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Experimental Cell Research 300 (2004) 109–120

Experimental Cell Research

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Cholesterol is essential for mitosis progression and its deficiency induces polyploid cell formation

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Received 9 March 2004, revised version received 24 June 2004
Available online 4 August 2004

Abstract

As an essential component of mammalian cell membranes, cells require cholesterol for proliferation, which is either obtained from plasma lipoproteins or synthesized intracellularly from acetyl-CoA. In addition to cholesterol, other non-sterol mevalonate derivatives are necessary for DNA synthesis, such as the phosphorylated forms of isopentane, farnesol, geranylgeraniol, and dolichol. The aim of the present study was to elucidate the role of cholesterol in mitosis. For this, human leukemia cells (HL-60) were incubated in a cholesterol-free medium and treated with SKF 104976, which inhibits cholesterol biosynthesis by blocking sterol 14α-demethylase, and the expression of relevant cyclins in the different phases of the cell cycle was analyzed by flow cytometry. Prolonged cholesterol starvation induced the inhibition of cytokinesis and the formation of polyploid cells, which were multinucleated and had mitotic aberrations. Supplementing the medium with cholesterol completely abolished these effects, demonstrating they were specifically due to cholesterol deficiency. This is the first evidence that cholesterol is essential for mitosis completion and that, in the absence of cholesterol, the cells fail to undergo cytokinesis, entered G1 phase at higher DNA ploidy (tetraploidy), and then progressed through S (rereplication) into G2, generating polyploid cells.

Keywords: Endoreplication; Cytokinesis; Cyclins; Cell cycle; Cholesterol

Introduction

Cholesterol is a major component of plasma membrane in mammalian cells. In addition to its structural requirement, cholesterol is important for other cell functions as bile acid and hormone biosynthesis, embryonic development, and cell proliferation. The requirement of cholesterol for cell growth and division of mammalian cells has been known for many years [1–3], but whether this is just a consequence of its use for membrane formation or whether it also plays a regulatory role in this process has not been clarified. We recently reported that cholesterol starvation produced by incubating the cells in a cholesterol-free medium and blocking cholesterol biosynthesis with SKF 104976, an inhibitor of lanosterol 14α-demethylase, resulted in cell cycle arrest specifically at G2-phase [4]. This arrest was associated with the inhibition of cyclin-dependent kinase 1 (Cdk1) activity, the protein kinase that regulates the transition to mitosis. The effect could be both prevented and reversed by cholesterol, suggesting this lipid acts as a regulator of cell cycle progression. In addition, other non-sterol mevalonate derivatives are required for the G1-S transition [5–8], thus confirming the relationships between the cholesterol synthesis pathway and cell proliferation.

Cell cycle is a regulated process during which alternating rounds of DNA synthesis (S-phase) and mitosis (M-phase) are coordinated by a series of checkpoints. Progression

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doi:10.1016/j.yexcr.2004.06.029

through the cell cycle is driven by a family of Cdks whose activity is regulated through binding to cyclin regulatory molecules [9,10]. In proliferating cells, phosphorylation events triggered by Cdks control the onset of both mitosis and S-phase. Mitotic control is mediated by B type cyclins complexes with Cdk1 [11–13]. Cyclin D/Cdk4 and cyclin D/Cdk6 complexes regulate early events in G1 phase of the cell cycle, while Cdk2 activity regulated by cyclins A and E appears to be critical for the initiation of DNA synthesis [14]. Endoreplicating cells (cells with extra copies of the genomic DNA) appear to have simplified this regulatory machinery by eliminating the expression of components that are no longer required. For instance, some endoreplicating cell types that bypass mitosis do not express Cdk1 or its activators, cyclin B1, cyclin A, and cdc25C [15]. Cell types in which mitosis is partially traversed but incomplete have been reported to have diminished levels of these G2/M regulators [16,17]. Moreover, in megakaryocytes, the best-known polyploid cell type in mammals, the endoreplication mechanism has been associated with reduced activity of Cdk1 [18,19]. This suggests that levels of mitotic Cdk1 activity may be responsible for determining the extent of mitotic functions retained in an endocycling cell. In addition, some studies revealed that endocycling cells can also lose structural components required for mitosis, such as centrosomes [20].

Little is known about the role of membrane lipids on the mitotic process. Recently, Emoto and Umeda [21] demonstrated that phosphatidylethanolamine is required for completion of cytokinesis in mammalian cells. The possible role of cholesterol, as an essential membrane lipid, on mitosis has been not examined so far. In the present work, we show that cholesterol starvation attained by incubating human leukemia HL-60 cells in a cholesterol-free medium in the presence of SKF 104976 induces a delay in G2-to-M transition followed by the formation of polyploid and multinucleated cells. This work is the first demonstration that cholesterol is essential for mitosis progression and its deficiency leading to aberrant mitosis and polyploid cell formation.

Materials and methods

Materials

HL-60 cells (human promyelocytic cells) were obtained from the ATCC (CCL 240). Cholesterol-free medium (DCCM-1) was purchased from Biological Industries. Antibiotics were provided by Gibco BRL. Cholesterol, indirubin-3′ -monoxime, and 5-fluordeoxyuridine were from Sigma. SKF 104976 was kindly provided by Dr. Ré, (SmithKline Beecham Pharmaceuticals). Antibodies were obtained from Santa Cruz Biotechnology (anti-human cyclin B1 monoclonal) and BD Pharmingen (anti-human cyclin E monoclonal, anti-human cyclin D1 monoclonal, and anti-human cyclin A monoclonal). The secondary antibody used was fluorescein isothiocyanate conjugated goat anti-mouse IgG from BD Pharmingen. [^{14}C]-acetate (53 mCi/mmol), [^{3}H]-cholesterol (45.6 Ci/mmol) were from Amersham Biosciences. The other chemical products were of analytical grade.

Methods

Cell culture

HL-60 cells were maintained in DCCM-1, supplemented with antibiotics (100 units of penicillin/ml, 100 μg of streptomycin/ml and 10 μg of gentamicin/ml) at 37°C in a humidified atmosphere containing 5% CO_2.

Proliferation assay

HL-60 cells were grown in DCCM-1 medium in the absence (control) or presence of 1.5 μM SKF 104976. At the indicated times, the viable cells were determined by Trypan blue dye exclusion using a hemocytometer.

Determination of cholesterol biosynthesis and total content by HPLC

To determine the biosynthesis of cholesterol, HL-60 cells (7.5×10^6) were preincubated for 2 h in 10 ml of DCCM-1 in the absence (control) or the presence of 1.5 μM SKF 104976, then the medium was supplemented with 40 μCi of [^{14}C]-acetate and incubation was prolonged for a further 8-h period. In other experiments, to determine the total cholesterol content, HL-60 cells (7.5×10^6) were incubated in the presence of SKF 104976 for 24, 48, 72, and 96 h in 10 ml DCCM-1. At the end of the incubation, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 0.5 ml of 10% (w/v) KOH. [^{3}H]-cholesterol was added as internal standard. The samples were treated sequentially with chloroform/methanol (2:1 v/v) and distilled water to obtain the lipidic and the aqueous-soluble fractions, as reported previously [22]. The lipid extract was further subfractionated into the saponifiable and non-saponifiable fractions. Non-saponifiable lipids were resuspended in hexane and used for sterol separation by HPLC and radioactivity counting as described previously [23]. Recovery of [^{3}H]-cholesterol was greater than 65% in every case. Sterol separation was accomplished by reverse-phase HPLC with Luna-Pack 5 μm pore size C18 (250 × 4.60 mm; Phenomenex). Lipids were eluted with acetonitrile/water (95:5 v/v) during the first 37 min, and then with methanol at a flow rate of 1.2 ml/min. The effluent was monitored simultaneously by UV-absorption spectroscopy (Beckman 168 variable-wavelength detector; Beckman Instruments) and online radioactivity detection using a LB-506 C-1 radioactivity detector (Berthold).

Cell cycle analysis

Asynchronous HL-60 cells (2.5×10^5 cells per ml) were cultured in DCCM-1 and supplemented with SKF 104976

and/or cholesterol. In other instances, cells were treated with the Cdk-1 inhibitor, indirubin-3′ -monoxime. At the end of the incubation, the cells were washed twice with ice-cold PBS, fixed in 70% cold ethanol, treated with 100 μg/ml ribonuclease A, and labeled with 50 μg/ml propidium iodide for 1h at 37°C. Cells were analyzed by flow cytometry (FACScalibur, Becton-Dickinson) using selective gating to exclude doublets of cells and subjected to MODFIT analysis (Verity Software House, Inc). To determine the actual DNA synthesis rate at the end of the experiments, the cells were incubated with 100 μM bromodeoxyuridine (BrdU) for 1 h at 37°C, then fixed in 70% cold ethanol and washed with PBS. After a 20-min incubation at room temperature with 2 N HCl, cells were washed with PBS and incubated for 15 min at room temperature in PBS containing 0.5% Tween 20 and 1%

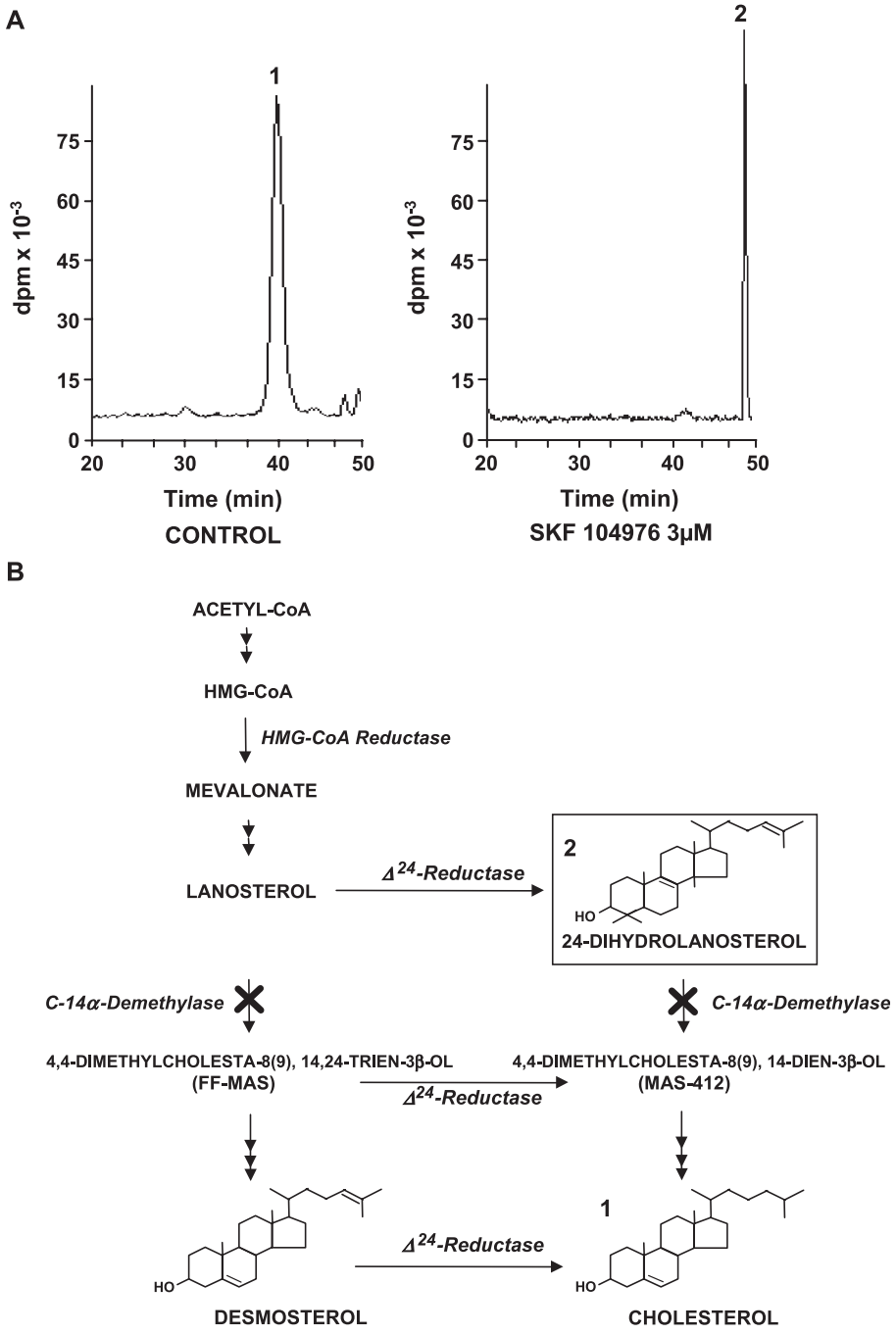

Fig. 1. Effect of SKF 104976 on [^{14}C]-acetate incorporation into sterols. (A) HL-60 cells were treated with 1.5 μM SKF 104976 or vehicle (control) and [^{14}C]-acetate for 8 h, and radioactivity incorporation into sterols was determined by HPLC. 1, cholesterol; 2, 24-dihydrolanosterol. (B) Scheme of the cholesterol biosynthesis pathway showing the enzyme inhibited by SKF 104976.

Table 1
Time-course of the effect of SKF 104976 on total content of cholesterol (μg cholesterol/mg protein)

	0 h	24 h	36 h	48 h	60 h	72 h
Control	5.56 ± 0.3	5.14 ± 0.2	5.48 ± 0.4	5.64 ± 0.3	5.36 ± 0.4	5.34 ± 0.4
SKF 104976 1.5 μM	–	2.61 ± 0.1	1.91 ± 0.1	1.21 ± 0.1	0.91 ± 0.05	0.81 ± 0.04

HL-60 cells were treated with 1.5 μM SKF 104976 or vehicle (control) and cholesterol was determined by HPLC. Data correspond to the means ± SEM of three experiments.

normal goat serum (NGS). Subsequently, cells were centrifuged, resuspended in 0.5% Tween 20, 1% NGS containing 20 μl of FITC-labeled anti-BrdU for 1 h at room temperature, and then stained with propidium iodide as before, to finally be analyzed by flow cytometry (FACScalibur, Becton-Dickinson).

Analysis of cyclin expression by flow cytometry

At the end of the incubation with the corresponding agents, the cells were washed twice with ice-cold PBS and fixed in p-formaldehyde (1% w/v in PBS) at room temperature for 2 min. The samples were then centrifuged, washed with PBS, and resuspended in methanol at 20°C for 10 min. Subsequently, the cells were washed twice with ice-cold PBS and treated with 0.1% Triton X-100 at room temperature for 2 min. After addition of PBS and centrifugation, the cells were resuspended in 500 μl of 1% NGS in PBS and incubated at 37°C for 15 min. Then, the cells were washed with PBS and resuspended in 500 μl of PBS containing 1% NGS and 0.125 μg/ml of monoclonal antibodies against human cyclins A, B1, D1, or E and incubated at 37°C for at least 1 h. Cells were then washed and incubated with FITC-conjugated goat anti-mouse IgG diluted 1:50 in PBS containing 1% NGS for 1h at 37°C. The cells were washed again, resuspended in 50 μg/ml propidium iodide and 100 μg/ml ribonuclease A in PBS, and then incubated at 37°C for 1 h before analysis. The isotypic control was processed as described above, except that the incubation with the first antibody was avoided.

Isolation and labeling of nuclei for flow cytometry analysis

HL-60 cells were cultured in DCCM-1 supplemented or not with SKF 104976 as above for 72 h at 37°C. At the end of incubation, the cells were removed from the flasks and washed twice with ice-cold PBS. After centrifugation, the cells were resuspended in cold nuclei extraction buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES, 1% Triton X-100, pH 7.4) to obtain a suspension of 1 million of cells/ml. The cells were gently vortexed for 10 s and incubated on ice for 10 min. Nuclei were then pelleted by centrifugation at 2000 × *g* and washed twice with nuclei wash buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.4). Nuclei yield and integrity were confirmed by microscopic examination with Trypan blue staining. This extraction procedure gives greater than 98% successful lysis with little debris and minimal clumping. At the end of this process, the nuclei were labeled with 50 μg/ml propidium iodide and treated with 100 μg/ml ribonuclease A to measure DNA content by flow cytometry.

Analysis of nuclear morphology by Hoescht 33342 staining

HL-60 cells were cultured on glass coverslips, previously treated with poly-D-lysine at a density of 5×10^4 cells per well in 12 well plates. For Hoescht 33342 staining, cells were washed three times with PBS and fixed with 1 ml of 4% p-formaldehyde in PBS at room temperature for 5 min, rinsed three times with PBS, and stained with 1 ml of Hoescht 33342 solution (final concentration 30 μM) at room temperature for 15 min. Then, cells were washed two times with PBS and analyzed with a UV filter package (BX,51; Olimpus, Inc.).

Electron microscopy

HL-60 cells were pelleted and embedded in epoxy resin following methods routinely used in our laboratory [24]. Briefly, cells were fixed for 2 h at 4°C in 4% p-formaldehyde, 1% glutaraldehyde, and 0.05% tannic acid in PBS, pH 7.4. Afterwards, samples were rinsed in PBS, postfixed in PBS containing 1% osmium tetroxide for 20 min at 4°C, washed in PBS again, and dehydrated in graded ethanol series. Samples were stained with saturated uranyl acetate during dehydration. After dehydration, cells were cleared in propilene oxide and embedded in Araldite. Samples were sectioned with diamond knives and serial ultrathin sections were collected on nickel grids. Sections were stained with

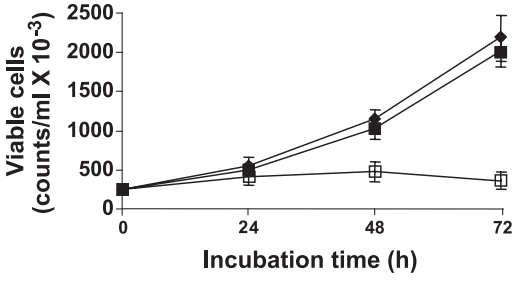

Fig. 2. Time-course of the effect of SKF 104976 on cell proliferation. HL-60 cells were cultured in cholesterol-free medium (DCCM-1) in the absence (control) (◆) or in the presence of 1.5 μM SKF 104976 alone (□) or in combination with 30 μg/ml free cholesterol (■). At the indicated times, the viable cells were counted. Data correspond to the means ± SEM of three experiments.

lead citrate and examined under a Zeiss EM10 electron microscope.

Results

Effect of SKF 104976 on sterol biosynthesis and total cholesterol content

We firstly determined the effect of SKF 104976 on cholesterol biosynthesis, as measured by the incorporation of [^{14}C]-acetate, and total cholesterol content in HL-60. As shown Fig. 1A, in control conditions most of [^{14}C]-acetate appearing in nonsaponifiable lipids corresponded to cholesterol. By contrast, in cells treated with SKF 104976, only [^{14}C]-dihydrolanosterol was detectable, which is consistent with the inhibition of sterol 14α-demethylase (Fig. 1B). Time course studies revealed that the cell content of cholesterol was significantly reduced at 24 h of treatment and continued to decline at longer incubations in the presence of SKF 104976 (Table 1).

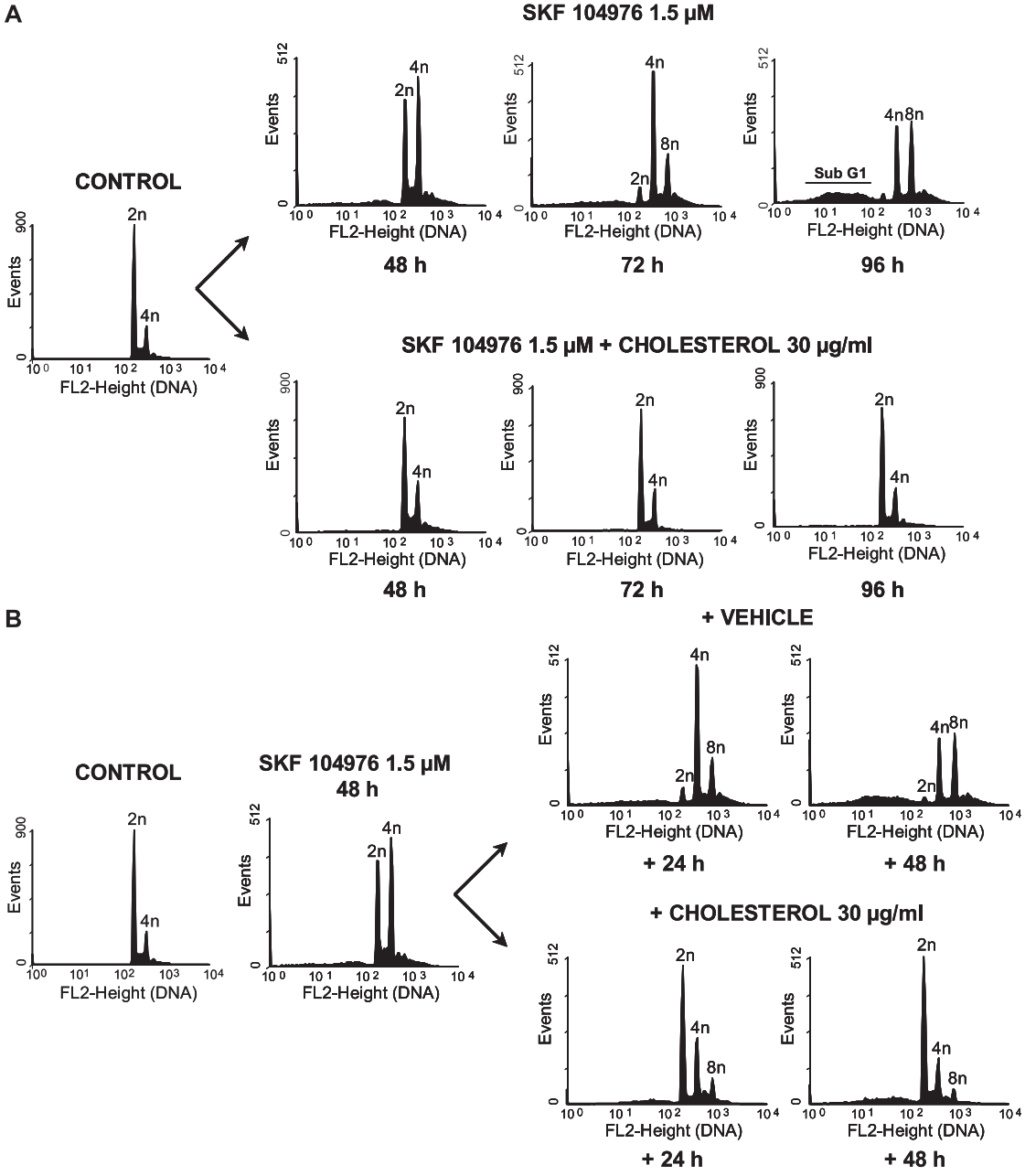

Fig. 3. Effects of SKF 104976 on cell cycle distribution. HL-60 cells were incubated in DCCM-1 in the presence of 1.5 μM SKF 104976 alone or in combination with free cholesterol 30 μg/ml. At the indicated times, cells were stained with propidium iodide and analyzed by flow cytometry. (A) Inhibition of cell cycle progression by SKF 104976 and effect of coincubation with free cholesterol. (B) Rescue of SKF 104976-induced cell cycle arrest by free cholesterol. Data correspond to a representative experiment out of three, which gave similar results.

Effect of cholesterol starvation on cell proliferation and cell cycle progression

To study the effect of decreasing cholesterol content on cell proliferation, we measured the viable cells at indicated times. As shown in Fig. 2, treatment of HL-60 cells with SKF 104976 markedly reduced cell proliferation at 48 and 72 h. These effects of SKF 104976 were abrogated when free cholesterol was added to the medium (Fig. 2).

We next determined the cell cycle in SKF 104976-treated cells and the effects of cholesterol addition. In control conditions, the distribution of the different phases of the cell cycle was normal (Fig. 3A). Treatment with SKF 104976 initially produced the accumulation of cells in G2/M, which was followed by a significant and progressive increment of polyploid cells thereafter (>4n DNA content) (Fig. 3A). The continuous exposure of cells to this cholesterol-starvation condition for 96 h or longer resulted also in generalized cell death, as indicated by the appearance of subG1 phase. These effects of SKF 104976 were totally abrogated by supplementing the medium with free cholesterol (Fig. 3A).

We then studied whether cholesterol provision could restore the normal cell cycle in cells previously arrested by cholesterol depletion. For this, HL-60 were preincubated with SKF 104976 for 48 h and then the medium was supplemented with vehicle or free cholesterol. As shown in Fig. 3B, the addition of free cholesterol markedly reduced the accumulation of cells with 4n DNA content, although some polyploid cells still remained.

Next, we determined the synthesis of DNA by incubating the cells in the presence of BrdU. As shown in Fig. 4, the accumulation of polyploid cells produced by cholesterol starvation did not affect the progress of the S phase, as indicated by the two inverted U-shape BrdU incorporation (S1 and, specially, S2).

Analysis of cyclin expression in the different phases of the cell cycle

To determine the mechanisms responsible for the effect of cholesterol starvation on cell cycle progression and polyploidization, we performed a bivariant analysis of cyclin expression in combination with DNA measurement by flow cytometry. This analysis makes it possible to relate the expression of each of these proteins with the actual cell cycle position [25,26]. This approach also allows the identification of mitotic cells and, hence, to distinguish between cell cycle arrest in G2 or M phases [27].

To establish the actual status of cells in G2/M phase, we analyzed the expression of the cyclin A and cyclin B1. Expression of cyclin A progressively increases during S phase and is maximal in cells at late G2 phase, its degradation occurring very quickly, in prometaphase or very early in metaphase. In contrast, the expression of cyclin B1 is essentially limited to late S and G2 phases, and its degradation occurs at transition to anaphase [14]. Thus, by measuring comparatively the expression of cyclin A and cyclin B1, it was possible to distinguish between G2 and mitotic cells. As shown in Fig. 5A, in cells treated with SKF 104976 plus cholesterol, cyclin A progressively increased during S phase and was maximal in G2/M cells. This pattern of expression was similar to that seen in control cells (data not shown). When cells in G2/M phase (4n DNA content) were analyzed, a population of cells, which were negative for cyclin A (R2 region) could also be observed. The proportion of the latter population was very similar in every time studied approximately 26–27% (2% of total cells) (Fig. 5A).

Incubation of cells in the presence of the SKF 104976 but in the absence of cholesterol resulted in an increased proportion of cells in G2/M phase (R1 region); this increase was attributable to cyclin A negative cells (Fig. 5B). The proportion of these cells increased progressively, reaching 88% at 72 h after the addition of SKF 104976. These results show that most of the cells arrested in G2/M by effect of cholesterol starvation actually had progressed at least to metaphase. To ascertain whether these mitotic cells were accumulating in anaphase or in a later phase in mitosis, we analyzed the expression of cyclin B1 [28,29]. As shown in Fig. 5B, on treatment with SKF 104876, the proportion of cells in G2/M expressing high cyclin B1 levels (R2/R1) progressively declined (from 0.42 – 0.44 in

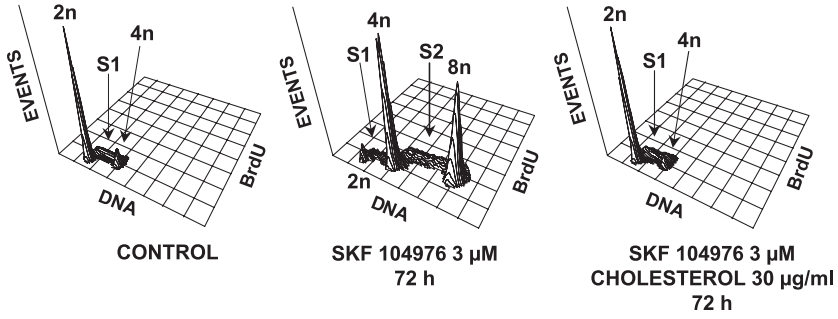

Fig. 4. Effects of SKF 104976 on cell cycle progression. HL-60 cells were incubated in DCCM-1 in the absence (control) or in the presence of 1.5 µM SKF 104976 alone or in combination with 30 µg/ml free cholesterol. After 72 h of culture, the cells received a 1 h-pulse of BrdU and then stained with propidium iodide and analyzed by flow cytometry. The three-dimensional histograms represent the number of events plotted against the DNA content and the incorporation of BrdU into DNA. Data correspond to a representative experiment out of three, which gave similar results.

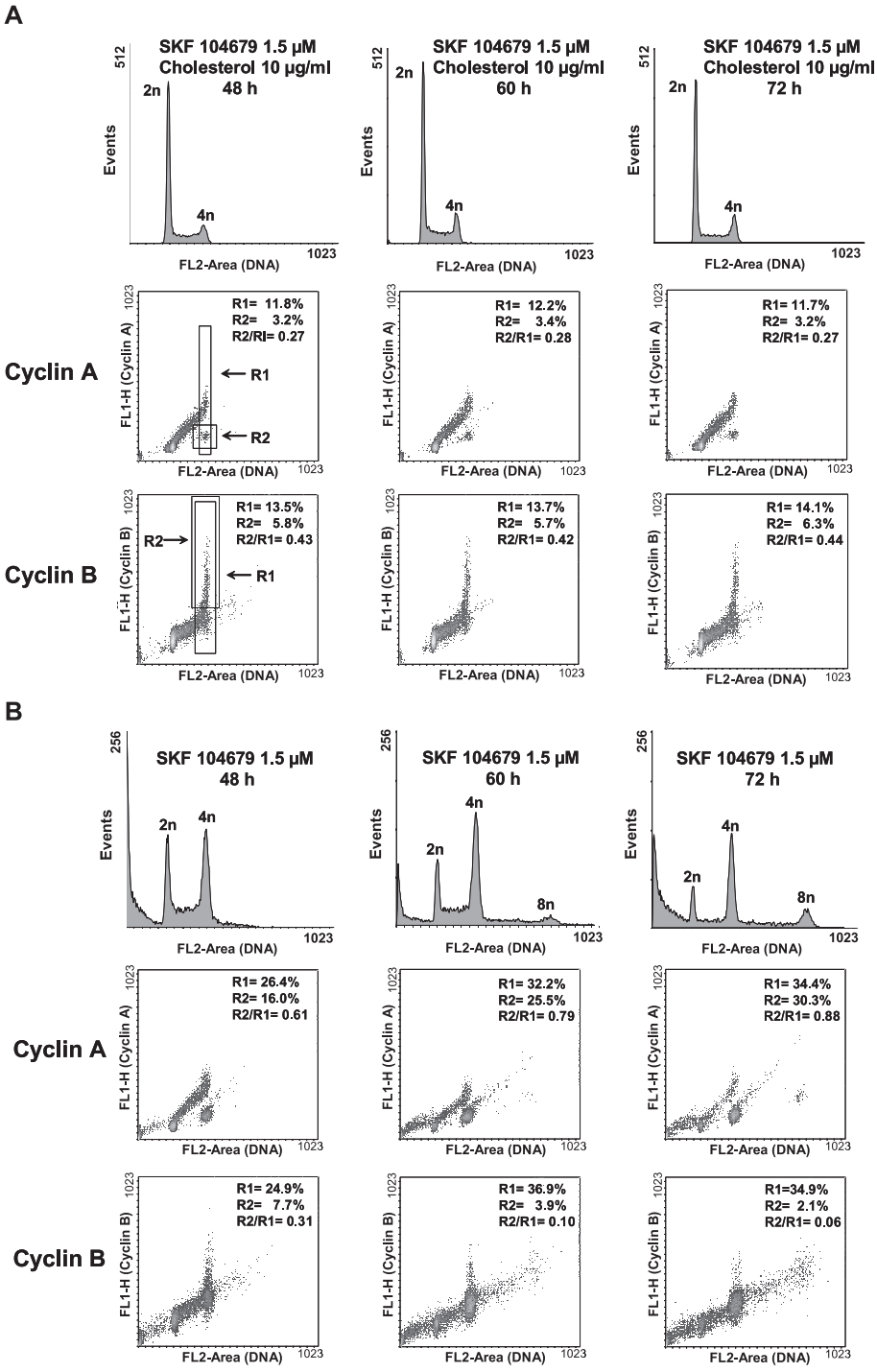

Fig. 5. Bivariant analysis of cyclin A and cyclin B1 expression versus DNA content. HL-60 cells were incubated in DCCM-1 in the presence of 1.5 μM SKF 104976 alone (B) or in combination with 30 μg/ml free cholesterol (A). At the indicated times, cells were stained with monoclonal antibodies against cyclin A or cyclin B1 and propidium iodide for DNA, and analyzed by flow cytometry. Density plot of propidium iodide (red fluorescence) versus fluorescein-labeled anticyclin A or B1 (green fluorescence) was obtained with MODFIT software (Verity Software House, Inc). DNA content histograms are displayed in the upper row of each section (A and B). Data correspond to a representative experiment out of three, which gave similar results.

the control, Fig. 5A, to 0.06 at 72 h of treatment with SKF 104876), indicating that most of the cells with a 4n DNA content had progressed into anaphase or later in mitosis but without undergoing cytokinesis. This was accompanied by an increase in the appearance of polyploid cells, which demonstrates that cells failing to divide reentered in a new cell cycle increasing their DNA content.

To differentiate between diploid G2/M and tetraploid G1 cells, both having a 4n DNA content, we studied the expression of cyclin E. The synthesis of cyclin E takes place in G1 and its degradation occurs during cell progression

through S phase [26]. As shown in Fig. 6A, when incubated in the presence of SKF 104976 plus cholesterol, the number of cells at G2/M phase expressing cyclin E was insignificant (R2 region). However, after treatment with the cholesterol biosynthesis inhibitor alone, the proportion of cells with a 4n DNA content expressing cyclin E progressively increased, reaching a R2/R1 ratio as high as 53% at 72 h of incubation (Fig. 6B). These results indicate that most of the cells accumulating in G2/M phase were actually tetraploid cells in a new G1 phase.

Finally, we measured the expression of cyclin D1. This cyclin, like cyclin E, is maximally expressed in G1 phase and it is complexed with Cdk2, as well as with Cdk4 and Cdk5 kinases, to regulate the transition of cells through G1

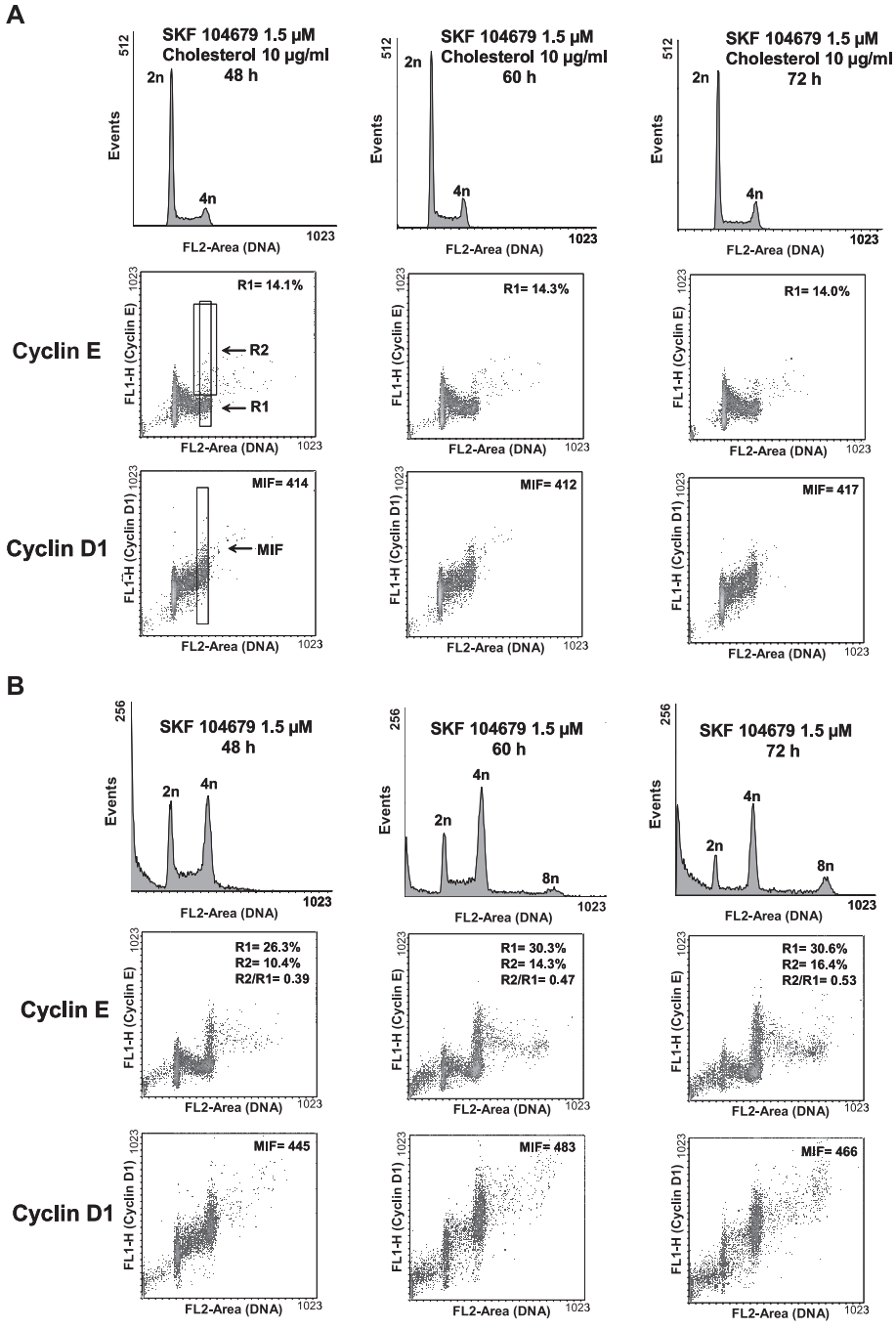

Fig. 6. Bivariant analysis of cyclin E and cyclin D1 expression versus DNA content. HL-60 cells were incubated in DCCM-1 in the presence of 1.5 µM SKF 104976 alone (B) or in combination with 30 µg/ml free cholesterol (A). At the indicated times, cells were and stained with monoclonal antibodies against cyclin E or D1 and propidium iodide for DNA, and analyzed by flow cytometry. Density plot of propidium iodide (red fluorescence) versus fluorescein-labeled anticyclin E or D1 (green fluorescence) were obtained with MODFIT software (Verity Software House, Inc). DNA frequency distributions are displayed in the upper row of each section (A and B). Data correspond to a representative experiment out of three, which gave similar results.

to S phase [30]. The expression of cyclin D1, as indicated by the mean of intensity of fluorescence (M.I.F.) of cells with a 4n DNA content, was substantially higher in cultures exposed to SKF 104976 alone (Fig. 6B) than in those supplemented with cholesterol (Fig. 6A), thus confirming that a certain proportion of cells in G2/M were actually in G1 of a tetraploid cycle.

All together, these results demonstrate that in the absence of cholesterol, cells failed to undergo cytokinesis, reentering G1 phase with a higher DNA ploidy (tetraploidy), and then progressing through S (rereplication).

Analysis of nuclei morphology by Hoescht 33342 staining and electron microscopy

To determine the nucleus morphology, standard immunofluorescence analysis with Hoescht 33342 dye was performed. As illustrated in Fig. 7A, after treatment with SKF 104976 for 72 h, the cells showed several nuclei. To ascertain the proportion of cells in G2/M phase that were actually multinucleated after cholesterol biosynthesis inhibition, at the end of the incubation aliquots of cells were processed in paralleled with either 70% ethanol, which maintains cellular physical integrity, or a buffer that allows nuclei isolation, and then analyzed by flow cytometry. As shown in Fig. 7B, the application of the latter method resulted in a reduction of the 4n peak (21% as compared to 43% in the case of cells processed with ethanol), which indicates that approximately 50% of the cells in G2/M after treatment with the drug were actually multinucleated.

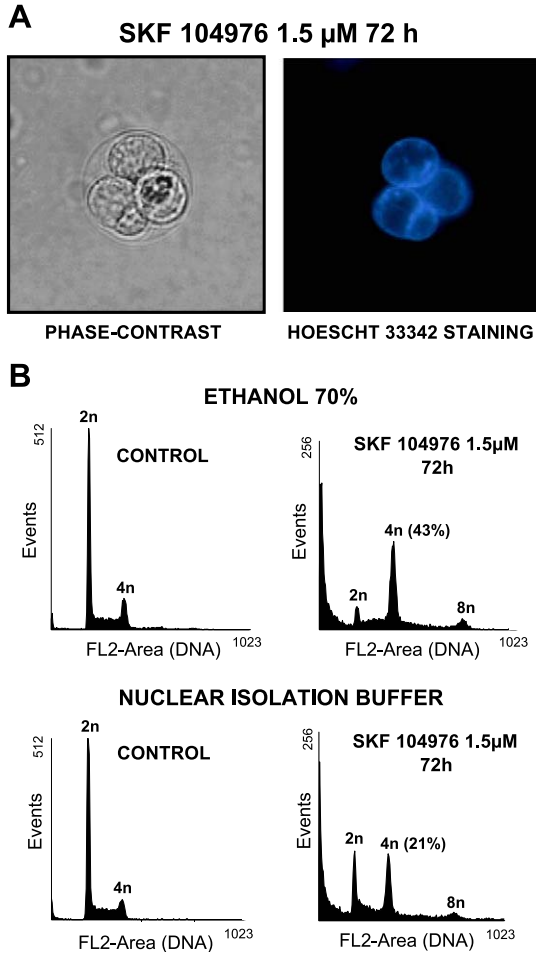

Fig. 7. Cell morphology and analysis of multinucleated cells by flow cytometry. HL-60 cells incubated with 1.5 µM of SKF 104976 for 72 h were photographed under phase-contrast microscopy and stained with Hoescht 33342 for 30 min and photographed under fluorescence microscopy (A). Other aliquots of the cells were processed with either 70% ethanol, which maintains cellular physical integrity, or with a buffer that allows nuclei isolation, and then were stained with propidium iodide and analyzed by flow cytometry (B).

Finally, we analyzed cellular structure by electron microscopy. Cells treated with SKF 104976 during 72 h showed several nuclei (Fig. 8A). Moreover, they had important structural alterations, such as abnormal number of centrioles (impair), which is also suggestive of an alteration in mitosis progression (Fig. 8B).

Effect of Cdk 1 inhibition on cell cycle progression

We previously demonstrated that cholesterol starvation resulted in a decrease of Cdk 1 activity in HL-60 cells [4]. To directly examine whether Cdk 1 inhibition results in polyploidization, HL-60 cells were treated with indirubin-3′-monoxime, a known specific inhibitor of Cdk 1 [31]. As shown in Fig. 9, treatment of cells with 10 µM indirubin-3′ -monoxime initially produced the accumulation of cells in G2/M, which was followed by a significant and progressive increment of polyploid cells thereafter (>4n DNA content).

Discussion

The requirement of cholesterol and other mevalonate derivatives to support cell growth is well established [1,7,8]. In the present work, we are reporting for the first time that cholesterol is essential for mitosis progression. To study the role of cholesterol in G2-M transition and mitosis progression, we used HL-60 cells, which upon cholesterol deprivation are initially arrested at G2 phase by decreasing Cdk1 activity [4]. Similar effects were observed in other human leukemia cells (MOLT-4) [4]. In other cell lines, however, cholesterol synthesis inhibition results in cell cycle arrest at G1 preferentially [32–34], hence being useless to study the effects of cholesterol starvation in mitosis. In the present work, by using that cell model, we have been able to demonstrate that cholesterol is required for cytokinesis and that sustained cholesterol starvation leads to the formation of polyploid, multinucleated cells with mitotic aberrations.

Suppression of cholesterol biosynthesis has been shown to inhibit cell proliferation, provided a source of exogenous cholesterol is not available [8]. In this work, we have used SKF 104976, an inhibitor of lanosterol 14α-demethylase that

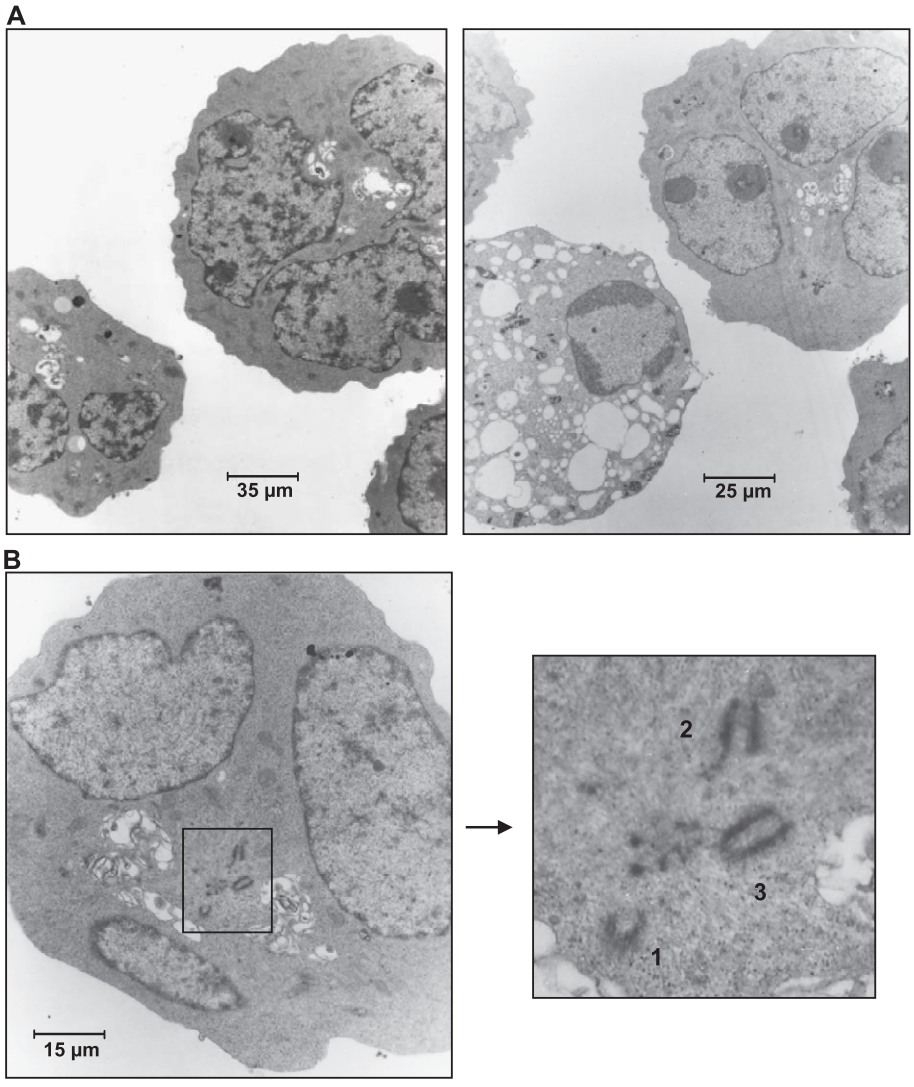

Fig. 8. Electronic micrographs of HL-60 cells after treatment with SKF 104976. Cells treated with 1.5 μM SKF 104976 for 72 h were photographed and analyzed by electron microscopy. (A) Multinucleated and apoptotic cells. (B) A multinucleated cell showing three centrioles (1, 2, and 3).

efficiently blocks cholesterol biosynthesis and significantly reduces cell cholesterol content. Paralleling these effects, SKF 104976 efficiently inhibited DNA synthesis and cell growth, which were prevented and restored by adding free cholesterol to the medium. These results fully confirmed our previous observations [4,35].

Flow cytometry analysis showed that SKF 104976 treatment resulted in a delay of G2-to-M transition, which

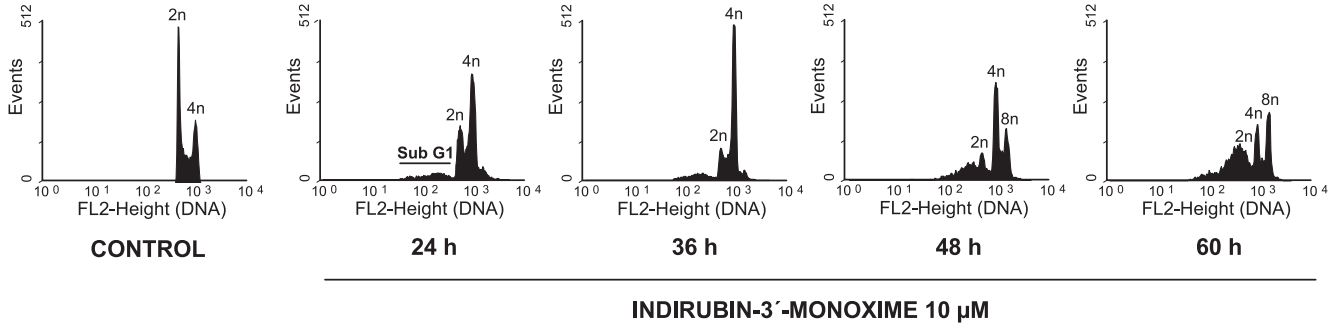

Fig. 9. Effect of Cdk 1 inhibition on cell cycle distribution. HL-60 cells were incubated in DCCM-1 in the presence of 10 μM indirubin-3′-monoxime. At the indicated times, cells were stained with propidium iodide and analyzed by flow cytometry. Data correspond to a representative experiment out of two, which gave similar results.

was followed by a progressive appearance of polyploid cells. A similar block in G2/M was observed in lanosterol 14α-demethylase-deficient cells when incubated in a cholesterol poor medium [36]. To distinguish between G2 and mitotic cells, we used a multiparameter (cellular DNA content versus cyclin expression) flow cytometry method. This approach has been used by others for the identification of the different phases of the cell cycle [27]. Based on the difference in time of synthesis and degradation of cyclin A (which is expressed in S and G2 phases and degraded in mitosis just before metaphase) versus cyclin B1 (which is expressed in G2 and degraded during transition from metaphase to anaphase), it was possible to distinguish between G2 and M phases. This approach showed that after treatment with SKF 104976, the cells were blocked in mitosis, as indicated by the accumulation of cells with 4n of DNA content being negative for both cyclin A and cyclin B1 expression. Furthermore, the concomitant expression of cyclin E, which is synthesized in G1, found in cells with 4n DNA content, indicates that these cells were in tetraploid G1 phase.

It is well recognized that G2-to-M transition in eukaryotic cells is controlled by the protein kinase complex Cdk1-cyclin B1 [13]. We previously demonstrated that cholesterol starvation resulted in a decrease of Cdk1 activity in HL-60 cells concomitant with the accumulation of cells at G2 phase [4]. This loss of Cdk1 activity in cholesterol-starved cells was associated with cyclin B1 rather than Cdk1 protein under-expression, as measured by Western blot [35]. In the present study, a decrease in the expression of cyclin B1 in 4n cells treated with SKF 104976 was also found by bivariant cytometric analysis (Fig. 5). All this is consistent with a G2 delay in cholesterol-starved cells because of low Cdk1 activity. However, these cells were able to partially traverse mitosis, although being unable to culminate it, and to rereplicate DNA, this way generating polyploid cells, as observed herein.

Analysis of nuclear morphology by Hoescht 33342 staining revealed that SKF 104976 treated, polyploid cells were actually multinucleated. This indicates that nuclear division took place, although the cells could not undergo cytokinesis. Electron microscopy analysis revealed that most of the polyploid cells had mitotic alterations, such as an impair number of centrioles. In consonance with these observations, some endoreplicating cell types have been reported to exhibit important alterations in mitosis, with loss of structural components required for mitosis, such as centrosomes [20]. Piel et al. [37] have recently demonstrated that the absence of centrosome leads to defects in cytokinesis, emphasizing the importance of centrosomes for normal mitosis.

As demonstrated by others, cells types in which mitosis is partially traversed but incomplete have low levels of Cdk1 or its activators, cyclin A, cyclin B1, or Cdc25C [16,17,38], and some endoreplicating cells, that apparently bypass mitosis, do not even express these G2/M regulators [15]. For instance, conditional inactivation of Cdk1 in HT2-19 human cells leads to extensive DNA rereplication in the absence of measurable mitotic events [39]. Moreover, in megakaryocytes, the best-known polyploid cell type in mammals, the endoreplication mechanism has been associated with reduced Cdk1 activity [18,19]. In the present work, we directly demonstrate that inhibition of Cdk1 by indirubin-3′-monoxime induces polyploidization in HL-60 cells (Fig. 9), which is in agreement with previous results by others in human breast epithelial cells [40]. Taken altogether, it is suggested that cell cycle perturbations and polyploidization observed in cholesterol-deficient cells are due to the reduced Cdk 1 activity.

Polyploidy can be induced by certain chemicals but not every cell type has the same predisposition to polyploidization [41,42]. A role for p53 and p21 in the prevention of the reinitiation of S phase in the absence of an intervening mitosis has been documented by different authors [42–46]. It is then possible that the p53-null condition of HL-60 [47] allows the reinitiation of S phase although cytokinesis is not completed by the cholesterol deficiency.

In summary, we have demonstrated that cholesterol is essential for mitosis completion and its lack causes mitotic aberrations, impedes cytokinesis, and induces the formation of polyploid and multinucleated cells.

Acknowledgments

This study was supported by grants from the Comunidad de Madrid (08.4/0037.1/2001) and the Instituto de Salud Carlos III (FIS 020241), Spain.

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