Modulation of Cellular Cholesterol and Its Effect on Cornified Envelope Formation in Cultured Human Epidermal Keratinocytes

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When cultured human epidermal keratinocytes (NHK) reach confluence they start to differentiate and an increase in the total cellular cholesterol content is observed. This increase parallels the appearance of a characteristic feature of terminal keratinocyte differentiation, the spontaneous formation of cornified envelopes (CE). Synthesis of CE is catalyzed by the plasma membrane-associated transglutaminase (TG_m). Supplementation of the medium with inhibitors of cholesterologenesis suppressed increase in cholesterol levels and CE formation but did not interfere with TG_m expression or TG_m activity. Modulation of the plasma membrane cholesterol-phospholipid ratio of confluent NHK cultures using either pure phospholipid liposomes or liposomes enriched in cholesterol strongly affected spontaneous CE formation. Pure phospholipid liposomes completely inhibited CE formation, whereas cholesterol-enriched liposomes ensured envelope formation, even in the presence of inhibitors of cholesterol synthesis. From these results we conclude that in differentiating NHK an increase in the cellular cholesterol level is part of the differentiation program and is essential for the spontaneous CE formation. *J Invest Dermatol 97:771–775, 1991*

Normal human epidermal keratinocytes (NHK) cultured under submerged conditions have a limited potential to undergo terminal differentiation. It is surprising therefore that confluent NHK spontaneously synthesize a cornified envelope (CE), which is one of the most characteristic features of epidermal keratinocyte differentiation [1–3]. CE synthesis requires the plasma membrane-associated transglutaminase (TG_m) [4–6], which cross-links in a Ca^{++}-dependent manner intracellular and plasma membrane proteins via the formation of isopeptide bonds [7–10].

Vitamin A and other retinoids are known to modulate the process of terminal differentiation in NHK, and in several different cell types affect cellular proliferation and differentiation [11,12]. In cultured NHK, retinoic acid acts as an inhibitor of differentiation by suppressing the expression of TG_m at a pre-translational level [5,13,14].

Some authors have reported that the cellular lipid composition is also related to keratinocyte differentiation [15,16] and that inhibitors of cholesterol synthesis significantly decrease CE formation [17].

In this paper we show that in differentiating NHK the cellular cholesterol content is related to the ability of the cells to synthesize cornified envelopes spontaneously, and that modulation of the plasma membrane cholesterol/phospholipid ratio by liposomes strongly affects CE formation.

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Abbreviations:
CE: cornified envelope
DMEM: Dulbecco's modification of Eagle's medium containing 5.5 mM glucose and 4 mM glutamine
DPPC: dipalmitoylphosphatidylcholine
DTE: dithioerythritol
EDTA: ethylenediamine tetraacetic acid
Enzyme: Transglutaminase (EC 2.3.2.13)
F12: Ham's medium F12
HPTLC: high-performance thin-layer chromatography
NHK: normal human keratinocytes
PBS: phosphate-buffered saline
SDS: sodium dodecylsulfate
TG_m: plasma membrane transglutaminase
TRIS: Tris (hydroxymethyl) aminomethane

MATERIALS AND METHODS

Materials Culture media and fetal calf serum were obtained from Flow Laboratories and GIBCO. Deoxyribonuclease and the calcium ionophore A23187 were purchased from Boehringer Mannheim. Dipalmitoylphosphatidylcholine (99% pure by TLC) and cholesterol were from Sigma. Twenty-two-ketocholesterol oxime (3 beta-hydroxycholest-en-22 one oxime) and 25-hydroxycholesterol were synthesized in our laboratories [18]. HPTLC plates (60 F 254) were obtained from Merck. Products for gel electrophoresis, dithioeythritol (DTE), and putrescine were from Bio-Rad, Sigma, and Serva. ^{125}I-labeled anti-mouse Ig, ^{14}C-labeled putrescine, ^{14}C-labeled sodium acetate, and 1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine were purchased from Amersham; the 4-^{14}C-labeled cholesterol was from NEN. Nitrocellulose paper was from Schleicher and Schuell. The monoclonal antibody BC.1 raised against human epidermal particulate transglutaminase (anti-epidermal TG antibody) was obtained from Dr. S. Thacher, A & M College, Texas.

Cells and Culture Conditions Normal human keratinocytes (NHK) were grown with the 3T3 feeder cell technique of Rhein-

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wald and Green [19]. NHK were used in their third or fourth passage. Before trypsination, remaining 3T3 cells were removed by incubating the culture in phosphate-buffered saline (PBS) containing 0.1% (w/v) glucose and 0.02% (w/v) ethylenediamine tetraacetic acid (EDTA).

Inhibitors of cholesterol synthesis were added to the culture medium shortly before the cells reached confluence.

Trypsination of NHK Cells were washed twice with PBS before being treated for 8 min at 37°C with PBS containing 0.04% (w/v) trypsin and 0.02% (w/v) EDTA. The trypsination was stopped by the addition of fetal calf serum to a final concentration of 20% (v/v).

Cornified Envelope Assay The procedure of Cline and Rice [20] was used with some modifications. To assess the degree of envelope competence, cells were trypsinized and 4×10^5 cells were incubated in 0.5 ml DMEM/F12 containing 10 μg of the calcium ionophore A23187. After 3 h, 50 ml of 20% (w/v) sodium dodecylsulfate (SDS) containing 1 mg DTE was added, before heating the suspension for 5 min at 100°C. After cooling, 10 μl of a DNase solution (1 mg/ml) was added to prevent aggregation of the envelopes, which were then counted with a hematocytometer.

Cell Fractionation Plasma membranes were purified by the microbead density perturbation technique [21] as described in [22].

Transglutaminase Assay Transglutaminase was assayed according to De Young and Balleron [23] with minor modifications [5].

Electrophoresis and Immunoblotting SDS polyacrylamide gel electrophoresis was carried out under reducing conditions as described [24]; 10% gels were used and 75 μg of plasma membrane protein were routinely applied on each slot.

For immunoblotting experiments, the electrophoretically separated plasma membrane proteins were transferred to nitrocellulose paper as described [25]. The sheets were saturated for 1.5 h at room temperature in 10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl and 3% (w/v) bovine serum albumin. They were incubated with the diluted antiserum in the same buffer. The sheets were washed and incubated with ^{125}I-labeled anti-mouse IgF(ab')2 fragment (0.25 mCi/ml). They were exposed for autoradiography to a Kodak XAR-5 film using the DuPont Cronex intensifying screen.

Lipid Extraction and Quantification of Cellular Cholesterol and Cholesterol Synthesis Upon trypsination, total cellular lipids were extracted by vortexing the cells for 1 min in a mixture of chloroform-methanol (2:1) containing 1 M KCl. After centrifugation ($500 \times g$ for 5 min), the lower chloroform phase was transferred into a glass tube to be evaporated under a gentle stream of nitrogen. The resulting pellet was dissolved in a small volume of chloroform-methanol (2:1), separated by HPTLC [26], and quantitated with a Berthold automatic TLC-linear analyzer. To assess the rate of cholesterol synthesis, the culture medium was supplemented with ^{14}C-acetate. Its incorporation into cholesterol was taken as a measure of cholesterol synthesis.

Preparation of Liposomes Multilamellar vesicles were prepared as described [27]. Briefly, 3.5 mg of dipalmitoylphosphatidylcholine (DPPC) were used without any further purification and dissolved in 2 ml chloroform. The solvent was removed by rotary evaporation under a stream of nitrogen followed by lyophilization overnight. The dried lipid film was mixed for 1 h with 5 ml PBS (without Ca^{++} and Mg^{++}) with vigorous vortexing at 50°C and thereafter sonicated at 50°C for 30 min in a bath sonicator in order to obtain a homogeneous liposome suspension.

Small unilamellar vesicles were obtained by sonicating the multilamellar vesicles [28] for 30 min at 50°C under a stream of nitrogen using a Branson type probe sonicator. To remove aggregates as well as titanium particles derived from the sonicator probe, the liposome suspension was centrifuged for 20 min at 20,000 rpm. Thereafter, the colloidal supernatant was sterilized by filtration (0.22 μm Millipore filter). Small unilamellar vesicles enriched in cholesterol were prepared according to the same protocol but using a DPPC/cholesterol ratio of 1:1.5. The cholesterol content of these vesicles was assayed by HPTLC [26]. The preparations of multilamellar vesicles and small unilamellar vesicles were controlled by light microscopy or electron microscopy after negative staining.

Radiolabeled liposomes were prepared as described above containing either 20 μCi ^{14}-C cholesterol (53.1 mCi/mmol) or 6.25 μCi DPPC (113 mCi/mmol).

Table I. Inhibition of Spontaneous CE Formation in Cultured NHK by Various Inhibitors of Cholesterologenesis

Compound	Concentration (μM)	Percent Inhibition
22-ketocholesterol oxime	0.1	98
25-hydroxycholesterol	5.0	90
Miconazole nitrate	5.0	98
Bifonazole	5.0	95
Mevinolin	3.0	95

RESULTS

When cultures of NHK reach confluence, many cells start to terminally differentiate and synthesize a CE. At day 7 of confluence, about 60% of the cells have spontaneously synthesized a CE. Concomitantly with the spontaneous CE formation, an increase in the total cellular cholesterol content is observed. Supplementation of the medium shortly before confluence with various inhibitors of cholesterol synthesis (Table I) abolished not only the increase in cellular cholesterol content but also spontaneous CE formation. Treatment of the cells with the Ca^{++} ionophore A 23187 induced CE formation in almost 100% of the cells well before spontaneous CE formation occurred. These results are summarized in Fig 1. The different inhibitors of cholesterol synthesis did not prevent ionophore-induced CE formation and had no effect on the rate of CE synthesis.

From Table I it can be seen that the most potent inhibitor of

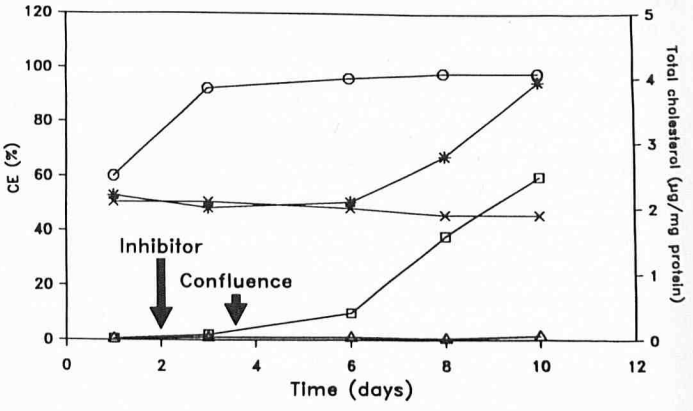

Figure 1. Total cellular cholesterol in the absence (*) and presence (×) of inhibitors of sterologenesis. Spontaneous CE formation in the absence (□) and presence (△) of inhibitors of sterologenesis. Ionophore-induced CE formation in the absence and presence of inhibitors of sterologenesis (○).

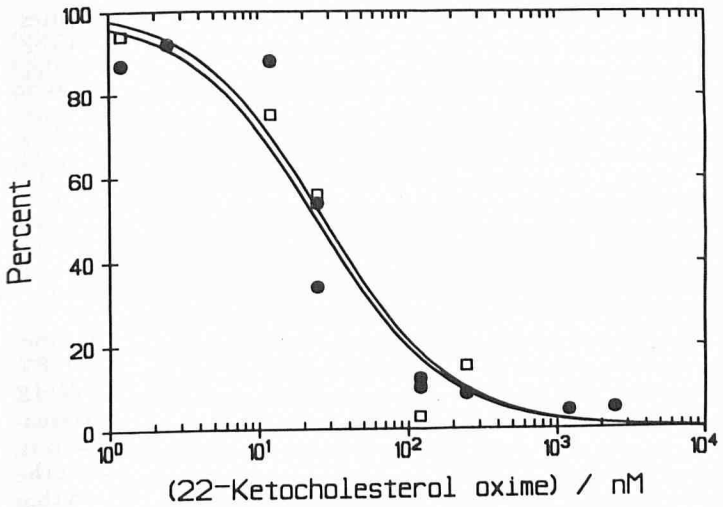

Figure 2. Dose-response curves to determine the inhibitory effect of 22-ketocholesterol oxime on CE formation (●) and cholesterol synthesis (□) in cultured NHK. (22-ketocholesterol oxime was added shortly before confluence; the assays were performed at day 7 of confluence.)

sterologenesis is 22-ketocholesterol oxime. As shown in the dose-response curves illustrated in Fig 2, the IC50 values (20 nM) were almost identical for the ability of this substance to inhibit either cholesterol synthesis or spontaneous CE formation.

Plasma membranes from confluent NHK (control) and from NHK cultured for 7 d in the presence of different concentrations of 22-ketocholesterol oxime were purified to examine the presence and specific activity of TG_m. Immunoblotting experiments with the TG_m-specific monoclonal antibody B.Cl revealed, after electrophoretic separation of the plasma membrane proteins, that 22-ketocholesterol oxime did not affect the presence of TG_m (Fig 3). No difference in the specific TG_m activity was observed between control and 22-ketocholesterol oxime-treated NHK (results not shown).

Incubation of NHK upon confluence with medium containing small unilamellar DPPC vesicles (0.1% v/v) completely inhibited spontaneous CE formation (Fig 4*A*) and a remarkable increase (220%) of the cellular cholesterol synthesis was observed (Fig 4*B*).

NHK in which spontaneous CE formation was suppressed by the presence of inhibitors of cholesterol synthesis completely regained their ability to spontaneously synthesize CE when the medium was supplemented with small unilamellar vesicles enriched in cholesterol (0.1% v/v) (Fig 5*A*). The addition of cholesterol-enriched vesicles did not antagonize the inhibitory activity of 22-ketocholesterol oxime on cholesterol synthesis (Fig 5*B*) but increased the total cellular cholesterol by incorporation of vesicular cholesterol (Fig 5*C*).

Using radiolabeled unilamellar DPPC vesicles, we revealed that after 12-h incubation the total lipid extract of the NHK contained about 5% of the vesicular DPPC. A more detailed study was performed to determine the effect of cholesterol-enriched vesicles on the plasma membrane cholesterol level. The lipid analysis of purified plasma membranes revealed, at a constant phospholipid level, that cholesterol was reduced by 50% when the cells were kept in the presence of 22-ketocholesterol oxime and that after 12-h incubation of these cells with cholesterol-rich vesicles, plasma membrane cholesterol had reached a level comparable to that found in untreated control cells. These results are summarized in Table II.

To provide evidence that it is cholesterol and not one of its metabolites such as cholesterol sulfate or a cholesterol ester that influences spontaneous CE formation, keratinocytes with blocked cholesterologenesis (and thus unable to spontaneously synthesize CE) were incubated with ^{14}-C cholesterol-enriched vesicles to induce spontaneous CE formation. Analysis of a total cellular lipid extract, obtained at the time spontaneous CE formation occurred (12 h later), revealed that the vesicular ^{14}-C cholesterol incorporated into the keratinocytes had not been metabolized.

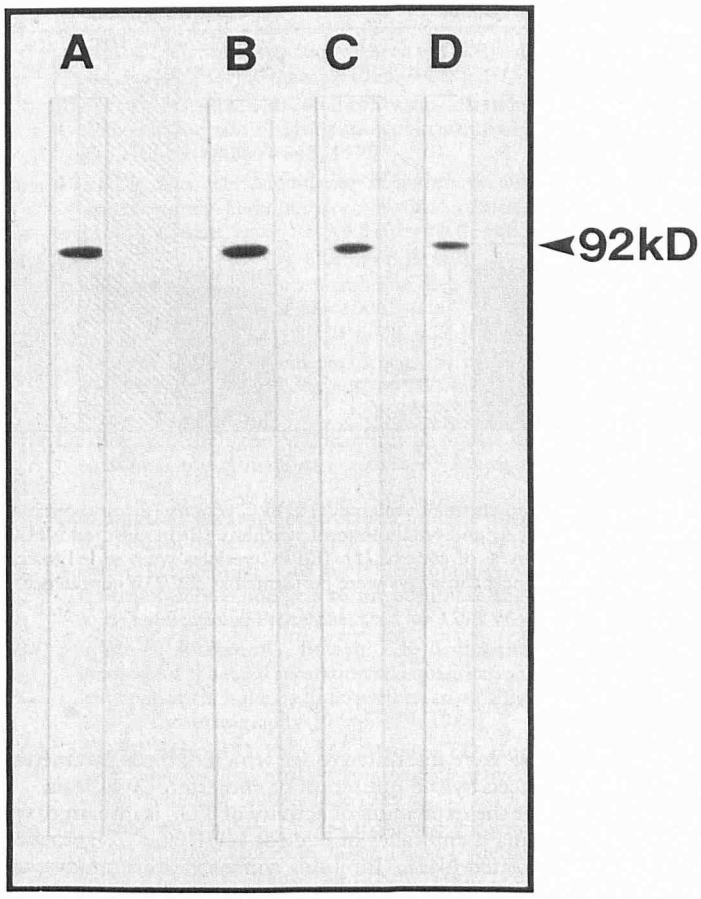

Figure 3. Immunoblot performed with the anti-epidermal TG_m antibody after electrophoretic separation (75 μg/lane) of plasma membrane proteins from control *(A)*, and from NHK cultured in the presence of 20 nM *(B)*, 50 nM *(C)*, and 100 nM *(D)* 22-ketocholesterol oxime. (22-ketocholesterol oxime was added shortly before confluence; the assays were performed at day 7 of confluence.)

DISCUSSION

In confluent NHK cultures, the increase in spontaneous CE formation is accompanied by an increase in the total cellular cholesterol content. To determine whether there is a relationship between the cellular cholesterol level and spontaneous CE formation, we supplemented the medium with various known inhibitors of sterologenesis shortly before the cultures reached confluence. The fact that inhibition of cholesterol synthesis abolished not only the observed increase of the cellular cholesterol but also the spontaneous CE formation (Fig 1) was a first indication that there might be a direct link between cholesterol and CE formation. This hypothesis received further support from the results obtained with 22-ketocholesterol oxime, a potent inhibitor of sterologenesis (Table I) that possesses almost identical IC_{50} values for cholesterol synthesis and spontaneous CE formation (Fig 2). It is interesting to note in this context the observation made by Kinsman et al [29] that subcutaneous injection of the cholesterol synthesis inhibitor ketoconazole prevented cornification of the vaginal epithelium in rats.

Spontaneous CE formation involves three essential factors: (i) the presence of the crosslinking enzyme TG_m, (ii) the availability of substrate protein, and (iii) sufficiently high intracellular Ca^{++} to

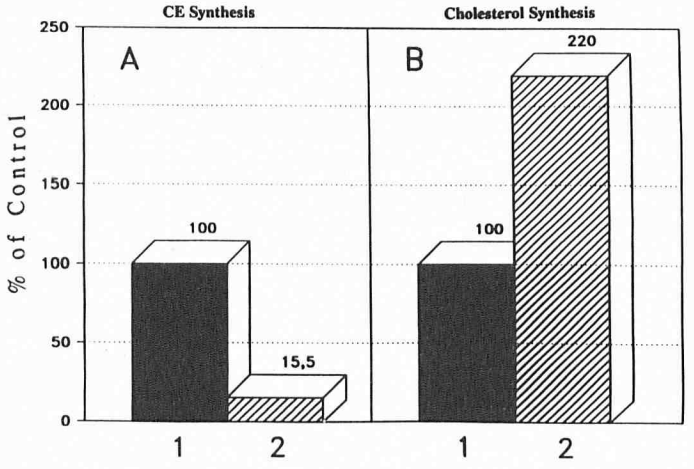

Figure 4. The effect of small unilamellar DPPC vesicles *(2)* on spontaneous CE formation *(A)* and on cholesterol synthesis *(B)* in cultured NHK. Results are given in % of control *(1)*. (DPPC vesicles were added to the medium at confluence; the assays were performed at day 7 of confluence.)

activate TG_m. We were interested to see which of these parameters would be influenced by the inhibition of cholesterol synthesis.

To see whether the expression or activity of TG_m is impaired, we purified the plasma membranes of control NHK and 22-ketocholesterol oxime-treated NHK. Inhibitor concentrations ranging up to 100 nM, a concentration at which spontaneous CE formation is completely inhibited, did not prevent expression and presence of TG_m in the plasma membranes, as revealed by immunoblotting with the TG_m-specific monoclonal antibody B.C1 (Fig 3). Furthermore, no difference in the specific activity of the enzyme was observed between control and 22-ketocholesterol-treated NHK. Thus, a direct effect of the inhibitor on TG_m is unlikely.

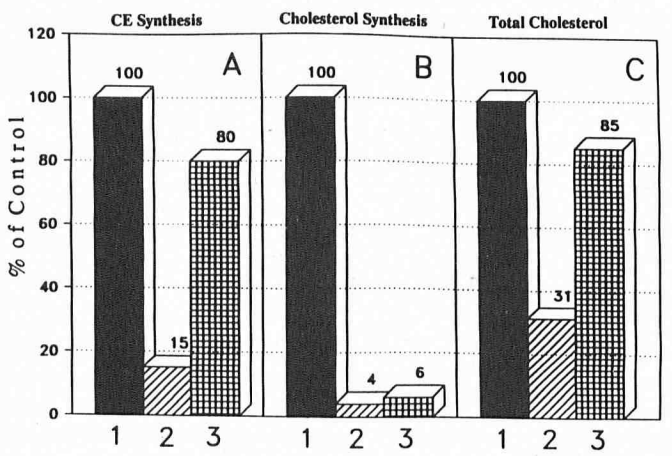

Figure 5. The effect of 25-hydroxycholesterol alone *(2)* and in combination with small unilamellar DPPC vesicles enriched with cholesterol *(3)* on spontaneous CE formation *(A)*, cholesterol synthesis *(B)*, and total cellular cholesterol *(C)* in cultured NHK. Results are given in % of control *(1)*. (25-hydroxycholesterol was added shortly before confluence and the vesicles at confluence. The assays were performed at day 7 of confluence.)

Table II. Cholesterol Content of Purified Plasma Membranes of Keratinocytes with Blocked Cholesterol Synthesis and After 12-h Incubation of These Cells with Cholesterol-Rich Vesicles

	Plasma Membrane Cholesterol (μg/mg protein)
Control cells	225
In the presence of inhibitors	115
In the presence of inhibitors and after treatment with cholesterol-rich vesicles	232

In a second set of experiments, we treated NHK at different times during growth and confluence with the Ca^{++} ionophore A23187. This treatment artificially increases the intracellular Ca^{++} of NHK and, if sufficient TG_m and substrate proteins are present, CE formation is induced [5]. In view of the fact that under these conditions, inhibitors of cholesterol synthesis did neither prevent nor affect the rate of CE synthesis, we deduce that TG_m is not the target and that these inhibitors of cholesterol synthesis do not interfere with the availability of TG_m substrate proteins.

The question remains how could cholesterol interfere with the supply of Ca^{++} necessary to activate the crosslinking enzyme TG_m in NHK? It is known that about 90% of the total cholesterol in mammalian cells is located in the plasma membrane [30]. Changes in the membrane cholesterol-phospholipid ratio alter its fluidity and interfere with the activity of plasma membrane-located ion-pumps [31]; it has been observed that an increase in the plasma membrane cholesterol stimulated the activity of the Ca^{++} ion pump [32]. Recently, several authors [33,34] found that epidermal differentiation is accompanied by a decrease in membrane fluidity, whereas hyperproliferating epidermal cells exhibit a relatively high membrane fluidity. It could thus be that in cultured NHK a reduction of the plasma membrane fluidity is part of the differentiation program and a prerequisite for spontaneous CE formation. If this were so, modulation of the cellular cholesterol and membrane fluidity by means other than inhibitors of cholesterol synthesis should provide similar results concerning spontaneous CE formation. We decided to use either pure phospholipid vesicles or those enriched in cholesterol, a known method to change membrane fluidity and cellular cholesterol without affecting cell viability [35,36].

The plasma membrane cholesterol level of keratinocytes that have been continuously treated with cholesterol synthesis inhibitors is reduced by about 50% and these cells are not able to spontaneously synthesize CE. However, they completely regain their ability to spontaneously synthesize CE when the medium is supplemented with cholesterol-rich vesicles (Fig 5*A*), a treatment that increases the plasma membrane cholesterol (Table II) without antagonizing the inhibition of cholesterol synthesis (Fig 5*C*). The fact that in the presence of inhibitors of cholesterol synthesis the incorporated vesicular cholesterol is not metabolized at the time CE are synthesized indicates that it is cholesterol and not one of its metabolites that influences spontaneous CE formation.

The possibility that due to the structural similarity between cholesterol and 22-ketocholesterol oxime the inhibitor itself affects the membrane fluidity and thus CE formation is unlikely because it is used at a thousandfold lower concentrations. Furthermore, other non-membrane active cholesterol synthesis inhibitors exhibit the same effect.

Incubation of NHK at confluence with medium containing pure phospholipid vesicles to reduce the plasma membrane cholesterol/phospholipid ratio completely abolished spontaneous CE formation (Fig 4*A*). It was interesting to note that this treatment induced a considerable increase in cholesterol synthesis (Fig 4*B*), as if the cells tried to maintain their original cholesterol/phospholipid ratio by higher cholesterol production.

From these results we conclude that in differentiating NHK there is a direct relationship between the observed increase in total cellular cholesterol and spontaneous CE formation. Although our results do

not elucidate the exact role of cholesterol in this process, they do not exclude that an elevated plasma membrane cholesterol level might be necessary to increase the internal Ca^{++} concentration to a level required for TG_m activation. Experiments to test this hypothesis will require a well controlled batch of keratinocytes because the membrane fluidity of submerged adult human keratinocytes can vary fivefold or more depending on the cell line, passage number, and growth conditions [37].

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