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Effect of estradiol and antiestrogens on cholesterol biosynthesis in hormone-dependent and -independent breast cancer cell lines

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The effects of estradiol and/or antiestrogens on cholesterol biosynthesis were studied in two breast cancer cell lines. Cholesterogenic activity was evaluated after labeling cells with sodium [^{14}C]acetate for increasing periods of time (up to 24 h) and measuring the incorporation of the radioactivity into nonsaponifiable lipids and into cholesterol, after separation from other labeled metabolites. We compared the effects of estradiol on cholesterogenesis with the well-known effects of this hormone on cell proliferation: estradiol stimulated both cholesterol synthesis and cell growth in MCF-7 cells, but stimulated neither in BT20 cells. The stimulation affected both the 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase step and the post-HMGCoA steps. Only the key enzyme step appeared to be mediated by the estrogen receptor. The hydroxytamoxifen and LY 117018 antiestrogens strongly inhibited cellular cholesterol production in both cell lines. Under the same conditions, cell growth is affected in MCF-7 cells, but not in BT20 (as shown by groups from other laboratories). This demonstrates that de novo synthesis of cholesterol is not essential for cell growth when cells are cultured in the presence of whole serum. The inhibition of cholesterol synthesis by antiestrogens mainly affected the lanosterol demethylation step and the C-27 sterol to cholesterol conversion. This inhibiting effect of antiestrogens was not mediated by the estrogen receptor.

Introduction

For several years, considerable attention has been focused on the relationships between the processes of cell growth and cholesterol biosynthesis. Extensive evidence indicates that cholesterogenesis is closely linked to cell growth [1–7]. When cells are synchronized, an increase in 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase activity (the key enzyme in the process) is observed at or just prior to the S phase of the cell cycle; in the absence of a cholesterol supply by the culture medium, inhibition of cholesterol biosynthesis at the key enzyme step totally prevents DNA replication [8–13].

In a preliminary work [14], we showed some effects of estradiol and tamoxifen on cholesterol biosynthesis in MCF-7 cells. MCF-7 is a breast cancer cell line [15], which is known to be highly sensitive to estrogens and antiestrogens [16–18]. Thus, the MCF-7 cell line appeared to be a convenient model for studying cholesterol biosynthesis and proliferation relationships. There was a

Abbreviations: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34); TLC, thin-layer chromatography; FCS, fetal calf serum; DSS, steroid-depleted serum; LPS, lipoprotein-poor serum; LDL, low-density lipoprotein; E2, estradiol; PBS, phosphate-buffered saline.

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parallelism between the effects on cholesterol biosynthesis which we observed and the effects on cell growth reported by others [16–18]: estradiol stimulated and tamoxifen inhibited cholesterol biosynthesis.

Thus, our objective was to elucidate the mechanism of these effects, and, especially, to investigate the possible role of estrogen receptors which are known to be involved in MCF-7 cell proliferation [16,17,19]. Moreover, we tried to determine which step of cholesterol biosynthesis is estradiol receptor dependent in cell lines the proliferation of which is hormone dependent. Therefore, we compared the effects of estrogens and antiestrogens at several steps of cholesterol synthesis in (a) the estradiol-dependent MCF-7 cell line and (b) the estradiol-independent BT20 cell line [20]. Cell proliferation of BT20 cells, which are devoid of estradiol receptors [21], is not regulated by either estradiol or by tamoxifen [19].

Materials and Methods

Materials

Sodium [2-^{14}C]acetate (45–55 mCi/mmol), was from C.E.A. (France); DL-3-hydroxy-3-methyl[3-^{14}C]glutaryl CoA (50 mCi/mmol) and [^{3}H]-mevalonic acid lactone were supplied by Amersham. Tamoxifen and *trans*-4-hydroxy-tamoxifen, (1,4-(2-dimethylaminoethoxy)phenyl-1-(4-hydroxyphenyl)-2-phenylbut-1-ene), were gifts from ICI, France. LY117018, 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(*b*)thien-3-yl-*p*-(2-(1-pyrrolidinyl) ethoxy)phenylketone was provided by Eli Lilly Co. (Indianapolis, IN, U.S.A.). Estradiol-17β was from Sigma. Kieselgel 60F_{254} plates (DC Alufolien) and kieselgel GF plates (on glass), were supplied by Merck. Reference samples of sterols were from Steraloids and culture media were from Gibco or Seromed.

Radiochromatograms were scanned on Berthold LB 2760 and Numelec PHAl analyzer; the radioactivity of solutions was measured with a Packard scintillation counter (TRI-CARB 460CD) in a toluene solution of 2,5-diphenyloxazole.

Methods

Steroid-depleted serum (DSS). Fetal calf serum was stripped of endogenous steroids by dextran/charcoal absorption [17]. Estradiol removal was controlled by radioimmunoassay and its concentration was less than 50 pM.

Lipoprotein-poor serum. Lipoprotein content was removed by a 3-day ultracentrifugation of fetal calf serum at $d = 1.20$ in a Beckman Model L3-50 ultracentrifuge with a rotor 35 [13].

Cell culture. Cells were cultured as monolayers; MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Seromed) supplemented with 5% fetal calf serum. BT20 cells [20] were grown in Eagle's medium with Earle's salt and non-essential amino acids (Seromed) and supplemented with 10% fetal calf serum (Gibco).

Addition of effectors. For the experiments, the proportion of serum was progressively decreased to 2% at least 2 days prior to exposure to the drug. In the presence of fetal calf serum: the effector (estradiol, hydroxytamoxifen or LY 117018) was added in ethanol solution (final concentration of ethanol 0,1%) to cells grown in the presence of 2% fetal calf serum (FCS). The cells were then incubated for 24 h. Ethanol alone was added to the control culture.

In the presence of steroid-depleted serum, cells were first grown in the presence of FCS, then in medium without Phenol red-enriched with 5% steroid depleted serum for 5 days and seeded in petri dishes. After 2 days, the medium was replaced by Phenol red-free medium with 2% steroid depleted serum, in which cells were again cultured for 2 days. Effector was then added as described above.

Determination of sterol biosynthesis

Four TLC systems were used: system I, (silicagel plate) hexane/ether/acetic acid, (50:50:0.5, v/v) a 2-h continuous run at room temperature, was used. System II, (glass precoated ($+NO_3Ag$) plate) carbon tetrachloride/benzene, (90:10) a 20-h continuous run at 4°C. System III, (glass precoated ($+NO_3Ag$) plate) carbon tetrachloride/benzene, (95:5) a 15 h continuous run at 4°C. System IV, (silicagel plate) hexane/ethyl acetate (97:3) one run.

The effector-containing medium was replaced by the labeling medium (25 μCi of [^{14}C]acetate per ml culture medium). After incubation, the cells were washed three times with PBS and trans-

ferred to glass tubes containing 2 ml of 10% sodium hydroxide. After 1 hour of saponification at 85–90 °C, the total ^{14}C incorporation into cells was measured (in an aliquot) and the non-saponifiable material was extracted with petroleum ether.

After evaporation of the solvent, the non-saponifiable material was analyzed according to Tabacik et al. [22–24]. Briefly, the non-saponifiable material was fractionated by thin-layer chromatography (TLC) in system I into the C-27 sterol zone, C-28 sterol + aliphatic alcohol zone, C-29 + C-30 sterol zone and front zone. The three sterol zones were reduced with BH_4Na in order to separate the bifunctional metabolites as more polar diols (TLC in system I).

For C-27 sterol analysis, the C-27 sterol zone was acetylated (pyridine + acetic anhydride) and the acetate mixture (supplemented with 10 μg of lathosterol acetate) was spotted on a NO_3Ag-impregnated plate. The plate was developed in system II. For preparation of the plates, glass precoated silicagel G plastes were dipped into a 10% aqueous NO_3Ag solution, then activated for 2 h at 120 °C. The front zone was fractionated on a silicagel plate in system IV with the reference samples: squalene, oxidosqualene, and cholest-7-en-3-one (as sterone). The sterone zone was reduced with BH_4Na (20 h in aqueous dioxan at room temperature) and the sterols obtained were analyzed by TLC in system I, then as acetates in system III. Cholesterogenic activity was expressed as a percentage: (dpm isoprenoids/dpm total incorporation) × 100. The *n*-fatty alcohol zone was oxidized by CrO_3 (in petrol ether/acetic acid, 70:30) for 18 h at room temperature, and then analyzed in system I.

HMG-CoA reductase activity

HMG-CoA reductase activity was measured according to the previous description [25] and modified as follows: after 24 h of culture in the presence of effectors, the culture medium was removed, cells were washed three times in PBS scraped and resuspended in 50 mM phosphate buffer (pH 7.4), 5 mM dithiothreitol and 1 mM EDTA. They were then homogenized by sonication (3 × 5 s at 0 °C). The reaction mixture contained 50–100 μg proteins, 5 mM dithiothreitol, 20 mM glucose 6-phosphate, 2.5 mM $NADP^+$, 0.1 M phosphate buffer and 0.14 IU of glucose-6-phosphate dehydrogenase (all from Boehringer Mannheim, F.R.G.) in a final volume of 75 μl. In blank assays, proteins were heated at 100 °C for 10 min. After preincubation at 37 °C for 15 min, the reaction was started by adding 1 nmol DL-3-hydroxy-3-methyl[3-^{14}C]glutaryl CoA ([^{14}C] HMG-CoA) and 5 nmol of unlabeled HMG-CoA. After a 40 min incubation, the reaction was stopped by 10 μl 6M HCl. Lactonization was performed at 37 °C for 30 min. 50000 dpm of [^3H]-mevalonic acid lactone and 250 μg of unlabeled mevalonic acid lactone were then added. Each sample was spun at 1600 × *g* for 15 min; the supernatant was spotted on a silica gel plate (Whatman LK5D) (1 run with ethyl acetate/acetone, 80:20). The percentage of synthesized [^{14}C]mevalonolactone was evaluated by scanning radioactivity on a Numelec scanner and counting the corresponding gel zones with a Packard scintillation counter. Proteins were measured according to Bradford's method using a Bio-Rad reagent, with bovine serum albumin as the standard.

Results

Kinetic study of cholesterol and cholesterol precursor biosynthesis: basal activity

First we compared cholesterogenic activity in the MCF-7 and BT20 cell lines in the absence of estradiol and antiestrogens. Cells were incubated in the presence of sodium [2-^{14}C]acetate for different times ranging from 0 to 24 h, and the labeled cholesterol was evaluated in the non-saponifiable material, according to a procedure developed earlier [22,23] and routinely used in our laboratory [24,26]. Since fatty acids remain in the alkaline aqueous phase after lipid extraction, the extracted non-saponifiable material consists exclusively of cholesterol and its precursors, except in MCF-7 cells which actively synthesize fatty alcohols. In this case, it was necessary to evaluate the part of aliphatic neutral metabolites: thus, the isoprenoid fraction represents the non-saponifiable material after substraction of fatty alcohols.

Table I shows the amount of total labeled isoprenoids, cholesterol, as well as the composition (%) of metabolites in the isoprenoid mixture.

TABLE I

TIME COURSE OF MCF-7 AND BT20 CELL LABELING: COMPOSITION OF THE RADIOACTIVE ISOPRENOID MIXTURE

Cells were grown in the presence of 2% fetal calf serum (FCS) or steroid-depleted serum (DSS). They were incubated with sodium [^{14}C]acetate for different periods and the labeled non-saponifiable lipids were analyzed as described in the Materials and Methods. The amount of each metabolite fraction is expressed as the percentage of total labeled isoprenoid lipids or as dpm·10^{-3} per mg cell protein (mean of duplicates).

Cell line: MCF-7				BT20		
Culture medium: DSS		FCS		DSS	FCS	
Incubation time (h): 6		6	24	2	2	24
% Apolar compounds (squalene, sterones)	15	8	7	17	15	3
% Methylsterols	18	29	14	19	22	32
% Bifunctional metabolites	15	6	2	19	35	12
% C-27 sterols including:	52	57	77	45	28	43
(cholesterol)	(15)	(21)	(57)	(24)	(18)	(39)
Amount (dpm·10^{-3}/mg protein) synthesized						
Isoprenoids	303.6	861.2	1505.2	2025.7	781.8	7691.6
Cholesterol	45.5	180.8	858.0	486.2	140.7	2999.7
% Transformation of total radioactivity						
into isoprenoids	1.4	3.4	5.5	28.7	16.2	32.7
into cholesterol	0.21	0.72	3.1	6.9	2.9	12.7

The percentage of C-27 sterols increased with labeling time, whereas the percentage of methyl sterols and bifunctional metabolites decreased. The latter compounds have been shown to consist mainly of oxymethyl sterol intermediates in the lanosterol demethylation [22,27].

In BT20 cells, the labeling of nonsaponifiable lipids reached a high level after 24 h (5-times higher than in MCF-7 cells) and the rate of transformation into cholesterol was higher than in MCF-7 cells, in spite of a lower percentage in nonsaponifiable lipids (39% versus 57%).

Culture conditions exerted an important effect on cell behaviour: in MCF-7 cells, steroid-depleted serum induced an important inhibition of cholesterogenesis (2.5-fold for isoprenoids, 3.5-fold for cholesterol). In BT20 cells, the effect was the reverse: when cells were cultured in steroid-depleted serum, a stimulation of both isoprenoid and cholesterol synthesis was observed.

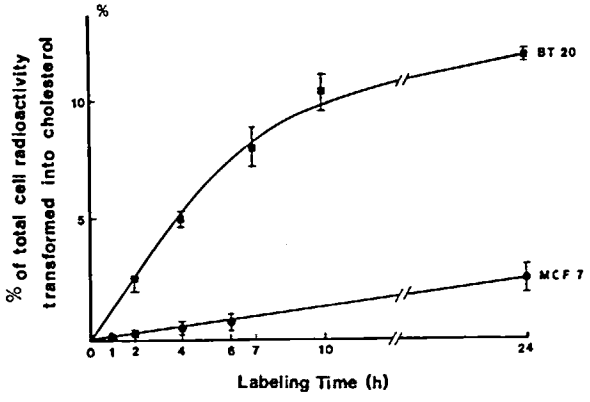

Fig. 1. Time course of cholesterol synthesis from acetate by MCF-7 and BT20 cell lines. Cells were cultured in 2% FCS-supplemented medium and incubated with sodium [2-^{14}C]acetate for different time periods, and the radioactivity incorporated into cholesterol and whole cells was determined. The results are expressed as a percentage of the first parameter versus the second: (■) BT20 cells, (●) MCF- cells (mean of duplicate determinations).

The variation of the cholesterogenesis time course in both cell lines are compared in Fig. 1. Cholesterogenic activity is expressed as the percentage of total cell-incorporated radioactivity transformed into cholesterol. By contrast with the absolute value of the metabolite radioactivity, this parameter takes into account the possible variations of cell permeability to labeled sodium acetate added to the culture medium. BT20 cells are highly cholesterogenic, whereas activity of MCF-7 cells is low. These data were necessary to determine the experimental conditions of the subsequent study: the effect of estrogens and antiestrogens on cholesterol biosynthesis. A short labeling time (1–2 h) was sufficient for BT20 cells, whereas MCF-7 required a longer incubation time (6–7 h).

Effect of estradiol and hydroxytamoxifen on cholesterol biosynthesis

In a preliminary study on MCF-7 cells [14], we observed a stimulation of cholesterol synthesis by estradiol and an inhibition by tamoxifen, which paralleled the effects on cell growth observed by others [16–18]. To assess whether this could be mediated by the estrogen receptor, we compared the action of estradiol and antiestrogens in estradiol-sensitive to -insensitive cell lines. The effects were evaluated at two levels: the transformation of total incorporated radioactivity (a) into isoprenoids (a parameter which varied in parallel with HMG-CoA reductase activity) and (b) into cholesterol. All data are expressed as percentages of the control experiment, performed with the vehicle (ethanol) alone.

MCF-7 cells. We studied in succession (a) the variation of the effects as a function of the effector concentration, (b) the competitivity of the effectors and (c) the reversibility of their action.

The first experiment was carried out in the presence of 2% fetal calf serum (FCS). In Fig. 2, isoprenoid synthesis is shown as a function of effector concentration. As expected, no estradiol effect on isoprenoid synthesis or on cholesterol synthesis was observed in this medium. Hydroxytamoxifen did not inhibit isoprenoid synthesis at concentrations lower than 10^{-8} M; a slight effect (20%) began only at 10^{-7} M. Cholesterol synthesis was inhibited by 50% at concentrations of 10^{-8} M and 100% at 10^{-7} M hydroxytamoxifen.

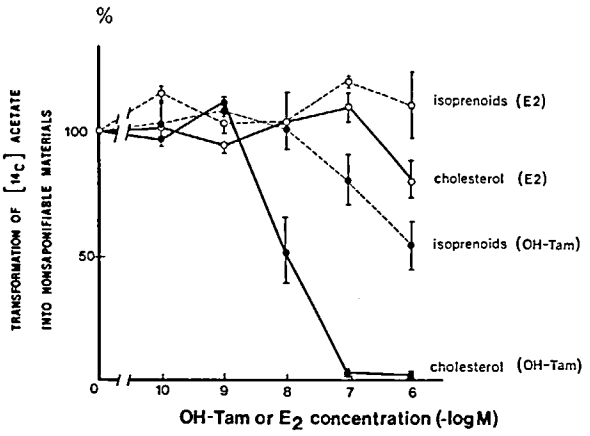

Fig. 2. Effect of estradiol and hydroxytamoxifen (OH-Tam) on cholesterol and isoprenoid synthesis in MCF-7 cells in the presence of fetal calf serum. Cells were cultured for 24 h in DMEM containing 2% FCS and different concentrations of estradiol (○) or hydroxytamoxifen (●). At $t = 0$, the culture medium was removed, replaced by fresh culture medium with sodium [2-^{14}C]acetate (25 μCi/ml) and cells were incubated for 6 h at 37° C. Transformation into isoprenoids (— — —) and cholesterol (———) was evaluated as described in the Materials and Methods. The results are expressed as a percentage of the control (mean of duplicates). Control values represent synthesized isoprenoids (250000 dpm/mg of cell protein) or synthesized cholesterol (106000 dpm/mg of cell protein).

The same study, performed in 2% steroid-depleted serum, showed a weak inhibition of isoprenoid synthesis (20% at 10^{-7} M) and a very strong inhibition of cholesterol synthesis, which began at concentrations as low as 10^{-9} M hydroxytamoxifen (over 50%) (Fig. 3). In this medium, the effect of estradiol was very high: isoprenoid synthesis was stimulated by 200% as a 'plateau' and cholesterol synthesis by 500%. There was a good correspondence between the effect of estradiol on cholesterol synthesis and that on DNA synthesis reported by others [16–18].

The competitive nature of the effectors was tested (Fig. 3) by simultaneous addition of increasing amounts of hydroxytamoxifen to a constant concentration of estradiol (10^{-8} M). Isoprenoid synthesis stimulation induced by estradiol was very weakly modified (except at 10^{-6} M hydroxytamoxifen). This is in agreement with the small effect observed with hydroxytamoxifen alone. Cholesterol synthesis stimulation was sup-

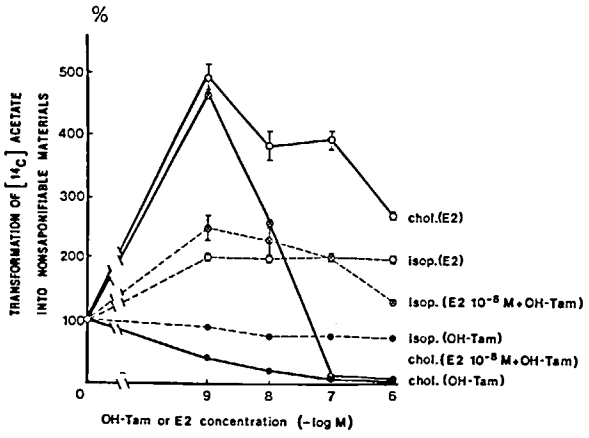

Fig. 3. Competitive effect of estradiol and hydroxytamoxifen (OH-Tam) on cholesterol and isoprenoid synthesis in MCF-7 cells in steroid-depleted serum. Cells were cultured for 24 h in DMEM containing 2% DSS and different concentrations of estradiol (○) or hydroxytamoxifen (●) or hydroxytamoxifen plus a constant concentration (10^{-8} M) of estradiol (⊗). At $t = 0$, the culture medium was removed, replaced by fresh culture medium with sodium [2-^{14}C]acetate (25 μCi/ml) and cells were incubated for 6 h at 37°C. Transformation into isoprenoids (— — —) and cholesterol (———) was evaluated as described in the Materials and Methods. The results are expressed as a percentage of the control (mean of duplicates). Control values represent synthesized isoprenoids (304000 dpm/mg of cell protein) or synthesized cholesterol (44000 dpm/mg of cell protein).

pressed at concentrations of hydroxytamoxifen higher than 10^{-8} M hydroxytamoxifen.

The reversal by estradiol of the effect of hydroxytamoxifen was tested as was previously done for cell proliferation and thymidine incorporation into DNA [16–18]. The stimulation of isoprenoid synthesis was completely restored by adding estradiol to the hydroxytamoxifen-treated cells (Fig. 4); the restoration required higher concentrations of estradiol (10^{-7} M) when the cells were cultured with a higher (10^{-7} M) concentration of hydroxytamoxifen. In cells cultured with 10^{-8} M hydroxytamoxifen plus estradiol, a moderate stimulation of cholesterol synthesis was observed, never reaching the value obtained with estradiol alone. In cells cultured with 10^{-7} M hydroxytamoxifen plus estradiol, a strong inhibition was observed. These results show that there was no restoration of cholesterol synthesis.

The experiments described above suggest that the effect of hydroxytamoxifen on estradiol-promoted isoprenoid synthesis is competitive and reversible, but that is not however, the case for cholesterol synthesis. This difference suggests that estrogen receptor is involved at the level of the HMG-CoA reductase regulation, but not on post-HMG-CoA steps. From analysis of labeled isopre-

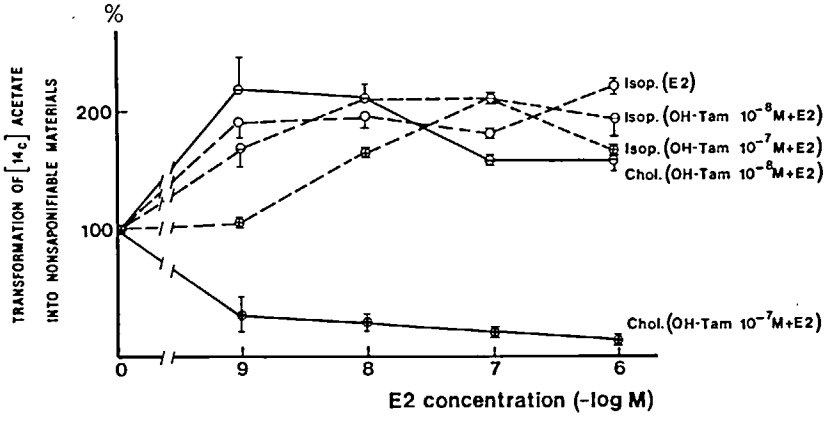

Fig. 4. Restoration by estradiol of cholesterol and isoprenoid synthesis inhibition in MCF-7 cells. Cells were cultured for 24 h in DMEM containing 2% DSS with no inhibitor (○) or with 10^{-8} M hydroxytamoxifen (OH-Tam) (⊖) or 10^{-7} M hydroxytamoxifen (⊕). On the second day, increasing concentrations of estradiol were added to each culture flask and cells were cultured for a further 24 h. The culture medium was removed, replaced by fresh culture medium with sodium [2-^{14}C]acetate (25 μCi/ml) and cells were incubated for 6 h. Transformation into isoprenoids (— — —) and cholesterol (———) was evaluated as described in the Materials and Methods. The results are expressed as a percentage of the control cultured with no inhibitor (mean of duplicates). Control values represent synthesized isoprenoids (280000 dpm/mg of cell protein) or synthesized cholesterol (28000 dpm/mg of cell protein).

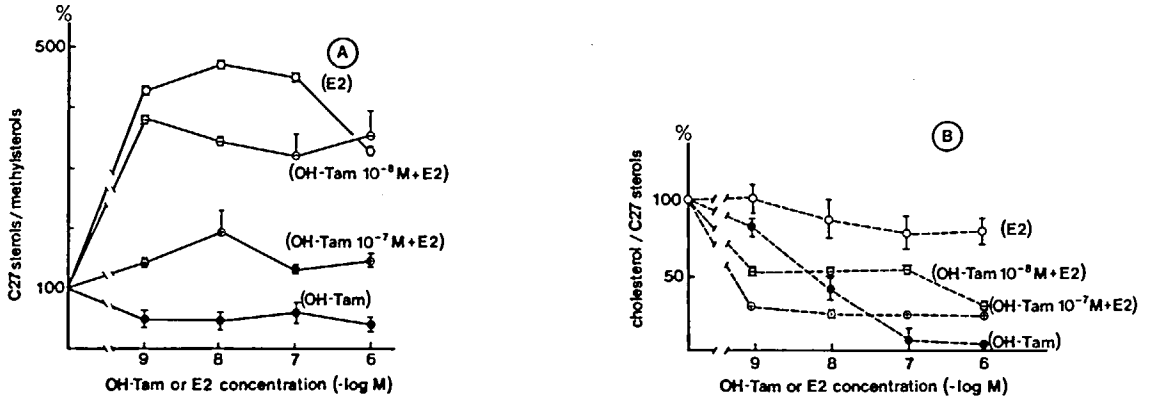

Fig. 5. Variation of the ratios C-27 sterols/methylsterols (A) and cholesterol/C-27 sterols (B) in restoration experiments in MCF-7 cells. Cells were cultured for 24 h in DMEM containing 2% DSS with no inhibitor or with 10^{-8} M hydroxytamoxifen (OH-Tam) (⊖) or 10^{-7} M hydroxytamoxifen (⊕). Increasing concentrations of estradiol were then added to each culture flask and cells were cultured for a further 24 h. Estradiol alone (○) or hydroxytamoxifen alone (●). The culture medium was removed, replaced by fresh medium containing sodium [2-^{14}C]acetate (25 μCi/ml) and cells were incubated for 6 h at 37°C. The ratios of C-27 sterols/methyl sterols (———) and cholesterol/C-27 sterols (— — —) were evaluated according to the procedure described in the Materials and Methods and are expressed as a percentage of the control cultured with no effector (mean of duplicates determinations).

noids, it was possible to localize which steps were involved. As shown in Table I, the isoprenoid mixture consists mainly of methyl sterols (arising from partial demethylation of lanosterol: C-28, C-29 and C-30 sterols) and C-27 sterols (isomers of cholesterol or less hydrogenated C-27 metabolites) the proportions of which were affected by estradiol and hydroxytamoxifen. The difference of sensitivity in the different steps of cholesterol biosynthesis is clearly viewed when considering the ratios C-27 sterols/ methylsterols and cholesterol/C-27 sterols. Fig. 5a shows the variation of the ratio C-27 sterols/methylsterols in relation to the control, in the rescue experiment (shown in Fig. 4). This ratio grows sharply with estradiol alone to five times the value of the control, and decreases with hydroxytamoxifen alone to 50% of the control at 10^{-9} M. This result indicates a high sensitivity of the demethylation process to estradiol or antiestrogens. The ratio cholesterol/C-27 sterols were by far the least affected (Fig. 5b) by estradiol, but a decrease was observed in the presence of hydroxytamoxifen at concentrations higher than 10^{-9} M.

Another non-steroidal antiestrogen, LY117018, which is more potent on cell growth inhibition than hydroxytamoxifen [28], was tested (Fig. 6). Its effect on cholesterogenesis was very similar to

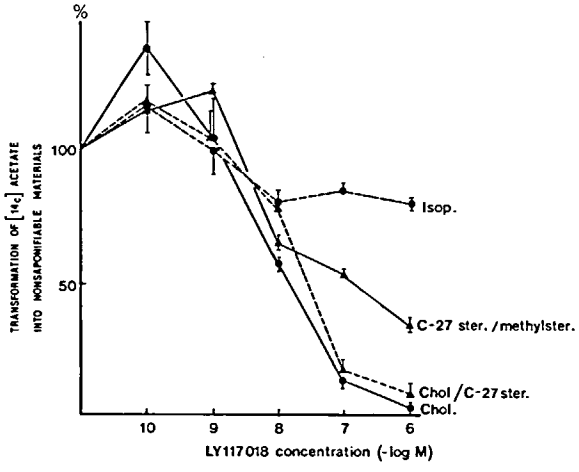

Fig. 6. Effect of LY 117018 on cholesterol and isoprenoid (isop.) synthesis and on the ratios C-27 sterols/methylsterols or cholesterol/C-27 sterols in MCF-7 cells. Cells were cultured for 24 h in DMEM containing 2% FCS and different concentrations of LY 117018. The culture medium was removed, replaced by fresh medium containing sodium [2-^{14}C]acetate (25 μCi/ml) and cells were incubated for 6 h at 37°C. Transformation into isoprenoids (●— — —●) and cholesterol (●———●) and the ratios of C-27 sterols/methylsterols (▲———▲) and cholesterol/C-27 sterols (▲— — —▲) were evaluated according to the procedure described in the Materials and Methods and are expressed as a percentage of the control cultured with no effector (mean of duplicate determinations). Control values represent synthesized isoprenoids (890000 dpm/mg of cell protein) or synthesized cholesterol (217000 dpm/mg of cell protein).

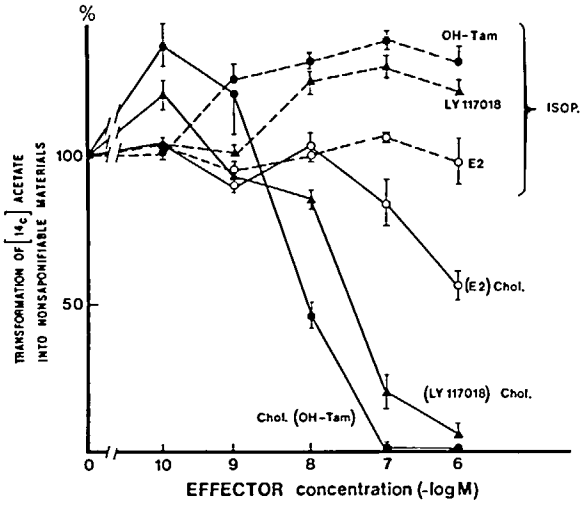

Fig. 7. Effect of estradiol, hydroxytamoxifen (OH-Tam) and LY 117018 on cholesterol and isoprenoid synthesis in BT20 cells. Cells were cultured for 24 h in Eagle's medium containing 2% DSS and different concentrations of estradiol (○) or in Eagle's medium containing 2% FCS and increasing concentrations of hydroxytamoxifen (●) or LY 117018 (▲). The culture medium was removed, replaced by fresh medium containing sodium [2-^{14}C]acetate (25 μCi/ml) and cells were incubated for 2 h at 37°C. Transformation into isoprenoids (— — —) and cholesterol (———) was evaluated as described in the Materials and Methods. The results are expressed as a percentage of the control (mean of duplicate determinations). Control values represent synthesized isoprenoids (946000 dpm/mg of cell protein) or synthesized cholesterol (165000 dpm/mg of cell protein).

that of hydroxytamoxifen: isoprenoid synthesis was weakly affected, whereas cholesterol synthesis was strongly inhibited (about 45% at 10^{-8} M and 90% at 10^{-7} M). This post-HMG-CoA reductase inhibition occurred both at the lanosterol demethylation and at the C-27 sterol to cholesterol conversion step.

Thus, as far as the post-HMG-CoA steps are concerned, estradiol strongly activated the demethylation step without affecting the conversion of C-27 sterol into cholesterol, whereas antiestrogens were active at both steps.

BT20 cells. The effect of estradiol was tested in the presence of steroid-depleted serum (Fig. 7): this cell line is devoid of estrogen receptors, and as expected, no stimulation of isoprenoid or cholesterol synthesis was observed. At 10^{-6} M, an inhibition occurred (45%) which may indicate that estradiol became cytotoxic.

The effect of hydroxytamoxifen was tested in the presence of fetal calf serum. A slight stimulation of isoprenoid synthesis was observed, with increasing concentrations of hydroxytamoxifen (30% at 10^{-8} M), whereas cholesterol synthesis was strongly inhibited (50% at 10^{-8} M, 100% at 10^{-7} M). LY117018 acts similarly, but less so than hydroxytamoxifen. The post-HMG-CoA step involved in this inhibition is partly lanosterol demethylation, mostly the transformation of C-27 sterols into cholesterol as shown by the decline of the ratio cholesterol/C-27 sterols (Fig. 8).

To test hydroxytamoxifen under exactly the same conditions as in cell growth experiments, BT20 cells were cultured in the presence of inhibitor for 9 days (Fig. 9). Under these conditions, inhibition of the cholesterol synthesis was more drastic than after 1 day of incubation (90% by contrast with 45% at 10^{-8} M), and the lanosterol demethylation step was more involved.

Effect of estradiol and hydroxytamoxifen on HMG-CoA reductase activity

To confirm the effects of the estradiol and antiestrogens on the transformation of cell-incorporated radioactivity into non-saponifiable lipids, we measured the activity of the key enzyme HMG-CoA reductase in MCF-7 and BT20 cells (Table II). The effect of estradiol was measured after 24 h of culture in the presence of hormone and steroid-depleted serum (DSS) and that of hydroxytamoxifen was measured after 24 h of culturing in the presence of inhibitor and fetal calf serum (FCS). To evaluate the possibility of a direct action of the effectors on the enzyme, we also measured the enzyme activity after simultaneous addition of these effectors to the substrate [^{14}C]HMG-CoA on cell homogenate. A supplementary control was run by culturing cells (for 4 days) in the presence of lipoprotein-poor serum (LPS).

Stimulation of MCF-7 cells was observed after culturing with estradiol, and inhibition occurred after culturing with hydroxytamoxifen, but not after a direct addition of the effector to the cell homogenate. Since there was no direct effect, the phenomenon apparently requires cell integrity. It was also noted that culturing in lipid-free medium, instead of enhancing HMG-CoA reductase activ-

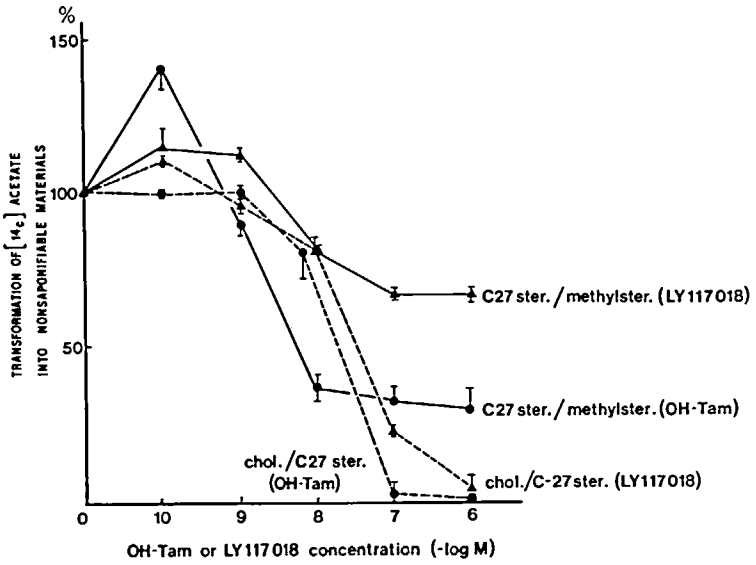

Fig. 8. Effect of hydroxytamoxifen and LY 117018 on the ratios of C-27 sterol/methylsterols and cholesterol/C-27 sterols in BT20 cells. Cells were cultured in Eagle's medium containing 2% FCS and increasing concentrations of hydroxytamoxifen (●) or LY 117018 (▲). The culture medium was removed, replaced by fresh medium containing sodium [2-^{14}C]acetate (25 μCi/ml) and cells were incubated for 2 h at 37 °C. The ratios of C-27 sterols/methylsterols (———) and cholesterol/C-27 sterols (— — —) were determined according to the procedure described in the Materials and Methods. The results are expressed as a percentage of the control (mean of duplicate determinations).

TABLE II

EFFECT OF ESTRADIOL (E2) AND HYDROXYTAMOXIFEN (OH-Tam) ON HMG-CoA-REDUCTASE ACTIVITY IN MCF-7 AND BT20 CELLS

After 24 h of culture with or without effectors, cells were washed and homogenized. For the evaluation of a direct effect, ethanol (control) or effectors were added to the incubation medium of some homogenated samples obtained from untreated cells. HMG-CoA reductase activity was measured in the homogenate as described in the Materials and Methods. The data are means of triplicate determinations.

Cell line	Culture medium	Effector added		HMG-CoA reductase activity pM/min per mg protein
		culture medium (indirect effect)	cell homogenate (direct effect)	
MCF-7	DSS	0	0	36.9±1.6
	DSS	0	10^{-9} M E2	37.2±1.0
	DSS	10^{-9} M E2	0	49.8±0.8
	FCS	0	0	47.3±1.5
	FCS	0	10^{-7} M OH-Tam	45.2±0.3
	FCS	0	10^{-6} M OH-Tam	45.6±1.4
	FCS	10^{-7} M OH-Tam	0	32.5±2.0
	LPS	0	0	33.7±1.1
BT20	DSS	0	0	43.5±1.4
	DSS	10^{-9} M E2	0	45.2±1.7
	FCS	0	0	28.1±0.7
	FCS	10^{-7} M OH Tam	0	45.3±1.0
	LPC	0	0	79.9±4.1

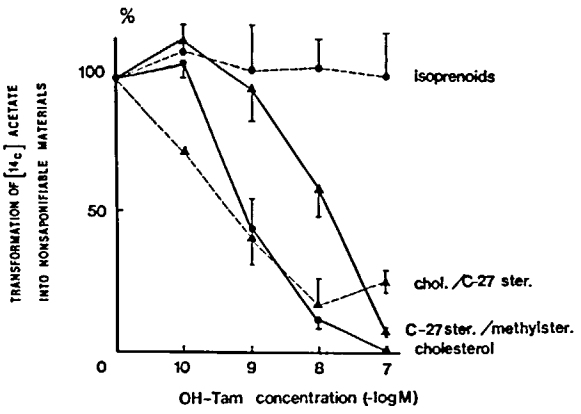

Fig. 9. Effect of hydroxytamoxifen on cholesterol and isoprenoid synthesis and on the ratios C-27 sterols/methylsterols or cholesterol/C-27 sterols after 9 days of culturing with BT20 cells. Cells were cultured for 9 days in Eagle's medium containing 5% FCS and increasing concentrations of hydroxytamoxifen. The culture medium was removed, replaced by fresh medium containing sodium [2-^{14}C]acetate (25 μCi/ml) and cells were incubated for 2 h at 37°C. Transformation into isoprenoids (●— — —●) and cholesterol (●———●) was evaluated as well as the ratios C-27 sterols/methylsterols (▲———▲) and cholesterol/C-27 sterols (▲— — —▲), according to the procedure described in the Materials and Methods. The results are expressed as a percentage of the control cultured with no inhibitor (mean of duplicate determinations). Control values represent synthesized isoprenoids (782000 dpm/mg of cell protein) or synthesized cholesterol (20000 dpm/mg of cell protein).

ity, lowers this effect. This could suggest that in MCF-7 cells, as in some other tumoral cells [29,30], cholesterol biosynthesis is not regulated by low-density lipoproteins. To our knowledge, no data are available on the possible lack of deficiency of LDL receptors in MCF-7 cells. If such is the case, the MCF-7 cell growth inhibition by hydroxytamoxifen could be related to endogenous sterol synthesis inhibition. Lowering HMG-CoA reductase activity by LPS might also result from a partial desteroidation of the serum during ultracentrifugation: as shown in Table III, the estradiol concentration of lipoprotein-poor serum was only 35% of that of fetal calf serum.

In contrast, cholesterol biosynthesis in BT20 cells seem to be regulated by lipoproteins, since there was a stimulation of HMG-CoA reductase activity (Table II) after culturing in lipoprotein-poor serum (79.9 versus 28.1 pM/min per mg protein). Stimulation did not occur when culturing

TABLE III

LIPID AND ESTRADIOL CONTENT OF FETAL CALF SERUM, STEROID-FREE SERUM AND LIPOPROTEIN POOR SERUM

Serum	Cholesterol (mM/l)	Triacylglycerols (M/l)	Estradiol (nM/l)
FCS	0.93±0.30	1.25±0.05	0.62±0.22
DSS	1.03±0.09	1.16±0.02	0.05±0.02
LPS	0	0.40±0.08	0.21±0.07

with estradiol, but did when culturing with hydroxytamoxifen. A comparison of the values of the control in fetal calf serum and in steroid-depleted serum reveals a stimulatory effect of steroid-depleted serum on HMG-CoA reductase activity.

The effect of steroid-depleted serum is visible not only on HMG-CoA reductase activity, but also on isoprenoid and cholesterol synthesis (Table I). The BT20 cell line is not estradiol-sensitive, and desteroidation did not modify the cholesterol and triacylglycerol content of the serum (Table III), suggesting that charcoal treatment removes some inhibitor of HMG-CoA reductase from FCS. This effect is masked in MCF-7 cells by estrogen removal which 'per se' lowers cholesterogenesis in this estrogen sensitive line.

Discussion and Conclusion

In the experiments described above, we studied the effect of estradiol on cholesterogenesis in the MCF-7 and BT20 breast cancer cell lines. When the results were compared with data from the literature concerning cell growth [16–18] we found a good correlation between these two effects: estradiol stimulates both cholesterogenesis and cell growth in the MCF-7 line, but stimulates neither of them in the estradiol-insensitive BT20 line. The stimulation occurred at several steps of the cholesterogenic process, i.e., at the HMG-CoA reductase and at two-post-HMG-CoA reductase steps. The reductase activity measurements were in good agreement with the rate of isoprenoid synthesis, which was restored by estradiol after inhibition by hydroxytamoxifen. This suggests involvement of the estradiol receptor in this effect,

by contrast with the effect on cholesterol synthesis.

Two antiestrogens (hydroxytamoxifen and LY 117018) were tested on the MCF-7 and BT20 cells. These acted very similarly, exhibiting a moderate effect on isoprenoid synthesis (negative in MCF-7 cells, positive in BT20 cells as confirmed by HMG-CoA reductase measurements with hydroxytamoxifen) and a strong effect on two post-HMG-CoA steps: lanosterol demethylation and C-27 sterol conversion into cholesterol. This inhibition did not correlate with the effect on cell growth, since antiestrogens affect the growth of MCF-7 cells [17,18], but not BT20 cells [19].

Hydroxytamoxifen completely inhibited the cholesterol synthesis in BT20 cells without impairing their proliferation; culture of BT20 cells in steroid-depleted medium increased the cholesterol synthesis without affecting the proliferation. These results are apparently in contrast with a previous report [26] in which one compound (pentadecane-2-one) inhibited both growth and cholesterol synthesis in a single type of cell, suggesting that newly synthesized cholesterol was required for cell division. This hypothesis should be corrected in the light of the present data, confirmed by other studies performed with a series of inhibitors on lymphocyte [36].

HMG-CoA reductase activity measurements showed that hydroxytamoxifen does not act as a direct enzyme inhibitor, but requires cell integrity to exhibit its effect. This effect is obviously not mediated by the estrogen receptor, since cholesterogenesis inhibition was observed even in cells devoid of this receptor. Antiestrogen binding sites were recently shown to be present in both MCF-7 and BT20 cells [32,32] and may mediate the effect of these antagonists on cholesterogenesis. Their possible role is discussed in another report [34].

The present study provides a clear answer to the problem of the relationships between cholesterogenesis and cell growth. In MCF-7 and BT20 cell lines, cholesterol synthesis was found to be drastically inhibited by antiestrogens, whereas in BT20 cells proliferation was not inhibited, showing that new synthesis of cholesterol is not 'crucial' for cell growth when culture is performed in whole serum.

However, the effect of hydroxytamoxifen on HMG-CoA reductase and isoprenoid synthesis in intact cells always remains relatively low. The existence of a mevalonic acid metabolite(s) differing from cholesterol and essential for cell growth has been demonstrated by others [7–10] and several isoprenoid derivatives of the branching pathway have been proposed [34,35]. This problem is now under investigation in our laboratory.

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