CHOLESTEROL REQUIREMENT OF PRIMARY DIPLOID HUMAN FIBROBLASTS

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ABSTRACT

Primary cultures of fibroblast-like cells were obtained from skin and articular cartilage of human donors in the age bracket of 1 to 15 years. For growth these cultures required 1 mg/liter of cholesterol added to Medium A2 plus acetyl choline, Na pyruvate, and D-galactosamine HCl (APG) containing 10% lipoprotein-free human serum. Established cell lines did not require cholesterol for growth. Eagle's medium could be used in place of Medium A2 plus APG with the same results. Desmosterol could replace cholesterol but lansterol or 7 dehydrocholesterol could not. Other cholesterol precursors were tested and found to be inactive. With the proviso that cholesterol precursors entered the cell and had to be converted to cholesterol to function, it was concluded that the particular primaries studied lacked a functional enzyme system to reduce the double bond at carbon 7.

INTRODUCTION

Cholesterol metabolism has been investigated in intact animals, tissue homogenates, and cell cultures (1, 2, 3, 5, 6, 7, 27). Many of the intermediates and enzymes associated with the conversion of acetate to cholesterol are known (3, 6, 26). Using liver homogenates, Dempsey has identified a number of naturally occurring setroids, formed from squalene, that are converted to cholesterol (6). She has also studied the effect of inhibitors on the various conversion steps.

The ability of intact established cell cultures to remove cholesterol from or excrete cholesterol to their environment has been investigated by Bailey (1, 2), and reviewed by Rothblat and Kritchevsky (27). The movement of cholesterol into and out of the cell is affected both by the serum protein and by noncholesterol lipids in the environment. In serum, cholesterol is found in the chylomicron, alpha- and beta-lipoprotein fractions. The quantity varies with the species, age, sex, and health of the donor (28). Though its function is not understood, cholesterol is found in the membranes of cells and is a precursor of the steroid hormones. To stimulate the growth, for more than one subculture, of primary human cells which are nonneoplastic, diploid, and exhibit a limited lifespan as described by Hayflick (14), serum is usually added to the medium as a supplement.

Our observations indicate that established heteroploid cultures differ from primary diploid cultures in their response to cholesterol and other lipids in the medium. Established heteroploid and neoplastic cultures can be adapted to grow in chemically defined media containing no cholesterol (10, 13), and in general, the media requirements are not as critical as they are for primary diploid cultures. Cristofalo, Howard, and Kritchevsky (5) have shown that the embryonic primary diploid human line designated W138 (14) incorporated labeled acetate into cholesterol. These investigators used Eagle's medium but did not determine whether the rate of cholesterol synthesis was adequate for growth. The composition of the chemically defined portion of the medium may

contain substances which affect cholesterol uptake or synthesis as in the case of certain amino acids (21). Primary diploid fibroblast-like cultures derived from both human skin and articular cartilage required 1 mg/liter of cholesterol when placed in medium A2 plus acetyl choline, Na pyruvate, and D-galactosamine HCl (APG) supplemented with lipoprotein-free human serum. These cultures were prepared from tissue of 25 different donors, ranging in age from 1 to 15 years. Upon removal of cholesterol, the appearance of these cells resembles that of the cells described by Geyer (12), Moskowitz (23), and MacKenzie et al. (22) with lipid accumulation in the form of droplets. The same results were obtained by using Eagle's medium.

With reference to the cholesterol pathway involving the naturally occurring beta hydroxysteroids determined by Dempsey (6), 5,24-cholestadienol (desmosterol) was found to support growth of our primary diploids and was comparable with cholesterol. It was also found that neither 5,7 cholestadienol (7 dehydrocholesterol) nor 5,24 cholestadienol (lanosterol) supported growth.

METHODS

Preparation of Human Serum

Freshly clotted human serum was obtained from fasting donors and stored at −70°C. Lipoproteins were removed by established methods (4). Potassium bromide (KBr) was added to portions of the serum to a density of 1.20 and centrifuged at 100,000 *g* for 48 hr. In some cases the centrifugation time was extended to 7 days. The tubes were sectioned and the bottom portion was dialyzed against 0.8% sodium chloride solution to remove KBr. The resultant, processed serum was referred to as lipoprotein-free serum. The top portion of the sectioned tubes containing the lipoproteins was relayered under 1.20 density KBr solution and recentrifuged for 24 hr at 100,000 *g* to remove residual nonlipoprotein serum. This lipoprotein fraction was then layered under 1.064 density KBr solution and centrifuged. The beta type lipoproteins were harvested from the top section of the tube, the alpha type from the bottom. Both the top and bottom fractions were dialyzed against saline. In some experiments 0.01% sodium versene was added during KBr treatment.

Acrylamide-Gel Electrophoresis

A modification (19) of a previously developed method (17) was used for comparative analysis of all sera.

Preparation of Lipid-Free Serum and Serum Lipids

Two methods were used, one for preparation of lipid-free serum and the other for the serum lipids.

Lipid-free serum was prepared by extracting serum according to the method of Scanu and Bumpus (29). Each ml was added to 25 ml of a mixture consisting of 2 vol of freshly distilled ethanol and 1 vol of ethyl ether precooled to −20°C. After 1 hr the protein was filtered and washed three times with 25 ml of precooled ethyl ether. Ether was removed under vacuum, and water was added to restore the extracted serum protein to its original volume.

Lipids were extracted from whole serum and fractions thereof, by use of Folch's solution mixture (11) consisting of 2 vol of chloroform and 1 vol of methanol. 24 vol of the mixture was used to extract 1 vol of serum. Total lipids were determined by the Sperry-Brand procedure (30). Composition of the lipids was determined by thin-layer silica gel chromatography. The developing solvent consisted of a mixture: hexane 90 vol, ethyl ether 10 vol, and acetic acid 2 vol (25). Developed plates were sprayed with 5% sulfuric acid, heated for 10 min at 110°C, and photographed in color immediately. In addition to examining the lipid extracts from serum, all commercially obtained materials such as squalene (Sigma Chemical Co., St. Louis, Mo.), cholesterol SCW (Nutritional Biochemicals Co., Cleveland, O.), desmosterol and lanosterol (California Biochemical Corp., Gaithersburg, Md.), and 7 dehydrocholesterol (Mann Research Labs, Inc., New York) were examined by thin-layer chromatography.

Preparation of Lipids for Tissue Culture Media

Total serum lipids or individual lipids free of solvent were dissolved either in methanol or acetone. The dissolved lipids were dispersed in vigorously agitated sodium cholate solution heated to 35°C. For example, 10 mg of cholesterol was dissolved in 20 ml of methanol which was added to 100 ml of 0.1% sodium cholate water solution at pH 7.2. The water-clear, sometimes bluish solution, depending upon the type of lipid dispersed, was placed in a rotary evaporator, reduced to 80 ml, and readjusted to 100 ml with distilled water. All solvents were spectrographic grade, and water was conductivity grade pyrogen-free. Nonbiological surfactants, such as Tween 80, commonly used in tissue culture, were avoided. Lipid solutions were sterilized through Morton glass or Coors porcelain filters pre-conditioned with 0.1% sodium bicarbonate solution. Nalge membrane filters were also satisfactory.

Tissue Culture Medium

The composition and preparation of Medium A2 plus APG has been previously described (15, 18). In some experiments cholesterol was removed from the formulation. Eagle's medium (8) was obtained from Grand Island Biological Co., Grand Island, New York.

Cell Cultures

Established cell lines and those adapted to grow in completely chemically defined medium have been described previously (18).

Primary diploid cells derived from human cartilage and skin were obtained from material kindly supplied by Dr. MacEwen, of the Surgical Department of the Alfred I. du Pont Institute. For example, patients were hospitalized for surgical correction of orthopedic problems and in many cases it was possible to obtain cartilage shaved from the outer surface of the knee joint. Small pieces of skin sterilized by standard surgical technics were obtained from the point of incision. The outer dermal areas were trimmed free of underlying fatty tissue. Falcon plastic flasks, 30 ml volume, were filled with 4 ml of Medium A2 plus APG supplemented with 10% human serum. The flasks were inverted and pieces of tissue which had been finely minced with scissors under a small volume of medium were placed on the inverted surface. After a 2 hr incubation the tissue attached to the surface of the flask; the flasks were then carefully restored to their normal position with the full volume of medium covering the pieces of tissue. Omission of the inversion step resulted in free floating tissue. The fibroblast outgrowth then encased the tissue and did not produce a satisfactory culture for our purposes. Within 10 days to 2 weeks the outgrowth of cells, fibroblast-like in appearance, was subdivided into additional flasks. Cells were loosened from the flask, by use of chemically defined medium containing 0.03% trypsin, and centrifuged. The pellet of cells was immediately dispersed in fresh medium containing either 10% human serum or the ingredients desired to be tested. As the use of trypsin was an undesirable feature, it was not possible to disperse the strongly adhering primary fibroblast cultures by mechanical scraping without massive cell destruction. This problem was not encountered with the established lines, all of which were dispersed with a silicone scraper. For purposes of maintaining cells as near as practical to their original metabolic state, all cultures were discarded after their 10th subculture, except in certain extended metabolic trials.

Growth was measured on replicate cultures as total cell protein (24), immediately after inoculation and just before subdividing the confluent culture. By using this procedure, it was recognized that some variation was to be expected in relating the total protein to cell numbers, due to changes in the individual cell's total protein during the course of the experiment.

EXPERIMENTAL

Serum Lipoprotein Requirements of Primary Cells

With the discovery of the alpha-one globulin growth factor (18), attempts were made to grow primary cell cultures both of skin and of articular cartilage under similar conditions. Growth beyond one subculture was not achieved, though no difficulty was experienced with replicate control cultures containing 10% dialyzed human serum. This led to investigation of the dialyzed human serum components required by primary diploid cells but not by heteroploid established cells.

Serum was centrifuged at a density of 1.20 to remove total lipoproteins (see Methods). Primary diploid cells placed in medium supplemented with this lipoprotein-free serum did not survive. The very characteristic appearance of degenerating cells in lipoprotein-free serum is shown in Fig. 1 and Fig. 2. Similar degeneration was observed with the use of reconstituted serum protein, precipitated, and extracted with ethanol and ethyl ether at −20°C (see Methods). Removal of lipoproteins by centrifugation caused a number of irreversible changes in the serum's electrophoretic pattern. These changes were due to the high concentration, density 1.20, of KBr (19). Typical alterations in the electrophoretic pattern of human serum resulting from KBr treatment are illustrated in Fig. 3. Despite this alteration, returning the total lipoprotein fraction to the lipoprotein-freed portion restored the growth-stimulating activity of the latter. Returning the lipoprotein did not restore the electrophoretic pattern to its original form.

To determine whether the components, which restored the growth-promoting activity of lipoprotein-free serum, resided either in the alpha-lipoprotein fraction, density 1.064–1.20, or the beta fraction, density less than 1.064, both were prepared and added separately to the lipoprotein-free serum. Either fraction was observed to restore the lipoprotein-free serum's activity, similar to the restoration observed when the total lipoproteins were returned. Fig. 1 shows the effect of returning the alpha-lipoprotein fraction. Cultures containing the alpha fraction showed less debris than those containing the beta fraction. When each of the

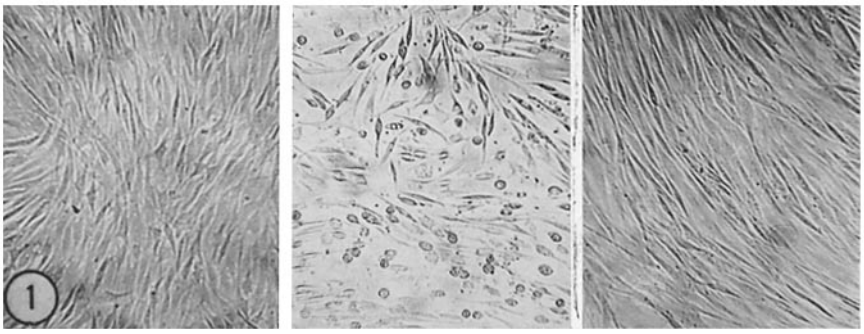

FIGURE 1 11-day-old replicate cultures of a third subculture of fibroblast-like cells obtained from articular cartilage grown in Medium A2 plus APG. The following supplements were added from left to right: 10% dialysed human serum; 10% lipoprotein-free human serum; and 10% lipoprotein-free human serum restored with alpha-lipoprotein fraction. × 80.

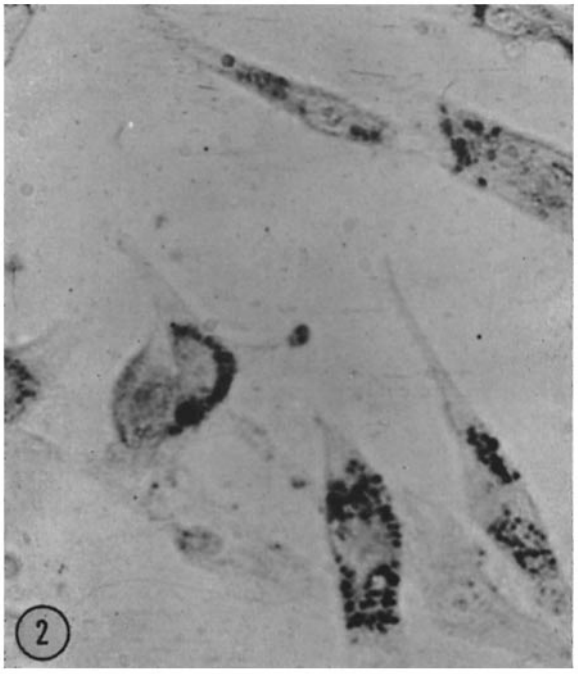

FIGURE 2 A duplicate of culture shown in Fig. 1 supplemented with 10% lipoprotein-free serum. Stained with 35% alcohol saturated with Sudan Black B to illustrate lipid-like character of droplet inclusions. × 800.

lipoprotein fractions were tested separately, the beta fraction was found to be toxic, causing cells to become pycnotic and to disintegrate. This toxicity, which was apparent within 12 hr, confirms a prior observation that showed the beta-lipoprotein fraction to be toxic for established cells (16). A similar toxic response was obtained with oleic acid at a concentration of 8 mg/liter as shown by Geyer (12) and Moskowitz (23). The toxic effect of oleic acid was prevented by the addition of lipoprotein-free serum, though the growth activity of the latter was not restored. The alpha fraction was not toxic and was not sufficient to maintain growth. The cells presented an appearance similar to that of the control on whole serum, but multiplication stopped after one subculture. The cells were not characterized by the accumulation of droplets as were cells placed in medium containing lipoprotein-free serum.

It was concluded that primary cells required both the lipoprotein fraction and the lipoprotein-free portion of serum in order to grow. Since the

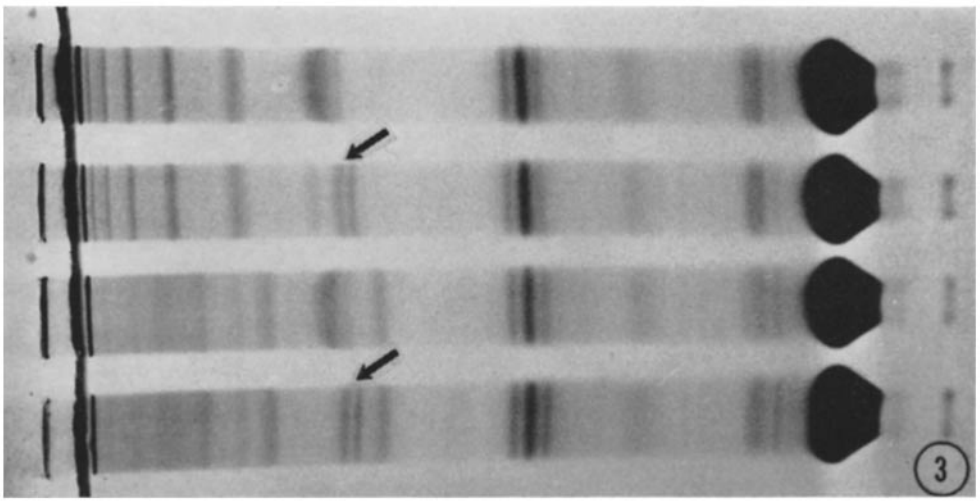

FIGURE 3 Acrylamide-gel electrophoretic patterns of human serum before and after removal of lipoproteins by ultracentrifugation, with use of KBr at a density of 1.20. From top to bottom: sample of dialysed group B Rh-positive human serum; same serum after removal of lipoproteins; a sample of dialysed group B Rh-negative human serum; same serum after removal of lipoproteins. Altered protein bands found after treatment with KBr are marked (arrows).

beta-lipoprotein fraction was toxic and the alpha fraction was not, one property of the lipoprotein-free serum was its ability to detoxify the beta-lipoprotein fraction. As this detoxifying action was not required in the case of the alpha-lipoprotein fraction, it was concluded that the detoxifying capability of lipoprotein-free serum was a reality, but was not necessarily its main function in stimulating cell growth unless the cells themselves produced toxic substances during growth. The substances in lipoprotein-free serum that are responsible for its detoxifying properties are being studied separately and will not be pursued further in this report. This report will concern itself with the growth-promoting substances in the lipoprotein of serum.

Lipid Requirements of Primary Cells

Since either the beta- or alpha-lipoprotein fraction restored the activity of the lipoprotein-free serum, it was considered a possibility that the activity resided in the lipid portion of the molecule, not in the protein portion. To determine whether this was the case, portions of the original serum and each lipoprotein fraction were extracted with Folch's mixture of chloroform and methyl alcohol, the solvent was removed, and the lipids were incorporated in a test medium containing lipoprotein free serum equivalent to 10% whole serum (see Methods). All three cultures continued to grow and could be subcultured without difficulty.

The amount and kind of lipids in whole and in lipoprotein-free serum was determined next. Considerable information is available in the literature on this subject (28). Quantitative determinations of total lipids in a number of sera yielded a value ranging from 500 to 900 mg %, similar to values reported elsewhere (28). When centrifuged for 48 hr to remove lipoproteins, human serum still contained 8% of the original total lipid. Continuous centrifugation at 100,000 *g* for one week at a density of 1.20 did not significantly lower this value.

The lipids extracted from whole serum and lipoprotein-free serum separated by thin-layer chromatography are shown in Fig. 4. A given volume of each serum type was solvent extracted and each pattern represents the proportion of lipid in each type, with one exception. One of the patterns of lipoprotein-free serum was increased fourfold, to more readily determine its composition. The free fatty acid component of the lipoprotein-free serum remained relatively high. This was not surprising, since a portion of the fatty acids is carried by albumin (20) and would not be removed by ultra-

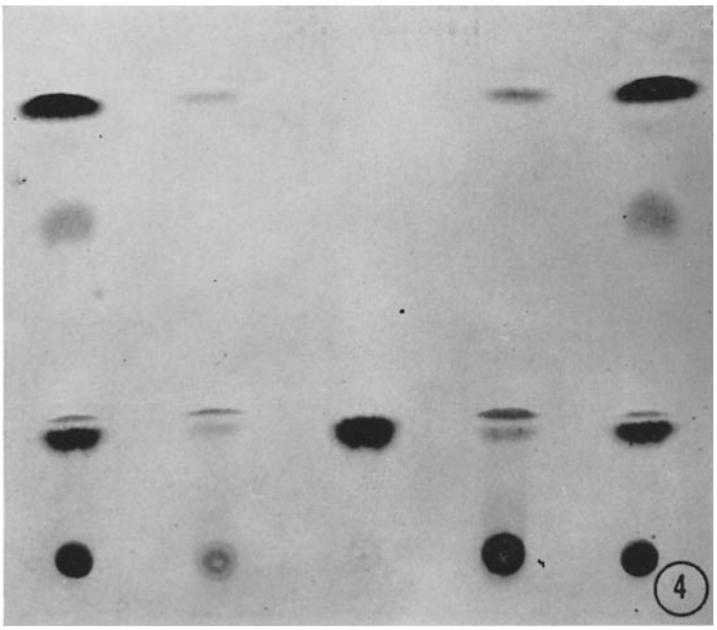

FIGURE 4 Thin-layer silica-gel chromatograms of serum lipids. Replicate volumes of respective sera extracted with chloroform-methanol mixture. Left to right: whole serum; lipoprotein-free serum; solution of 1% SCW cholesterol; lipoprotein-free serum concentrated fourfold; duplicate whole serum. × 0.5.

centrifugation. Cholesterol either was not present in the lipoprotein-free extract or was present only in trace amounts. When individual lipids were tested, cholesterol was found to restore the growth-promoting activity of the lipoprotein-free serum. Fig. 5 shows the effect of adding increasing amounts of cholesterol to replicate cultures of articular fibroblast-like cells. No further beneficial action was observed with concentrations of cholesterol greater than 1 mg/liter. In Fig. 6 the growth rate of replicate cultures in medium containing cholesterol is compared with the growth rate of those placed in medium containing no cholesterol. Cells grown with the use of cholesterol have been successfully subcultured 18 times; however, when the cholesterol was removed, the cells stopped growing. It was concluded from the above observations (see Fig. 6) that, irrespective of the age, in reference to the subculture of the cells used to conduct the test, there was sufficient cholesterol carry-over to permit the first of the test cultures to approach confluency. Fig. 7 compares growth rates of skin fibroblasts under conditions similar to those for the articular fibroblasts shown in Fig. 6. No other lipid fraction, including the cholesterol esters, recrystallized from acetone and checked for composition and purity by thin-layer chromatography, was effective. In view of these results, negative control cultures of lipoprotein-free serum, used in all experiments, were prepared using Medium A2 plus APG without the 0.2 mg/liter of cholesterol normally included in its formulation. When this was done, the negative controls on each experiment became very regular, failure occuring promptly upon the first transfer and often apparent before they became confluent in the first cultures.

Utilization of Cholesterol Precursors by Primary Cells

The above observations suggested that in the primary fibroblast-like cultures there might be a key phase in cholesterol synthesis that for one reason or another was non-functional. To test this possibility, special media were formulated which contained either beta hydroxy methyl-glutarate, mevalonate, squalene, lanosterol, desmosterol, or cholesterol. Each of the commercial materials used was tested for purity by thin-layer chromatography. Squalene, for example, showed several spots but

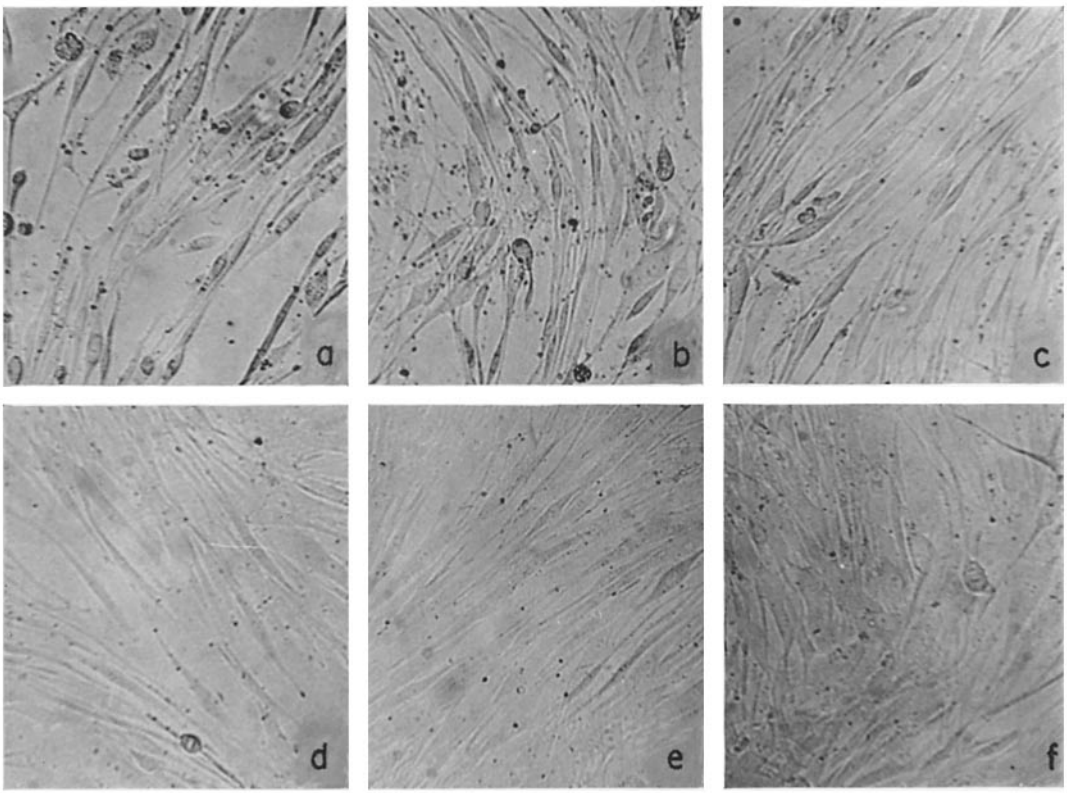

FIGURE 5 Replicate cultures of 12-day-old articular fibroblast-like cells grown in Medium A2 plus APG supplemented with 10% lipoprotein-free serum. (*a*) through (*f*) indicate the effects of increasing amounts of cholesterol starting with 0.0; 0.2; 0.4; 0.6; 0.8; and finally 1.0 mg/liter. × 400.

no cholesterol. Some of the other materials were not entirely pure, but showed no evidence of cholesterol. Cholesterol SCW yielded one major spot with a minute trace of a second component when the plate was developed with acetone-chloroform (1:4 vol). Only two materials proved to be active, cholesterol and desmosterol. Since desmosterol is identical to cholesterol except for a double bond at carbon 24, it was decided to test 7 dehydrocholesterol, which is identical with cholesterol except for an extra double bond at carbon 7. It has been shown by Dempsey (7) that this particular steroid represents an alternate pathway to cholesterol. The 7 dehydrocholesterol was not active. It was concluded that the functional pathway to cholesterol in primary cells, fibroblast-like, obtained from human donors in the age bracket between 1 and 15 years of age, was probably via desmosterol rather than 7 dehydrocholesterol. Furthermore, the pathway to desmosterol was blocked at some point between lanosterol and desmosterol. The validity of these conclusions rests on the assumption that the intermediates tested are able to enter the cell and that it is cholesterol that restores the growth activity of lipoprotein-free sera.

Lipid Requirements of Established Cells

A variety of established heteroploid cell lines, Chang's conjunctiva and Girardi's human heart, whether they had been adapted or not to grow in chemically defined medium (18), did not require the addition of cholesterol for growth. Gey's HeLa cells, Eagle's KB cells and our own neoplastic human lung cells, all permanent strains, grew without added cholesterol. Cristofalo et al. reported that WI38 synthesized cholesterol from acetate in Eagle's medium (8). Consequently, Eagle's medium was used in place of medium A2 plus APG and tested on primary skin cultures. All of these cultures required the addition of cholesterol in order to grow.

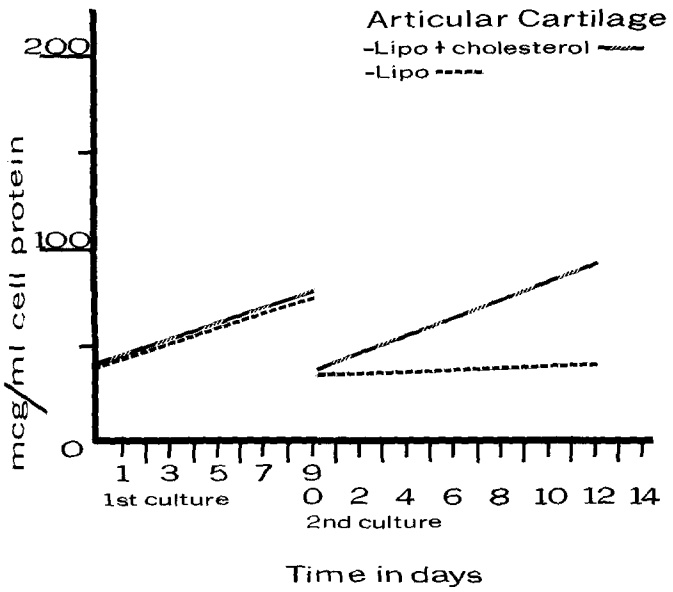

FIGURE 6 Shows the change in total protein of replicate cultures of articular fibroblast-like cells grown in lipoprotein-free serum and in lipoprotein-free serum supplemented with 1 mg/liter of cholesterol.

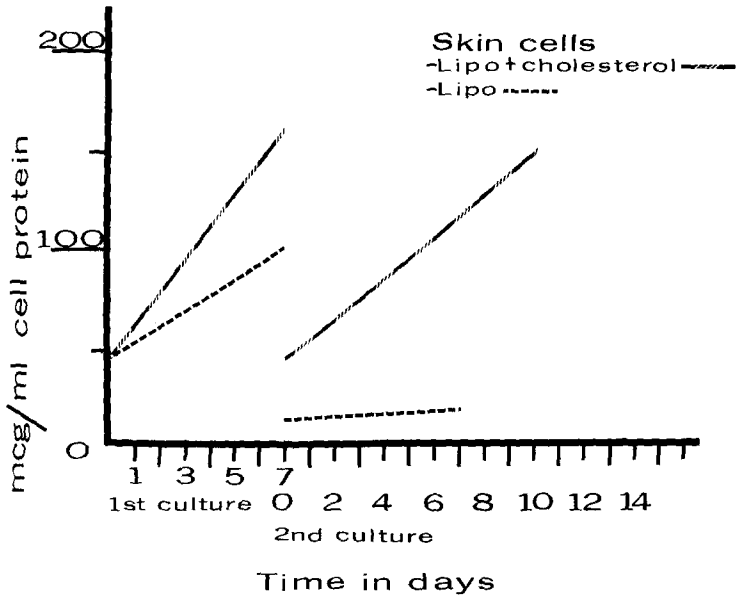

FIGURE 7 Same as Fig. 6, except that the fibroblast-like cells were obtained from human skin.

DISCUSSION

It is recognized that established cell lines and primary diploid cell lines are metabolically different (9). According to Hayflick (14), primary diploid cells differ from established cells in that they exhibit a limited lifespan under present conditions of culture. Within the group tested, it is now apparent that one of the differences between primary and established cell lines is the ability of the latter to synthesize cholesterol from acetate in amounts sufficient for growth.

Although primary cultures of both skin and articular cartilage exhibit a requirement for choles-

terol, one may not draw the conclusion that all primary cells require cholesterol. Cells selected from other tissues, for example liver, or cells of embryonic origin not fully differentiated, may behave differently.

We have limited our studies to primary cultures not older than ten subcultures. Some metabolic changes that are not immediately apparent may have occurred even during this period. There was no evidence of transformation to produce an established strain in cultures used in any of the experiments. Transformation has been observed previously and has always been characterized by the appearance of epithelial-like cells of increased growth potential as described by Eagle (9). Our results were consistently reproduced, using both skin and articular cartilage fibroblast-like cultures from some 25 different donors in the age bracket of 1 to 15 years. Different batches of human sera were processed to remove the lipoproteins, and similar results were obtained with each batch.

The lipids in the lipoprotein-free serum were lowered with the exception of the free fatty acids which remained disproportionately high. Investigation is in progress to determine why the primary cultures failed to grow in the lipoprotein-free serum medium. Though the pH of the cultures was carefully adjusted, the fatty acids in unbalanced form may be causing a type of acidosis similar to that described by MacKenzie et al. (22), or they may be accumulated in the cell as shown by Geyer (12) and Moskowitz (23). Bailey has also shown that lipids other than cholesterol, as well as lipoproteins, can affect cholesterol uptake and excretion (2). Irrespective of cause, in these initial studies, it seemed significant that such a small amount of cholesterol, 1 mg/liter, consistently could reverse the effect. There may be differences in membrane structure and permeability between established and primary cultures. The possibility exists that there is an inhibitor either in the defined portion of the medium or in the serum which specifically affects cholesterol metabolism in primary cultures.

Further experimentation is required to confirm that desmosterol is converted to cholesterol and that 7 dehydrocholesterol is not. If this is confirmed then there is a possibility that the particular cell cultures used in the present study lack the ability to reduce the double bond at carbon 7. This would explain why lanosterol, which is converted to Δ 5, 7,24 cholestiendiol before conversion to desmosterol (25), is nonfunctional. An inhibitor of this enzyme may be present.

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