought a similar number of cells into the S-phase. These effects were completely abolished by ICI. The analyses

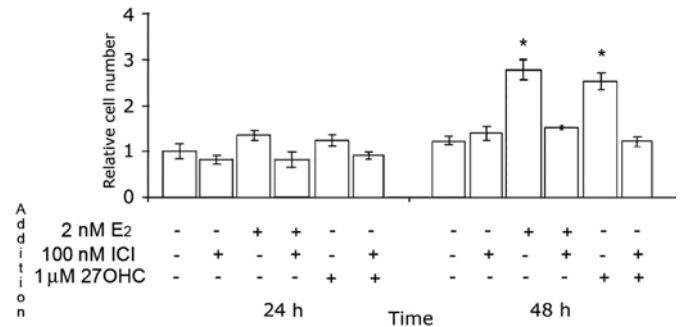


Figure 3. Effect of ICI 182,780 (ICI) on the proliferation in culture of MCF7 cells exposed or not to E₂ or 27OHC. The proliferation of non-exposed cells or of cells exposed to 100 nM ICI, 2 nM E₂ alone; 2 nM E₂ and 100 nM ICI, 1 μ M 27OHC alone, or 1 μ M 27OHC and 100 nM ICI, is depicted. The proliferation rate was expressed as the relative cell number calculated in relation to the number of control cells at 24 h after seeding, which was set to 1. Values are the mean \pm SD of 3 experiments performed in triplicate. * P<0.05 at 48 h for differences between E₂ vs. E₂ + ICI and 27OHC vs. 27OHC + ICI. Differences between the other pairs were non-significant.

also indicated that the extent of apoptosis was not significantly altered within the tested exposure duration. After 24 h of stimulus, E₂ and the novel SERM significantly increased the percentage of cells in the S-phase. In this case, the effects were completely abolished by 100 nM ICI (data not shown).

As depicted in Fig. 5, the effect of 27OHC was completely abolished by simvastatin. The maximal effect of this HMGCR inhibitor was obtained at 1 μ M; notably, the proliferation of MCF7 cells under estradiol stimulation was not affected by the addition of simvastatin at this concentration. Also of note, 2 μ g/ml AFPeP inhibited the proliferation of MCF7 cells stimulated with 2 nM E₂, but did not affect the influence of 27OHC on cell growth.

As compared to control non-stimulated MCF7 cells, EGFR2 immunoreactivity increased after a 15-min stimulation with 2 nM E₂. This effect was abolished by 2 μ g/ml AFPeP. No effects on EGFR2 reactivity were observed upon exposure to 1 μ M 27OHC, independent of the presence of AFPeP or

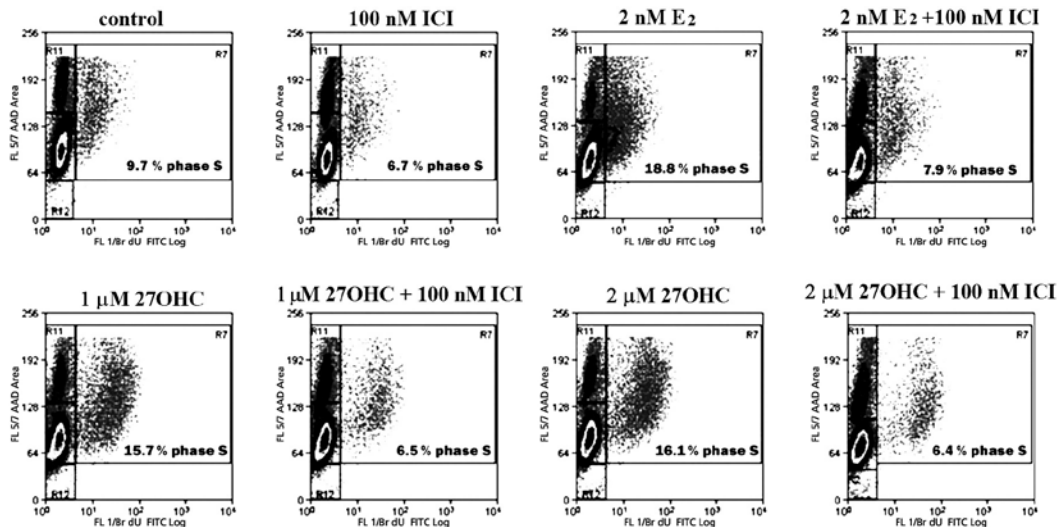


Figure 4. Cell cycle analysis of MCF7 after stimulation with E₂ or 27OHC. The panels depict representative dot-plots. Labels indicate the percentage of cells found at the S-phase after a 48-h long exposure to vehicle or to 2 nM E₂, 1 μ M 27OHC or 2 μ M 27OHC, in the absence or presence of 100 nM ICI.

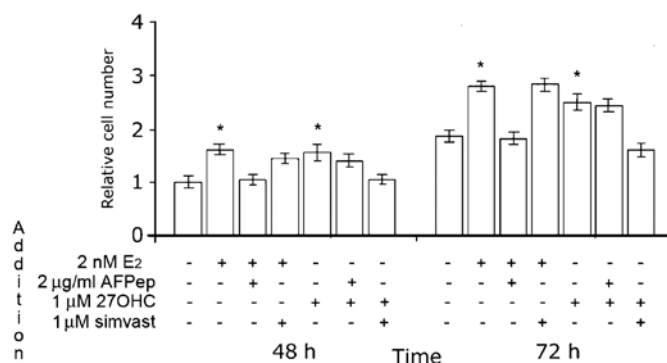


Figure 5. Differential effects of AFPep and simvastatin on the proliferation of unstimulated and stimulated MCF7 cells in culture. The proliferation of non-stimulated MCF7 cells or of cells exposed to 2 nM E₂ or to 1 µM 27OHC in the absence or presence of either 2 µg/ml AFPep or 1 µM simvastatin, is depicted. The proliferation rate was expressed as the relative cell number calculated in relation to the number of control cells at 24 h after seeding, which was set to 1. Values are the mean ± SD of 3 experiments performed in triplicate. * P<0.05 at 48 and 72 h for differences between AFPep + E₂ vs. E₂ and 27OHC + simvastatin vs. 27OHC.

Table I. Comparative effects of 27OHC and cholesterol on the proliferation of MCF7 cells.

Treatment	Proliferation	
	48 h	72 h
Control	1.00±0.06	1.51±0.05
1 µM 27OHC	1.78±0.07 ^a	2.63±0.07 ^a
1 µM 27OHC + 1 µM simvastatin	1.04±0.08	1.33±0.09
5 µM cholesterol	1.03±0.05	1.56±0.09
5 µM cholesterol + 1 µM simvastatin	0.96±0.05	1.36±0.09
20 µM cholesterol	1.10±0.03	1.61±0.02
20 µM cholesterol + 1 µM simvastatin	1.06±0.12	1.66±0.09

MCF7 cells (15,000/cm²) were seeded on P24 plates and incubated for 24 h in medium containing 10% fetal bovine serum. The attached cells were incubated for 24 h with medium containing 1% charcoal-treated serum, 3% HAES and ITS, and then exposed for the indicated times to either 27OHC or cholesterol. The proliferation rate was expressed as the relative cell number calculated in relation to the number of control cells at 48 h after seeding, which was set to 1. Values are the mean ± SD of 3 experiments performed in triplicate. The medium was either used directly as the control or adjusted to 1 µM 27OHC or 5 or 20 µM cholesterol in the absence or presence of 1 µM simvastatin. ^aP<0.05 at 48 or 72 h for differences between the control vs. 1 µM 27OHC.

simvastatin. Fig. 6 depicts representative fluorescence images of these experiments.

Unlike 27OHC, cholesterol added at concentrations of up to 20 µM did not show any stimulatory effect on MCF7 cell proliferation, as summarized in Table I.

Discussion

Cholesterol is abundantly distributed in eukaryotes. In higher animals, the main sources of circulating cholesterol are the uptake from fat rich foods and endogenous synthesis (12).

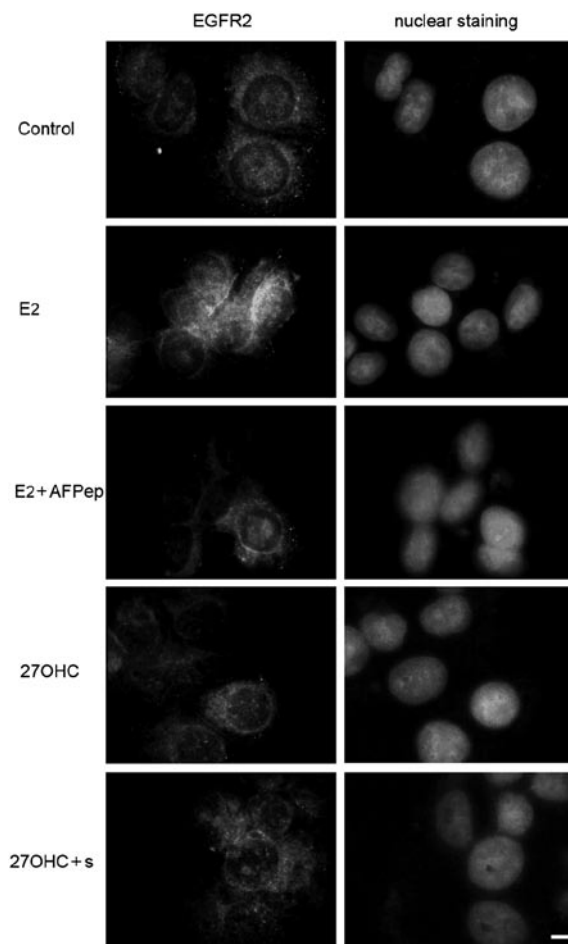


Figure 6. Immunofluorescence analysis of EGFR2 in MCF7 cells exposed to estradiol or 27OHC. Representative fluorescence images of EGFR2 in MCF7 cells grown for 48 h in medium containing 1% charcoal-treated serum and 3% HAES, then stimulated for 15 min with 2 nM E₂ alone, 2 nM E₂ + 2 µg/ml AFPep, 1 µM 27OHC alone or 1 µM 27OHC + 1 µM simvastatin (s), or maintained in medium alone (control). Images on the left depict EGFR2-immunofluorescence; images on the right depict nuclear staining of the respective cells. Scale bar, 20 µm.

The blood of normal human subjects contains several oxidized metabolites of cholesterol (oxysterols). Of these, 27OHC is the most abundant species, reflecting cholesterol saturation in the body and predicting to some extent the responsiveness to dietary cholesterol (13). Circulating 27OHC levels increase in cardiovascular disease (14), and this metabolite is easily detected in macrophages isolated from atherosclerotic lesions (15). Due to their higher polarity and limited stuffing in cell membranes, oxysterols share the ability to translocate faster than cholesterol across membranes, allowing their participation in cell signaling, lipid metabolism and vesicle transport (16). 27OHC affects some cellular functions in macrophages (17); in fact, the oxysterol exhibits a concentration-dependent regulation in human macrophages: low concentrations of 27OHC, favor cell survival, while high concentrations induce apoptosis (18). Some authors have reported that, in macrophages and in certain intestinal cancer human cell lines, 27OHC activates the liver orphan receptor α (LXRα), inducing the expression of efflux transporters ABCA1 and ABCG1 and thus promoting the detoxification of cholesterol metabolites (19). Many other cells are affected by 27OHC, including

vascular smooth muscle cells (20) and endothelial cells (21). Certain effects of 27OHC are probably a consequence of its potent HMGCR inhibitory activity (22).

In the present study, we compared the effects of 27OHC and estradiol on the proliferation of mammary tumor cells in culture. The results confirmed earlier evidence regarding a mitogenic activity of 27OHC in estrogen-responsive breast tumor cells. Similarly to estradiol, 1 μ M 27OHC was found to stimulate the proliferation of MCF7 cells, promoting significant changes in the fraction of cells entering the DNA-synthesis phase in the cell-cycle. The effects of 27OHC and estradiol on MCF7 cells were counteracted to the same extent by the pure estrogen-antagonist ICI 182,780. Neither estradiol nor 27OHC or ICI affected the proliferation of non-tumorigenic MCF10 cells. The stimulatory effect of 27OHC differed from that from E₂ in relation to the sensitivity to 1 μ M simvastatin and to AFPep, respectively. In the first case, obtained at the optimal concentrations of 27OHC and simvastatin, the results suggest that these HMGCR inhibitors may act additively, most probably causing a decrease in the isoprenylation capacity. At the same concentration of simvastatin, no major changes were observed in estradiol-induced MCF7 proliferation, probably because isoprenylation was not dramatically affected (Sierralta *et al.*, unpublished data).

In ER-positive human and canine mammary tumor cells, AFPep is an effective inhibitor of estradiol-stimulated proliferation (23,24). This cyclized nonapeptide disturbs membrane receptor-tyrosine kinase signaling pathways regulated by growth factors (25,26). By indirect immunofluorescence, we detected an increase in EGFR2 reactivity after 2 nM estradiol. This was abolished by AFPep; however, upon exposure to 1 μ M 27OHC, no changes in EGFR2 immunoreactivity were detected, suggesting that estradiol and 27OHC have different mechanisms of action.

27OHC is a low-affinity ER ligand, and there remain some doubts regarding its actual physiological relevance. As pointed out by DuSell and McDonnell (27), during estrogen-depletion, 27OHC affects ER by acting as a type of intracrine/paracrine modulator, and not as a classical endocrine agent. In mammary cancer cells, 27OHC works as a partial ER agonist that recruits ER α to target gene promoters and controls the transcription of ER target genes (11). The ability of 27OHC to stimulate, at physiological concentrations, the proliferation of ER α -positive mammary-cancer cells would appear to be of major physiological importance.

Occasionally, resistance develops during the treatment of breast cancer with anti-estrogens and aromatase inhibitors. Many therapy-resistant breast cancers do maintain ER expression, indicating the continued operation of estrogen signaling pathways. A permanently activated ER may well be the consequence of an association with endogenously produced SERMs, such as 27OHC. Through the metabolism of profusely available plasma membrane cholesterol, many cells produce and secrete 27OHC in their neighborhood, as demonstrated in the case of fibroblasts (28). The infiltration of macrophages is an indicator of poor prognosis in patients with a mammary gland affected by a tumor (29). It has been demonstrated that macrophages actively increase the local formation of 27OHC (30). Taken together, these findings suggest that, in tissues infiltrated by cancer, a constant stimu-

lation of tumor cells may be caused by the activity of local macrophages. It was previously thought that the increased risk of breast cancer caused by obesity was mainly due to enhanced aromatase capacity in adipose tissue, which generates higher local concentrations of estradiol. Since obesity is associated with hypercholesterolemia and increased 27OHC production, a proliferative effect of this oxysterol on ER-positive cancer cells is likely to be more intense in obese patients.

Besides binding to ER, 27OHC is considered a potential ligand of orphan receptor LXR (31). This interaction should be taken into consideration, since Vedin *et al.* (32) have reported the growth-inhibitory effects of LXR agonist GW3965 in ER-positive breast cancer cell lines. This anti-proliferative effect was absent in ER-negative cell lines, suggesting that ER plays an important mediator role in the process (32).

A complete understanding of whether the final cell response depends on the proportions of ER and LXR and the ratios of SERM and SLXRM present at a given time in a particular tissue requires further study. In the meantime, there is agreement that an ample characterization of all the mechanisms involved in estrogen-stimulated breast cancer growth is required to improve diagnosis and the ability to predict the response of a tumor to a specified therapy (33). As would be expected due to the complexity of any biological system, it is safe to predict that many more as yet unknown interacting factors are involved in the modulation of cancer cell growth.

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