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CHANGES IN PHOSPHOLIPIDS FROM CHICK FIBROBLASTS DURING EMBRYO DEVELOPMENT

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SUMMARY

The content of cholesterol and total phospholipids was assayed in 8- and 16- day old chick embryo fibroblasts, harvested at subconfluence after a 48- and 96-hour primoculture, respectively. Cholesterol content did not change during embryo development, whereas the amount of total phospholipids decreased (28%) from the 8th to the 16th day of development, giving an increase of the cholesterol/phospholipid ratio. Studies of the fatty acid composition of the predominant membrane phospholipids indicated that there was no significant change in phosphatidylcholine, whereas phosphatidylethanolamine was depleted in the myristate, as the embryo grew older. These findings demonstrate that the lipid contents are modified during embryo development and suggest that the fluidity of chick embryo cell membranes decreased during development.

INTRODUCTION

Embryo cells during differentiation, and normal and transformed cells (1-2) exhibit differences in plasma membrane-mediated biological properties, e.g cellular adhesivity and recognition (3-4), hexose transport (5-6) and the effects of lectin on them (7-10). This suggests that membrane components such as proteins, glycoproteins and lipids undergo changes during cell differentiation. Differences in protein and glycoprotein patterns have been reported between normal and transformed cells (7,11) and in embryo cells at various stages of development (12-13). Changes in phospholipid composition have also been noted between normal and transformed cells (14) related to changes in membrane fluidity (15-16). It therefore seemed interesting to investigate the possible modification of membrane lipid composition in a cell system like normal embryo differentiation. To this end, 8 and 16-day old chick embryo fibroblasts were harvested at subcon-

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fluence after a 48- and 96-hour primoculture, respectively, taking into account the growth rate difference observed between these two stages of development (8). The levels of cholesterol and phospholipids were measured. The fatty acid composition of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) was also determined, taking into account the fact that the patterns of molecular species alterations in total phospholipids reflect those of total cell membranes as well as of plasma membranes (17-19).

MATERIALS AND METHODS

Cells and cultures. The fibroblasts were obtained from 8- and 16-day chick embryos (Société Jourdain, France) by the method of Rein and Rubin (20) modified as previously described (21). Primary monolayer cultures were made in 600 cm^2 single trays (Nunc Denmark) in 120 ml of Eagle's minimum medium supplemented with 1% glutamine, 1% antibiotics (penicillin, streptomycin) and 10% foetal calf serum. The initial seeding concentration was 10^6 cells/ml. Cultures were grown in humidified air containing 5% CO_2, at 37°C. Growing fibroblasts from 8- and 16-day embryos were harvested using a rubber policeman at the identical step of culture, i.e at subconfluence after 48- and 96- hours. A sample of cells was counted in a haemocytometer (1 to 1,5 x 10^8 cells per culture) and the cell viability was shown to be greater than 95% as estimated by trypan blue exclusion.

Lipid extraction and analysis. Assay of phospholipid content was done on the cytosol fraction after the cells had been disrupted according to the method of Stone et al (22). The phospholipids in the cytosol fraction amounted to less than 2% of the total cell content. For subsequent experiments, cells were therefore extracted (23-24) with a chloroform-methanol mixture (2:1, v/v) followed by phase partition. Neutral and polar lipids were then collected for cholesterol and choline-containing phospholipid assays and samples were dried and dissolved in propanol-2. The cholesterol content was determined by an enzymatic method using the CHOD-PAP kit (Boehringer, W. Germany). The choline-containing phospholipids were estimated by an enzymatic method using the Wako phospholipid B-test (Biolyon, France). The amount of total phospholipids was calculated from the quantity obtained by phosphate assay (25) using the average molecular weight of 775.

Lipids were separated by thin layer chromatography on silica plate (TLC) (Merck, Reference 7451). About 200 µg of lipid extract were submitted to TLC into two directions (26) : 1) chloroform-methanol-water (65:25:4, v/v/v) and 2) butanol-acetic acid-water (3:1:1, v/v/v). After chromatography, lipids were revealed by iodine vapor and identified by comparison with known standards (Supelco Inc. Bellefonte, USA) run on similar plates. Fatty acid analysis was carried out on the PC and PE species. After evaporation of the iodine from the TLC plates, the spots were scraped off the plate, eluted with chloroform-methanol (2:1, v/v) dried and saponified at 65°C in 2 ml of 1 N KOH for 10 min. The fatty acids released were esterified (27) with 2 ml of methanol containing 10% BF_3 (Carlo Erba, Italy) at 65°C for 5 minutes. Methyl

TABLE I : Total cholesterol and phospholipid content of 8- and 16- day chick embryo fibroblasts. Extraction and assay procedures are described in Materials and Methods. Results are expressed as $\mu g/10^6$ cells. Each value is the average of 4 separate assays from 3 separate experiments. Variations in percentage are given from the 8th to the 16th day. C/P ratio was calculated as a molar ratio, assuming that the average molecular weight for phospholipids is 775.

	Age of embryo in days		Variation in perc
	8	16	%
Total phospholipids	64,6 ± 7,9	46,3 ± 9,7	- 28
Choline-containing phospholipids	26,7 ± 2,2	19,2 ± 1,6	- 28
Others	37,9	27,1	- 28
Cholesterol	7,8 ± 1,1	7,2 ± 0,9	0
C/P ratio	0,242	0,311	+ 28

Others are estimated as the difference between total and choline-containing phospholipids.

esters were extracted into pentane and concentrated. Fatty acid methyl esters were analysed by gaz liquid chromatography (GLC) on a Carlo Erba Fractovap 2100 Chromatograph at 185°C with a 2 m-long column using chromasorb WAW 80/100 mesh as support and 10% diethylene-glycol succinate as stationary phase. Weight percentage were calculated by a Hewlet Packard 3800 A integration system.

RESULTS

I - Total Lipid content

The results for total cholesterol and phospholipid contents are summarized in Table I. The cytosol fraction did not contain a significant amount of phospholipid (less than 2%). For the total cell membranes, there was no significant difference in cholesterol content between the 8th to the 16th day of development. By contrast, the total phospholipid content associated with the total cell membranes was decreased by 28% in fibroblasts from 16 day embryos as compared to those from 8 day embryos. This decrease was similar in choline containing phospholipids. The cholesterol/phospholipid ratio (C/P ratio) thus increased by 28% from the 8th to the 16 th of development.

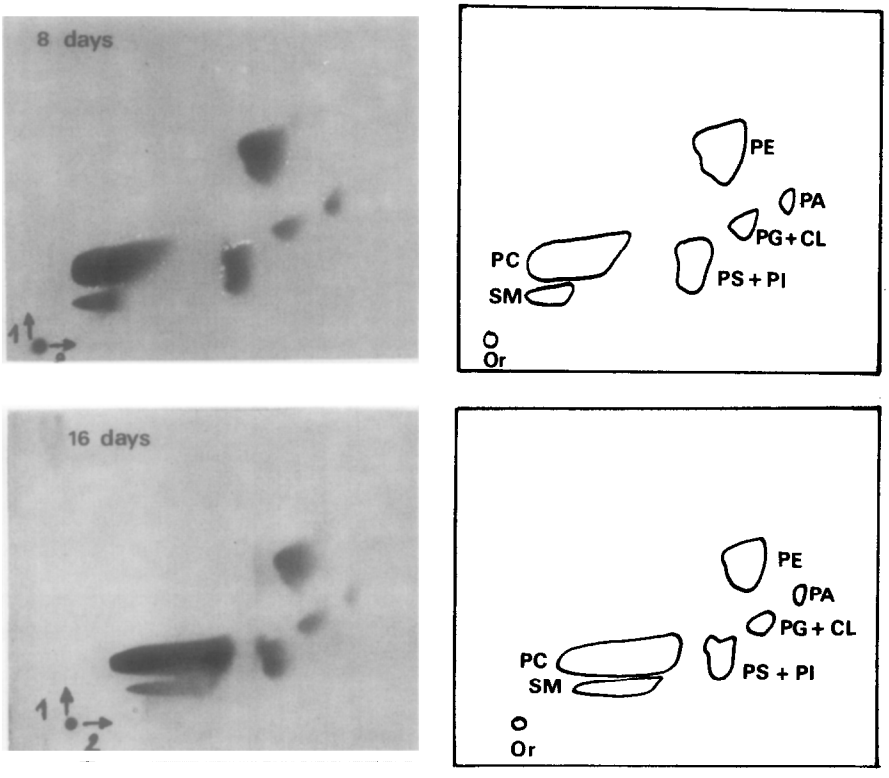

Figure 1 : Typical TLC-Chromatograms of total phospholipids from fibroblasts of 8- and 16- day chick embryos after iodine staining.
The experimental procedures are given in Materials and Methods.
The phospholipids were identified by comparison with known standards.
SM : Sphingomyelin ; PC : Phosphatidylcholine ;
CL : Cardiolipin (diphosphatidylglycerol) ;
PE : Phosphatidylethanolamine ; PI : Phosphatidylinositol
PA : Phosphatidic Acid ; PS : Phosphatidylserine ;
PG : Phosphatidylglycerol.

II - Fatty acid composition of PC and PE

The lipids extracted were separated by TLC and Fig.1 shows the typical patterns for phospholipids from fibroblasts from 8- and 16- day embryos. For both stages the two major components were phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and the minor components sphingomyelin, phosphatidic acid, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylserine and phosphatidylinositol. The fatty acid composition of the two major components was analysed by G.L.C. As can be seen in Table II, there was no significant change in the fatty acid composition

TABLE II : Fatty acid composition and percentage distribution of phosphatidylethanolamine and phosphatidylcholine in 8- and 16- day chick embryo fibroblasts. Preparation and quantification of fatty acid methyl esters are described in Materials and Methods Each value is the average of 3 separate experiments.

	Fatty acid composition						
	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	>C 18:2
Phosphotidylethanolamine							
8-day	13.2	35.2	5.4	24.1	19.0	3.0	traces
16-day	3.9	37.5	6.2	29.2	22.8	traces	traces
Phosphotidylcholine							
8-day	3.8	41.2	7.0	19.1	28.6	traces	traces
16-day	4.2	40.6	8.1	17.3	29.4	traces	traces

of PC between the 8th and the 16th day. However marked differences were observed in PE, particularly in the percentage of myristate (C 14:0) which decreased by 70% from the 8th to the 16th day (Table II). The percentage of stearate (C 18:0) and oleate (C 18:1) increased slightly with the age of embryos. Linoleate (C 18:2) was detectable in a significant amount at day 8 whereas only traces were noted at day 16.

DISCUSSION

Variations in membrane fluidity through transformation and normal embryo differentiation were first suggested by the mobility of lectin binding sites in relation to the cell agglutinability (7, 15, 28-30). The agglutination rate of cells increases with increased membrane fluidity, as estimated by the spin label method (15). From a molecular point of view the results reported here support this notion.

Cholesterol and phospholipids are almost exclusively located in the cell membrane, PC and PE being the two predominant membrane phospholipids (31). The lack of variation in the cholesterol content associated with the decrease in phospholipids during embryo development could be explained by a compensatory increase in plasma membrane proteins (13), since no significant modification has been detected in fibroblast volume (6) as measured either with a Coulter particle Counter or by the method

of Kletzien et al (32). We have shown that the cholesterol/phospholipid ratio (C/P ratio) in subconfluent primary cultures of chick embryo fibroblasts increases by 28% from the 8th to the 16th day of embryo development. Assuming that the C/P ratio constitutes the major determining factor in the microviscosity of the cell membranes (16, 32-33), this finding indicates an increase in the microviscosity of the cell membranes and thus a decrease in their fluidity as the embryos grow older. These results are to be compared with those obtained by Alderson and Green (34) who demonstrated that sterol-enriched Ehrlich ascites cells exhibit an higher C/P ratio associated with a decrease in membrane fluidity than to control cells. Our results also suggest that PE could play a role in the decrease in fluidity of the cell membrane since this was depleted in the myristate species during embryo development. It is important to note that the pattern of alteration in molecular species of total phospholipids (including PC and PE) associated with the changes in membrane fluidity has been detected by Shiga et al (17) and Freter et al (18), in total membranes ; this would agree with the notion that these membranes arise partially from Golgi apparatus membranes and from the endoplasmic reticulum and that they are re-utilised in the production of secretory vesicles and plasma membranes (35).

Finally, the decrease in cell membrane fluidity during embryo development are compatible with the previously noted modifications in the properties of fibroblasts, such as lectin binding, agglutinability and hexose transport.

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