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Aging of rat heart fibroblasts: relationship between lipid composition, membrane organization and biological properties

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The relationship between age-related alterations in the lipid composition of cultured rat-heart fibroblasts and several biochemical and biophysical parameters was investigated. Aged (14–15-day-old) cultures displayed higher mole ratios of sphingomyelin to phosphatidylcholine, as well as elevated cholesterol levels. A concomitant increase was observed in the total protein content of the cells and in the V_{max} values of both membranal and cytoplasmic marker enzymes. Fluorescence photobleaching recovery was employed to study the lateral mobility of the lipid probe NBD-phosphatidylethanolamine and of membrane glycoproteins that bind succinylated concanavalin A The mobile fractions of both probes were higher in aged cultures, while the lateral diffusion coefficients were lower To further demonstrate the dependence of the above parameters on the cellular lipid composition, we have manipulated the lipid composition of old cultures by treatments with liposomes (small unilamellar vesicles) of specific compositions Treatments which reversed the lipid composition towards that of young (5–6-day-old) cultures caused a concomitant reversal of the measured biochemical and biophysical parameters to the values observed in young cultures. These findings suggest that alterations in the organization and mobility of cell membrane constituents are involved in mediating changes in cellular functions In view of our previous findings on cultures of rat-heart myocytes (Yechiel, E., Barenholz, Y. and Henis, Y.I. (1985) J. Biol. Chem. 260, 9132-9136), it appears that the modulation of cellular properties through the membrane lipid composition may be a general phenomenon in many cell types.

Introduction

The lipid composition of cell membranes plays an important role in defining various membrane properties Changes in the membrane lipid composition can be related to physiological processes (for example, aging), they may also be exerted as a result of various diseases or through alterations in the surroundings of the cells [1–5] The changes in the lipid composition include alterations in the content of cholesterol, degree of phospholipid acyl-chain saturation, and mole ratio and level of phosphatidylcholine (PC) and sphingomyelin [6] Apart from affecting the dynamic properties of membrane constituents, these alterations may

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Abbreviations PC, phosphatidylcholine, TMR, tetramethylrhodamine, FPR, fluorescence photobleaching recovery, NBD-PE, *N*-4-nitrobenzo-2-oxa-1,3-diazolylphosphatidylethanolamine, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, *R*, mobile fraction, *D*, lateral diffusion coefficient

interfere with the organization of the lipids in distinct domains [7–11]

We have recently demonstrated that cultured rat heart myocytes undergo changes in several parameters during aging, and that these changes may be manipulated by treatment with liposomes of various compositions [6,7] These changes include alterations in the lipid composition of the myocyte membranes, in the level of both membrane and cytoplasmic enzymes, and in the beating rate of the cultured myocytes [6] In parallel, we detected modulations in the lateral mobility and organization of lipids and proteins in the myocyte membrane [7], the latter changes could serve as mediators between membrane lipid composition and cellular functions [6]

In order to examine whether the above phenomena exist also in other cell types and are not unique to newborn rat myocytes, we have now performed analogous studies on cultures of the nonmuscle cells derived from rat heart [12,13] These cells are referred to as endothelial cells [14] or as fibroblasts [12] The term fibroblast will also be used here Our results demonstrate that all the above phenomena (changes in content of protein, altered levels of marker enzymes, and changes in the lateral mobility and organization of membrane lipids and proteins) occur also in the cultured fibroblasts in response to alterations in their membrane lipid composition Such alterations accompany the aging of the cells in culture, and can be reversed by treatment with PC liposomes The modulation of membrane and cellular properties through the membrane lipid composition may thus serve as a general mechanism in many cell types

Materials and Methods

Reagents

PC and sphingomyelin (over 99% pure) were purified from egg yolk and bovine brain, respectively, according to established procedures [15,16] Cholesterol (over 99% pure) was obtained from Sigma (St Louis, MO) *N*-4-Nitrobenzo-2-oxa-1,3-diazolylphosphatidylethanolamine (NBD-PE) prepared from egg PE was purchased from Avanti (Birmingham, AL) Succinyl concanavalin A was prepared according to Gunther et al [17] and conjugated with tetramethylrhodamine (TMR) isothiocyanate by standard procedures [18] Medium (F-10 HAM) and sera (fetal calf serum and horse serum) was obtained from either Gibco (Grand Island, NY), or from Biological Industries, Kibbutz Beth Haemek, Israel All other chemicals were of the highest purity available

Cells

Two types of culture were prepared – mixed cultures containing both heart myocytes and fibroblasts, and cultures of heart fibroblasts only A mixed cell suspension containing the two cell types was prepared according to Kasten [14], as described by us earlier [6] The plating of this cell suspension yielded a myocyte-fibroblast mixture referred to as 'mixed cultures' Alternatively, fibroblast cultures were prepared using a minor modification of the differential attachment technique (Method A of Kasten, Ref 14) The mixed cell suspensions (5 ml of 8 10^5 cells/ml) were plated in 60×15 mm 3002 Falcon tissue culture plates (Falcon, Cocksville, MD) and incubated (37°C, 5% CO_2, 1 h) to allow the adherence of the fibroblasts The nonattached cells were removed, and the adhered cells were referred to as fibroblast cultures Under these conditions the fibroblasts divided only during the first three days, after which the cell number and the DNA level remained constant (50 ± 4 μg DNA per plate) The medium employed both for plating and for growing the cells was F-10 HAM medium supplemented with additional calcium and $NaHCO_3$ (1 2 mM and 30 mM final concentrations, respectively) 10% fetal calf serum, 10% horse serum, and antibiotics (200000 IU/liter penicillin, 200 mg/liter streptomycin) The two types of heart cell monolayer were grown in an incubator at 37°C in 4–5% CO_2 and high humidity for 21 days, changing growth medium every 48 h Under these conditions, spontaneous beating (which is a good indicator for the recovery of cultured myocytes) was observed 24–48 h after seeding Only 3% of the cells in young fibroblast cultures demonstrated beating, as compared with more than 95% in young myocyte cultures [6] This suggests very little contamination of the fibroblasts with myocytes

Treatment of cells with liposomes

13 days after plating, the cells were treated with

liposomes prepared from PC, sphingomyelin, or a mixture of PC and cholesterol Liposomes (small unilamellar vesicles) were prepared by ultrasonic irradiation [16], sterilized by filtration through a 0 22 μm filter, and added to the medium in the dish to give a final phospholipid concentration of 1 mM This procedure was repeated with every medium change For more details, see Ref 6

Analytical methods

Determination of lipid composition, protein content, DNA content and the level of the seven marker enzymes described in Table II were performed according to established procedures as described in the miniprint supplement of Ref 6 The results of each experiment represent an average on two or three plates For statistical evaluation of the experimental results, each experiment was repeated ten times

Fluorescent labelling of cells

The cells were washed twice in F-10 medium (without Phenol red and sera) containing 20 mM Hepes For labelling with NBD-PE, the cells in a 35 mm 3001 Falcon dish were incubated 15 min at 22°C in 1 ml of this medium containing 2 10^{-5} M NBD-PE The fluorophore was introduced by 1 100 dilution from a 2 mg/ml solution of NBD-PE in ethanol Excess NBD-PE was removed by washing twice with the same medium This procedure results in a uniform fluorescent labelling on the cell membrane This labelling is not due to formation and absorption of NBD-PE liposomes, since labelling the cells with liposomes from unlabelled lipids whose inside contained carboxyfluorescein resulted in a few patches of fluorescence stuck on the cells, which were immobile in fluorescence photobleaching recovery experiments

For labelling with TMR-succinyl concanavalin A, the cells were incubated for 15 min at 23°C with 10 μg/ml of the label, followed by a similar washing procedure 2 ml of the Hepes-buffered solution were added back into the dish, which was taken for the FPR experiments

Fluorescence photobleaching recovery

Lateral diffusion coefficients and mobile fractions were measured by the FPR method [19,20] at 23°C using the instrument described previously [21] The attenuated beam of an argon ion laser (488 nm at 0 03 μW for NBD-PE, 529 7 nm at 0 3 μW for TMR-succinyl concanavalin A) was focused through the microscope to a spot of 2 0 μM Gaussian radius with $\times 40$ water immersion lens A brief intense pulse of the beam (39–80 ms, 0 3 mW for NBD-PE, 3 mW for TMR-succinyl concanavalin A) bleached about 50–70% of the fluorescence in the illuminated region, and the attenuated beam was employed to monitor the recovery of fluorescence in that region due to the entry of unbleached fluorophores Two parameters can be derived from the fluorescence recovery curves – D, the lateral diffusion coefficient of the mobile fluorophores, and R, the fraction of fluorophore which is mobile on the experimental time-scale [20] The values of these parameters were independent of the extent of bleaching It should be noted that recent experiments have demonstrated that the bleaching conditions employed in FPR do not affect the diffusion measurements, yielding values similar to those described from experiments which do not employ bleaching [45,46]

Results

Alteration of cellular properties due to aging and liposome treatments

Table I demonstrates the gross chemical changes in cultured rat heart fibroblasts following aging or treatment of aged cultures with small unilamellar vesicles of various lipid compositions The results resemble those obtained previously for cultured myocytes [6]

The aging of the cells in culture was accompanied by changes in the lipid composition of the cells and in their total protein content, while the DNA content (which is proportional to the number of cells) was unaffected The total phospholipid content of the cells was essentially constant with the culture's age, however, aged cultures displayed a much higher sphingomyelin to PC mole ratio, mainly due to a large increase in the sphingomyelin level and a smaller decrease in PC (Table I) The cholesterol level in old cultures displayed a net increase, and thus a 23% increase in the cholesterol/(cholesterol + phospholipids)

TABLE I

THE GROSS CHEMICAL COMPOSITION OF NEWBORN RAT FIBROBLASTS EFFECT OF CULTURE AGE AND TREATMENT WITH LIPOSOMES

Myocytes were prepared, grown and their gross chemical compositions was determined as described in Materials and Methods (A) 5–6-day-old cultured defined as 'young cultures', (B) 14–15-day-old cultured defined as 'old cultures', (C) 'old cultures' treated with egg PC liposomes, (D) 'old cultures' treated with liposomes made of egg PC cholesterol 1 0 9 (mol mol) (E) 'old cultures' treated with bovine brain sphingomyelin (SM) liposomes All treatments (systems C–E) started at the culture's 13th day and went on for at least 48 h using 1 mM phospholipid All values are given per one plate as an average of ten experiments three plates in each experiment

System	DNA content (μg)	Total protein (μg)	Total phospholipid content (nmol)	Cholesterol content (nmol)	SM content (nmol)	PC content (nmol)
A	49 ± 5	2800 ± 250	133 ± 14	25 ± 2	34 ± 5	54 ± 3
B	48 ± 5	4150 ± 500	144 ± 20	37 ± 3	72 ± 8	40 ± 3
C	48 ± 4	3400 ± 400	129 ± 17	20 ± 2	28 ± 3	51 ± 4
D	53 ± 7	3450 ± 350	131 ± 15	45 ± 5	33 ± 4	52 ± 5
E	46 ± 6	3900 ± 400	142 ± 20	22 ± 2	70 ± 8	41 ± 3

mole fraction was observed in aged cultured In parallel with the lipid composition of the cells, their protein content had increased upon aging in culture (Table I)

In order to examine further the relations between these parameters, the lipid composition of aged cultures was altered by treatment with liposomes of specific compositions When PC and PC/cholesterol liposomes were present in the growth medium during the 13th and 14th days in culture, the PC/sphingomyelin mole ratio and content returned to the values observed in young cultures, in the case of PC liposome treatment, the sphingomyelin level was reduced even below that observed in young cultures (Table I) These phenomena were not observed upon treatment with sphingomyelin liposomes, since the changes in the phospholipid composition of the cells depend on the rate of their release from the liposomes and on the ability of the latter to serve as phospholipid acceptors, the sphingomyelin release rate from sphingomyelin liposomes at 37°C is very low [22] and bovine brain sphingomyelin liposomes are also poor phospholipid acceptors at 37°C, which is well below their transition temperature (Ref 3 and Barenholz, unpublished data) On the other hand, all the liposome treatments were efficient in producing either cholesterol depletion or enrichment, depending on the cholesterol content of the liposomes (Table I), thus, PC and sphingomyelin liposomes reduced the cholesterol level in old cultures to values even below those observed in young cultures, while treatment with PC/cholesterol liposomes (1 0 9) (mole ratio) increased the cholesterol level by a factor of 1 22 It is worth noting that higher increases in the cholesterol level may be obtained by incubation with liposomes containing higher cholesterol/PC mole ratios (data not shown) The dependence of the cholesterol depletion or enrichment on the cholesterol gradient between the cells and the liposomes resembles previous reports on other systems [23–25] Interestingly, both PC and PC/cholesterol liposome treatments (but not sphingomyelin liposome treatment) reduced the total protein content of the cells close to the level observed in young cultures, demonstrating a correlation between this parameter and the cellular lipid composition

In order to examine the dependence of biochemical properties of the cells on the cellular lipid composition, the kinetic parameters (V_{max}/μg DNA and K_m) of seven marker enzymes in fibroblast cultures were measured as a function of age and following treatment with egg PC liposomes (Table II) These enzymes include 5′-nucleotidase, alkaline phosphatase, $(Na^+ + K^+)$-ATPase, and Mg^{2+}-ATPase as plasma membrane markers, glucose-6-phosphatase as an endoplasmic reticulum marker, acid phosphatase as a marker for lysosomes, and creatine phosphokinase which

TABLE II

KINETIC PARAMETERS (V_{max}/DNA AND K_m) OF SEVEN MARKER ENZYMES OF FIBROBLASTS IN CULTURE EFFECT OF CULTURE AGE AND INTERACTION WITH EGG PC LIPOSOMES

Myocyte cultures were homogenized and the enzymatic activity (V_{max}, K_m) of seven of their enzymes (marker enzymes) were determined as described in Materials and Methods (A) 5–6-day-old cultures ('young cultures'), (B) 14–15-day-old cultures ('old cultures'), (C) Old cultures treated with egg PC liposomes V_{max} is given as μmol product per μg DNA per h and K_m as substrate concentration (mM) For more details see Methods and Results

System	V_{max}						
	Acid phosphatase	Alkaline phosphatase	Glucose-6 phosphatase	Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase	5′-AMP nucleotidase	Creatine phosphokinase
A	0 070±0 005	0 014±0 0011	0 040±0 0028	0 090±0 0072	0 82±0 07	0 060±0 005	0 13±0 02
B	0 172±0 013	0 034±0 0024	0 073±0 006	0 200±0 0160	2 05±0 17	0 14 ±0 011	0 29±0 03
C	0 081±0 0058	0 010±0 0008	0 039±0 002	0 085±0 0063	0 8 ±0 08	0 055±0 006	0 04±0 003
	K_m						
A	1 4±0 15	2 3±0 33	1 0 ±0 08	2 7±0 5	6 4±0 8	0 09±0 007	n d [a]
B	1 0±0 35	2 2±0 3	1 1 ±0 15	3 5±0 7	5 5±0 5	0 11±0 01	n d
C	1 1±0 15	2 5±0 4	1 15±0 2	2 5±0 4	6 9±0 9	0 12±0 01	n d

[a] K_m of creatine phosphokinase was not determined (n d) because commercial diagnostic kits were used under conditions where only V_{max} but not K_m can be determined

is found in mitochondria and in the cytosol [6] The changes in the kinetic parameters of the marker enzymes resemble those previously reported by us in rat myocytes [6], namely, changes occurred only in V_{max}, and not in K_m, suggesting increases in the enzyme levels upon aging The latter increases were beyond those observed in the total protein content of the cells (Tables I, II) The V_{max} values were reduced to the levels observed in young cells (or even more for creatine phosphokinase) by the treatment with egg PC liposomes Sphingomyelin liposomes did not have any effect (data not shown)

Membrane organization and dynamics

Fluorescence photobleaching recovery experiments yield two parameters – the lateral diffusion coefficient, and the fraction of mobile fluorophores Significant changes were observed in the R values of the fluorescent phospholipid probe NBD-PE in the plasma membrane of the cultured fibroblasts as a function of the culture's age and in the liposome treatments (Table IIIA) The R values observed in aged (14–15-day-old) cultures were higher than in young (5–6-day-old) ones (0 89 vs 0 78) Treatment of aged cultures with PC liposomes, which increased the PC/sphingomyelin ratio and decreased the cholesterol level (Table I), induced a decrease in R of NBD-PE to 0 66, a value below that obtained for young untreated cultures

Treatment of old cultures with sphingomyelin liposomes, which induced cholesterol depletion, but did not alter the PC/sphingomyelin ratio (Table I), induced a much smaller decrease in R of NBD-PE (to 0 78) Thus, the effect of the PC liposome treatment cannot be solely due to cholesterol depletion This point is further demonstrated by the effect of treatment with liposomes containing both PC and cholesterol (at 1 0 9 mol mol ratio), this treatment increased the PC/sphingomyelin ratio without depleting cholesterol (in fact, the cholesterol level had increased by about 10% – see Table I) These liposomes were also effective in reducing R (from 0 89 to 0 74), although not below the level encountered in young (5–6-day-old) fibroblast cultures The effect of the liposome treatments on R of the lipid probe is also not due to 'fluidization' of the membrane, since incubation of old cultures with 0 1% pentanol, which is considered a 'fluidizer' [26], did not alter the R value (data not shown)

The effects of aging and liposome treatments on the R value could be due to alterations in the

TABLE III

LATERAL ORGANIZATION (R) AND DYNAMICS (D) IN MEMBRANE OF RAT HEART FIBROBLASTS EFFECT OF CULTURE AGING AND TREATMENT WITH LIPOSOMES OF VARIOUS COMPOSITION

IIIA Mobile fractions of NBD-PE and TMR-succinyl concanavalin-receptor complexes S E was determined from measurements performed on 10–20 cells The cultures were treated as follows (A) Young untreated 5–6-day-old cultures (B) old untreated 14–15-day-old cultures, (C) old cultures treated with 1 mM egg PC SUV, (D) old cultures treated with 1 mM bovine brain sphingomyelin SUV, (E) old cultures treated with liposomes (SUV) composed of 1 mM egg PC and 0 9 mM cholesterol IIIB Lateral diffusion coefficients of NBD-PE and TMR-succinyl concanavalin A-receptor complexes S E was determined from measurements performed on 10–20 cells For systems A–E see IIIA For more details see Materials and Methods

IIIA System	Mobile fraction (R)	
	NBD-PE fibroblasts	TMR-SConA fibroblasts
A	0 78 ± 0 02	0 35 ± 0 02
B	0 89 ± 0 03	0 44 ± 0 03
C	0 66 ± 0 04	0 22 ± 0 03
D	0 78 ± 0 08	0 30 ± 0 03
E	0 74 ± 0 04	0 35 ± 0 04
IIIB	Lateral diffusion coefficients (D)	
	NBD-PE ($\times 10^9$) fibroblasts	TMR-SConA ($\times 10^{10}$) fibroblasts
A	5 8 ± 0 27	6 2 ± 0 70
B	4 5 ± 0 20	7 4 ± 0 80
C	5 4 ± 0 50	6 4 ± 0 55
D	3 2 ± 0 22	5 2 ± 0 30
E	3 3 ± 0 22	4 5 ± 0 50

organization of the membrane lipids in domains [7] In such a case, a parallel change should also be observed in the R values of membrane proteins, since if part of the lipid domains are separated from the rest of the lipid pool by lateral phase separation, the membrane proteins in these domains will also appear as immobile in FPR studies [7] This is indeed the case, as shown by experiments on concanavalin A receptors labeled with TMR-succinyl concanavalin A, a dimeric concanavalin A derivative which does not induce excessive crosslinking and immobilization effects [27–29] The pattern of the changes in R (Table IIIA) is qualitatively similar to that observed with the lipid NBD-PE, the only difference is in the size of R, which is lower for TMR-succinyl concanavalin A receptors under all the conditions employed (0 21–0 43 vs 0 66–0 89 for NBD-PE) This phenomenon is not surprising in view of the rather large immobile populations observed for a variety of membrane proteins on many cell types [30–32], most likely due to interactions with cellular structures such as the cytoskeleton [33–36] In spite of the lower absolute values of R for TMR-succinyl concanavalin A receptors, the percentage of the changes observed in this parameter upon aging and in response to the various liposome treatments are rather similar to those observed for NBD-PE (Table IIIA) The only exception is in the case of PC-liposome treatment, where the decrease in R was more significant for the TMR-succinyl concanavalin A receptors As in the case of the lipid probe, pentanol treatment was ineffective (data not shown)

The FPR experiments yield the diffusion coefficient characterizing the lateral motion of the fluorophore population which is mobile on the experimental time scale The effects of the culture's age and of the various liposome treatments on D of NBD-PE are summarized in Table IIIB The highest D was observed on young cultures (5 8 10^{-9} cm^2/s), this value dropped to 4 5 10^{-9} cm^2/s in old cultures These values are in the same range reported earlier for several lipid probes in numerous cell types [30–32] Treatment of old cultures with PC liposomes raised D back to the value obtained on cells in young cultures (5 4 10^{-9} cm^2/s)

In order to differentiate between the contributions of alterations in phospholipid composition and in the cholesterol level, old cultures were treated with either sphingomyelin liposomes or with PC cholesterol (1 0 9) liposomes (see previous section) In both cases, a decrease (to 3 2 10^{-9} and 3 3 10^9 cm^2/s respectively) rather than an increase in the D value of NBD-PE was observed (Table IIIB) It therefore follows that the increased D value following PC liposome treatment is not due simply to cholesterol depletion The simplest explanation for the differential effects of cholesterol in these cases is that it acts as an 'averager' and its effect depends on the membrane lipid composition [37] This point is dealt with extensively in the Discussion

The effects of the culture's age and of the various liposome treatments on the lateral diffusion rate of membrane proteins were also investigated by FPR studies on TMR-succinyl concanavalin A receptors (Table IIIA) Only minor differences were observed between the D value of the membrane protein marker in young and old cultures treated with PC liposomes On the other hand, treatment of cells in old cultures with sphingomyelin liposomes or with PC cholesterol (1 0 9) liposomes, which induced a significant decrease in D of NBD-PE (Table IIIB), resulted in a parallel and significant decrease in D of the TMR-succinyl concanavalin A receptors (from 7 4 10^{-10} to (5 2 and 4 5) 10^{-10} cm^2/s, respectively)

Discussion

Cellular membranes play a major role in many cellular functions, and their dynamics and organization may serve as key factors in the expression and control of these functions The dynamic properties and organization of membranes depend on the membrane lipid composition [2,3,23,38] We have recently demonstrated such a relationship in cultured rat heart myocytes, which undergo changes in their lipid composition during aging [6] These changes were accompanied by alterations in the lateral mobility and organization of membrane components, which were reflected in changes in the lateral diffusion coefficient and in the mobile fraction (R) of a membrane lipid probe, as well as in R of a membrane protein marker [7] In parallel with these changes, physiological and biochemical properties of the cultured myocytes were also affected [6] Moreover, the alteration in both membrane dynamics and in the physiological-biochemical properties of the cells could be modulated by specific liposome treatments [6,7], demonstrating the dependence of the effects on the lipid composition The purpose of the present communication was to examine the generality of these phenomena by investigating their existence in cell types other than myocytes For this purpose we have employed the fibroblastic nonmuscle cells derived from rat heart These primary cells differ from myocytes in their morphology, and they lack beating

The general features of the age-related changes in heart fibroblasts resemble qualitatively those observed in myocytes [6,7] As in the case of myocytes, the culture heart fibroblasts undergo changes in their lipid composition during aging, which are paralleled by alterations in their biochemical properties (Tables I, II) Thus, old (14–15 days) cultures display increased levels of sphingomyelin and cholesterol per DNA, but lower PC levels (Table I) In parallel, the total protein content per DNA increases in old cultures (Table I), and the V_{max} (but not K_m) of the seven marker enzymes tested is elevated, demonstrating higher enzyme levels (Table II) The alterations in the biochemical properties of the cells upon aging could thus appear in response to the changes in their lipid composition This notion is supported by the finding that when the phospholipid composition and the cholesterol content of aged cultures were brought close to the values observed in young cultures by treatments with liposomes of specific compositions, the cellular protein content and the levels of the marker enzymes had also approached those of young cultures (Tables I, II) In this context, it should be noted that the V_{max} values of the seven marker enzymes (Table II) were sensitive mainly to changes in the PC/sphingomyelin mole ratio, and were affected to a much smaller degree by the cholesterol level A similar pattern of sensitivity was observed in myocytes [6]

The changes in the lipid composition of the cultured fibroblasts are accompanied by alterations in the dynamic properties and organization of their plasma membrane lipids and proteins These alterations resemble qualitatively those observed in cultured myocytes [7] However, there are quantitative differences between the two cell types In the case of the lipid probe (NBD-PE), the mobile fraction is always higher for the fibroblasts (Table IIIA) as compared with myocytes under similar conditions [7] Moreover, the scale of the effects on R of NBD-PE is smaller in fibroblasts They vary between 0 66 and 0 89, as compared with 0 35–0 75 in myocytes which have been subjected to identical treatments [7] The changes in D of NBD-PE are in the same scale in the two cell types ((3 2–5 8) 10^{-9} cm^2/s in fibroblasts (Table IIIA) and (2 8–4 9) 10^{-9} in myocytes [7]), demonstrating that R and D may

be modulated independently of each other As for the membrane protein marker (TMR-succinyl concanavalin A), the scale of the effects on R is very similar in the two cell types 0 21–0 43 in fibroblasts (Table IIIA), as compared with 0 21–0 39 in myocytes [7] However, there is a clear difference between the fibroblasts and the myocytes regarding the modulations in the D value of TMR-succinyl concanavalin A receptors while D of this probe is not significantly altered in myocytes under any of the conditions employed [7], it is significantly reduced in fibroblasts concomitantly with the lipid probe following treatment with sphingomyelin liposomes or with PC cholesterol (1 0 9) liposomes (Table IIIB) The lack of sensitivity of the D value of TMR-succinyl concanavalin A receptors on myocytes to the altered dynamic properties of the lipid bilayer was explained by the additional restriction on membrane protein mobility by interactions with other cellular structures, e g , the cytoskeleton [7,33–36] However, this does not mean that as a general rule the lateral motion of membrane proteins is insensitive to the viscosity of the membrane lipid bilayer This is exemplified by the increase in D of epidermal growth factor receptors on A-431 cells in parallel with the increase in temperature, which decreases the viscosity of the lipid bilayer [39] Such a dependence on the state of the membrane lipids is encountered in the cultured fibroblasts following treatment with sphingomyelin or PC cholesterol (1 0 9) liposomes (Table IIIB) The difference between the fibroblasts and the myocytes in this respect may reside in different organization of their cytoskeletal structure, since interactions with cytoskeletal elements were demonstrated to retard the lateral motion of membrane proteins [33–36], moreover, the cytoskeleton-dependent modulation of the lateral motion of certain membrane proteins was shown to depend on the cell type [29] An alternative explanation is that TMR-succinyl concanavalin A labels different groups of membrane glycoproteins on fibroblasts and on myocytes, and the group labeled on fibroblasts is more sensitive to changes in the state of the membrane lipids

The changes in D and R of NBD-PE and of TMR-succinyl concanavalin A receptors on the cultured fibroblasts are correlated with changes in the lipid composition of the cells Thus, young cultures display a higher PC/sphingomyelin mole ratio and lower cholesterol levels than old cultures, and treatment of aged cultures with specific liposomes alters these parameters (e g , liposome treatment increases the PC/sphingomyelin mole ratio and induces cholesterol depletion – Table I) The effect of lipid composition on the dynamic properties of membrane constituents cannot be attributed simply to changes in membrane fluidity, since treatment of old cultures with the fluidizer pentanol [26] had no effect on the R values in fibroblasts (Table IIIA), in accord with our previous findings in myocytes [7]

The simplest explanation for the effect of the membrane lipid composition on the mobility and organization of membrane components is provided by alterations in the organization of lipids in domains in response to changes in their composition The formation of specific lipid domains whose lipids cannot interchange freely with lipids outside these regions would lead to the appearance of an immobile lipid fraction in EPR experiments (unless the domains are small enough to enable lateral motion of whole domains on the experimental time scale) Changes in the proportion of lipids organized in domains are, therefore, expected to result in altered R values for the lateral diffusion of lipid probes, as well as in changes in the D values due to the variations in the membrane lipid composition outside of the domains This, indeed, is the experimental situation (Table III) The finding that R of a membrane protein marker changes concomitantly with that of the lipid probe supports this hypothesis, since membrane proteins localized in such nonexchangeable lipid domains should also appear as immobile in the FPR studies, as is indeed the case (Table IIIA) The notion that changes in the membrane lipid composition may result in altered organization of the lipids in domains is supported by studies on model membranes under isothermal conditions, where lateral phase separation was shown to depend on the phospholipid composition and cholesterol level [37,40–42]

Some idea on the respective roles of phospholipid composition and cholesterol level may be gained by examination of the effects of the various

liposome treatments on the membrane lipid composition (Table I) and on the dynamic parameters of membrane components (Table III) As observed earlier in myocytes [7], it is evident that cholesterol depletion alone cannot explain the effects of the liposome treatments of old cultures Thus, if one examines the R values (Table IIIA), it can be seen that treatment of old cultures with sphingomyelin liposomes (which induce cholesterol depletion but do not alter the PC/sphingomyelin mole ratio) induces only about half of the effect of treatment with PC liposomes (which increase the PC/sphingomyelin mole ratio in addition to depleting cholesterol – Table I) It therefore appears that the cholesterol level may modulate the effects of the PC liposome treatment, which are induced primarily through alterations in the PC/sphingomyelin mole ratio The modulating effect of cholesterol is evidenced in the effect of treatment with PC cholesterol (1 0 9) liposomes on R of both NBD-PE and TMR-succinyl concanavalin A receptors (Table III), this treatment decreases R to the level observed in young cells, but not below (as occurred following PC liposome treatment) Thus, cholesterol appears to act as an 'averager', weakening the effects induced by the alterations in the PC/sphingomyelin mole ratio

Elevated cholesterol levels could interfere with the membrane lipid organization by intermixing of domains with the rest of the lipid bilayer The observed effects of cholesterol are in line with studies on model membranes, which demonstrated that changes in the dynamics of the lipids are strongly affected by the cholesterol level [37] The 'averaging' effects of cholesterol appear to depend on the membrane phospholipid composition [37,40–42] Thus, in membranes enriched in sphingomyelin (e g , old cultures – see Table I), cholesterol depletion induces a decrease in D (Table IIIB), most likely since the original high level of cholesterol in the old cultures reduces the tendency of sphingomyelin to make the membrane less fluid On the other hand, high PC levels make the membrane more fluid, thus, in the case of old cultures treated with PC liposomes (a treatment which increases the PC/sphingomyelin ratio – Table I), the accompanying cholesterol depletion induces an increase rather than a decrease in D of the lipid probe (Table IIIB) Furthermore, elevation of the cholesterol level in old cultures enriched with PC (following treatment with PC cholesterol (1 0 9) liposomes) induces a decrease in the D value Thus, in cases where the membranes are more 'fluid' (due to higher PC/sphingomyelin mole ratios), the addition of cholesterol tends to diminish the 'fluidizing' effects of PC Similar effects were observed in model systems [43,44]

In view of the fact that the primary target for the effects of the liposome treatments is the cellular plasma membrane lipid composition, it is tempting to suggest that the resulting changes in the mobility and organization of membrane lipids and proteins are an initial response which is involved in mediating latter alterations in cellular functions and enzymatic activities (Table II) either directly or through changes in total plasma membrane area and area to volume ratio, as was proposed for the myocytes [6]

The studies presented in this communication demonstrate that the various phenomena that accompany changes in the lipid composition of cells (i e , changes in the organization and dynamics of membrane components, alterations in enzymatic activities) are not special to myocytes, and can be encountered in other cell types Thus, the modulation of cellular properties through the lipid composition of the cell membrane may be a general phenomenon in many (if not all) cell types

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