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Lipid and membrane fluidity abnormalities in platelets and megakaryocytes of the hereditary macrothrombocytopenic Wistar Furth rat

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Biochemical and functional abnormalities of megakaryocytes and platelets were studied in Wistar Furth (WF) rats which have genetically determined macrothrombocytopenia and megakaryocytopenia, and were compared with their counterparts in Sprague-Dawley (SD) rats. Both megakaryocytes and platelets synthesized phospholipids from [^{14}C]acetate. WF and SD megakaryocytes incorporated 0.27 and 0.29 nmol acetate per 10^6 cells, respectively. Phosphatidylcholine (PC) accounted for 64% and 58% of the PL radioactive label in megakaryocytes of SD and WF rats, respectively, ($P < 0.05$), while 69% of labeled activity was associated with PC of SD platelets compared to 60% found in PC of WF platelets ($P < 0.01$). In WF platelets a significant increase in the levels of lysophosphatidylcholine (6.1% vs. 3.0%) was observed. WF platelets had substantially higher levels of esterified cholesterol, triglycerides, ceramides and a 3-fold increase in the total protein per platelet compared to SD platelets. The fatty acid composition of WF platelet PC showed quantitative abnormalities. Plasma lecithin–cholesterol acyl transferase activity and platelet function monitored by the uptake and release of [^{14}C] serotonin showed nonsignificant variations between SD and WF rats. Compared with the control, platelet membrane fluidity, measured by fluorescence polarization using platelets labeled with 1,6-diphenyl-1,3,5-hexatriene, was significantly decreased in the WF rats.

Introduction

Platelet cholesterol and phospholipids are elements that play a significant role in maintaining the functional and biochemical integrity of the membrane. Several mechanisms that regulate the concentration and organization of these critical platelet elements have been proposed [1,2]. Among these possibilities, the least explored one seems to be the contribution of megakaryocytes in the production of lipid components of their progeny, the platelets. It has been reported [1] that megakaryocytes could modulate platelet lipid synthesis by combining the processes of endogenous lipid formation and incorporation of plasma lipids. The cholesterol and protein content of platelets is known to critically depend on megakaryocytes, since platelets have relatively little or no ability to synthesize either one. The Wistar Furth rat, with a genetically determined macrothrombocytopenia and megakaryocytopenia [3,4], similar to certain platelet disorder, may be useful in understanding if platelet abnormalities are determined by abnormal megakaryocytopoiesis. We have used the Wistar Furth rat, with a special emphasis on the platelet membrane, to see if such a relationship exists. This study has examined the effects of such

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Abbreviations: CE, cholesterol esters; DPH, diphenylhexatriene; FA, fatty acid(s); FC, free cholesterol; FC/PL, free cholesterol/phospholipid molar ratio; LCAT, lecithin–cholesterol acyltransferase; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; MK, megakaryocytes; NL, neutral lipid(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid(s); PPP, platelet-poor plasma; PS, phosphatidylserine; SpH, sphingomyelin; PRP, platelet-rich plasma; SD, Sprague-Dawley; TLC, thin-layer chromatography; U.I., unsaturation index; UL, unidentified lipids; VLDL, very-low-density lipoprotein; WF, Wistar Furth.

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deficiencies on the membrane components and function, with a special emphasis on the platelet membrane.

Materials and Methods

Animals

Wistar Furth rats (Harlan Sprague Dawley Inc., Indianapolis, ID) and Sprague-Dawley rats (Bantam and Kingman C., Fremont, CA) aged 9 to 10 weeks were used.

Megakaryocyte and platelet preparation

Rat megakaryocytes were prepared from bone marrow according to the procedure outlined by Leven and Nachmias [5]. The megakaryocytes isolated by this procedure were on the average 85% pure. 64000 and 36000 megakaryocytes per animal were isolated from the Sprague Dawley and Wistar Furth rats, respectively. Rats were bled by cardiac puncture under anesthesia. 9 volumes of blood were collected into a plastic tube containing 1 volume of 0.11 M sodium citrate. Platelet-rich plasma (PRP) was prepared according to the procedures described by Dalal et. al. [6]. Platelets were counted by phase microscopy. Platelet recovery was $80 \pm 14\%$ in the WF group and $81 \pm 11\%$ in the control group. White cell and red cell contamination in the PRP gradient was negligible.

Incubation of cells with [^{14}C]acetate

Freshly prepared megakaryocytes were divided into aliquots of $2 \cdot 10^4$ cells and suspended into 1 ml of Dulbecco's modified Eagle medium with 10% fetal calf serum containing 6 μCi [^{14}C]acetate, spec. act. 57 mCi/mmol (Amersham, Arlington Heights, IL), final concentration 0.1 mM. Incubation was carried out at 37°C for 1.5 h [7]. The platelet number was adjusted to $1 \cdot 10^9$ in 1 ml of incomplete Tyrode solution containing 9 μCi [^{14}C]acetate; they were incubated at 37°C for 90 min, centrifuged at $2000 \times g$ for 10 min at 4°C and washed three times in 3 ml cold incomplete Tyrode (pH 6.8) [8].

Separation of megakaryocytes and platelet lipids

Lipids from both cells were extracted and fractionated by the techniques outlined elsewhere [6]. In each determination total lipids representing 5000–15000 cmp were applied to each TLC plate. The cholesterol (free and esterified) was estimated by the modified method of Pearson et al. [9]. Total protein was determined by the procedure of Lowry et al. [10]. Extraction, transmethylation and quantitation of fatty acids of platelet PC was carried out according to the methods of Dalal et al. [6].

The LCAT assay was performed according to Stokke and Noram [11]. The method to obtain plasma free of very-low-density lipoprotein (VLDL) and low density lipoprotein (LDL) was modified [12] to maximize the removal of VLDL and LDL. The following final concentrations of heparin/manganese chloride were used: heparin, 1.5 mg/ml; $MnCl_2$ 40 mM for SD plasma and 60 mM for WF plasma. Treated plasma was centrifuged at 7500 rpm for 40 min, the clear supernatant was removed and used for further analysis. The measurement of [^{14}C]serotonin uptake and release was carried out according to the procedure outlined elsewhere [6].

Fluidity measurements

Platelet membrane fluidity was determined by measuring fluorescence polarization [13,14]. 1,6-Diphenyl-1,3,5-hexatriene (DPH) (Sigma Diagnostic, St. Louis, MO) was used as the hydrophobic probe. Modified features of the procedure are as follows: DPH, 1 mM in tetrahydrofuran, was diluted 1:500 into platelet suspension medium with vigorous stirring, immediately before use. One ml of this diluted DPH solution was added to 1 ml of the platelet suspension having final platelet concentration of $1 \cdot 10^8$ platelets/ml. The mixture was incubated at 37°C for 1 h. Steady-state fluroscence polarization measurements were carried out at 37°C using a Perkin-Elmer Luminescence spectrometer LS-5B equipped with Glan-Thompson prism polarizers. The excitation and emission wavelengths were set at 360 and 430 nm, respectively. The steady-state fluroscence anisotropy (V_s^0) was calculated from the relative intensities recorded at parallel and perpendicular positions of the excitation polarizer axis to that of the emission polarizer [14]. In order to minimize signal fluctuations, the samples were measured once and then remeasured in reverse order, and a mean V_s^0 was calculated.

Statistical analysis

The results were expressed as mean ± S.E. and were compared using Student's *t*-test for unpaired samples.

Results

The incorporation of radioactivity into PL was relatively similar in both SD and WF MK (0.29 ± 0.08 and 0.27 ± 0.12 nmol/10^6 cells, respectively $n = 6$ determinations, each determination consisted of pooled cells from two or three animals). The phospholipids contained a substantial portion of the total lipid radioactivity. The most extensively labelled PL was PC. There was a significant difference in the synthesis of PC by the SD and WF MK (64.0 ± 0.26 vs. $58.0 \pm 1.9\%$ of total PL activity: $P < 0.05$). Synthesis of other phospholipids in both groups of rat MK revealed non-significant differences. (PE: 23.0 ± 1.6 vs. 26.0 ± 0.9; PS + PI + SpH: 12.8 ± 2.0 vs. $15.0 \pm 1.0\%$ of total PL activity).

TABLE I

Incorporation of [^{14}C]acetate into platelet phospholipids

Each value is the average ± S.E. n = Number of determinations, each determination consisted of pooled platelets from two or three animals. [^{14}C]Acetate uptake (nmol/10^9 platelets): in SD-PL = 0.10 ± 0.010; in WF-PL = 0.11 ± 0.015.

	% of phospholipid radioactivity	
	SD	WF
LPC	3.0 ± 0.80 [a]	6.1 ± 1.3 [a]
PS PI	7.0 ± 1.60	8.0 ± 2.0
SpH	4.0 ± 0.70	6.0 ± 1.5
PC	69.0 ± 2.50	60.0 ± 2.7 [a]
PE	15.0 ± 1.00	17.0 ± 1.0
UL	1.8 ± 0.22	3.0 ± 1.0
Platelet count (1·106/cu mm)	0.95 ± 0.022	0.32 ± 0.017
n	6	6

[a] Significantly different from control value $P < 0.01$.

The uptake of radioactivivity into PLs of SD and WF platelets is shown in Table I. Of the PL identified by TLC technique, WF platelets showed significant increase in the level of LPC with a subsequent decrease in the level of PC when compared with the SD platelets. The lipid composition of both SD and WF platelets is shown in Table II. In WF platelets, total phospholipids when expressed in terms of absolute amounts per cell

TABLE II

Lipid distribution in Sprague Dawley (SD) and Wistar Furth (WF) rat platelets

n = six determinations, each determination consisted of pooled platelets from two or three animals. Each value is the average of ± S.E. TPL, total phospholipid; LPC, lysophosphatidylcholine; PI, phosphoinositol; PS, phosphatidylserine; SpH, shingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PC/PL, free cholesterol/phospholipid molar ratio; FC, free cholesterol; CE, cholesterol esters; TG, triglycerides; UL, unidentified lipids.

	UG/10^9 platelets	
	SD	WF
TPL	285 ±13	277 ±33
LPC	8.5 ± 1.2	17.0 ± 2.0
PI	16.0 ± 3.0	11.0 ± 4.5
PS	28.3 ± 4.5	38.0 ± 3.7
SpH	69.0 ± 6.0	66.0 ± 4.0
PC	119 ±12	83.0 ± 10.5
PE	40.0 ± 3.0	44.0 ± 4.5
UL	4.0 ± 0.9	18.0 ± 3.5
FC/PL molar ratio	0.48	0.49
FC	71.0 ± 2.0	72.0 ± 3.0
CE	3.0 ± 1.2	9.0 ± 2.1
TG	12.0 ± 1.6	14.0 ± 1.9
Ceramides	5.0 ± 0.9	2.0 ± 1.8
n	6	6

TABLE III

Fatty acid composition of Sprague Dawley (SD) and Wistar Furth (WF) platelet phosphatidylcholine (PC)

Two determinations, each determination consisted of pooled platelets from two or three animals.

Fatty acid	Wt. %	
	SD	WF
16:0	28.2	27.5
18:0	20.0	20.8
18:1	8.4	18.9
18:2	32.6	22.4
20:4	6.2	8.8
Unidentified FFA	4.4	2.2
Unsaturation index (U.I.)	98.4	102.1

showed only moderate decrease when compared to the SD platelets. Among individual PL, levels of LPC, PS and unidentified lipids (at the origin of TLC plate) were markedly increased, while those of PI and PC were considerably reduced. Among neutral lipids, cholesterol esters and triglyceride levels were increased, and ceramide level was reduced in the WF platelets. However, WF platelet phospholipids, when expressed in terms of μmole PL/mg protein revealed pronounced increase (LPC; 0.0052 vs. 0.0088) and decreases (TPL; 0.180 vs. 0.055, PC; 0.0735 vs. 0.0165) compared to their control counterparts. Total protein content of WF platelets was 3-fold greater than that of SD platelet (3.76 ± 0.40 vs. 1.21 ± 0.17 mg/10^9 platelets). The fatty acid composition of platelet PC is shown in Table III. There was a substantial accumulation of 18:1 and 20:4 and reduction of 18:2 in the PC of WF platelets. The increase in 18:1 and 20:4 caused a moderate elevation in the unsaturation index (U.I.) of the PC molecule. In Table IV the FC and LCAT activity of the whole plasma and VLDL LDL free plasma are shown. FC concentrations of SD and WF plasma, both treated and untreated, were the same. LCAT activity in terms of μmol of cholesterol esterified/h per l showed small differences in plasma (both

TABLE IV

Free cholesterol concentration and LCAT activity determined in Sprague Dawley (SD) and Wistar Furth (WF) rat plasma

Each value is the average ± S.E. of five determinations, each determination consisted of pooled plasma from two or three animals. FC, free cholesterol (mg/dl of plasma); LCAT activity, μmol of cholesterol esterified/h per l.

	Plasma		Supernatant of $MnCl_2$ precipitated plasma	
	FC	LCAT activity	FC	LCAT activity
SD	47.0 ± 3.1	67.5 ± 4.3	18.5 ± 0.5	12.5 ± 0.6
WF	52.0 ± 3.4	61.8 ± 3.9	20.0 ± 0.5	15.8 ± 0.5

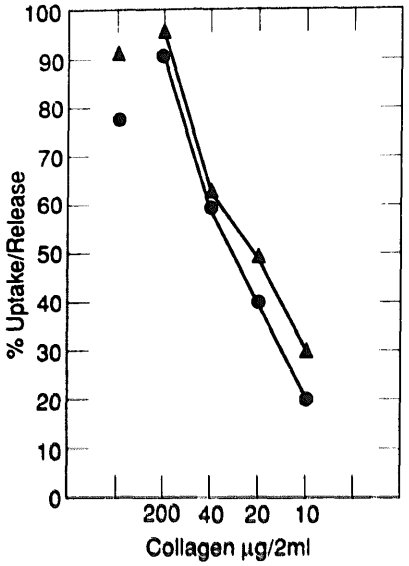

Fig. 1. Effects of various concentrations of collagen on the uptake and release of radioactivity from ^{14}C-labelled PRP from Wistar Furth (▲) and Sprague-Dawley (●) rat platelets (unconnected points represent % uptake) Each point (average of two determinations) is the result obtained with pooled PRP from two or three animals.

treated and untreated) of SD and WF animals. The LCAT activity in the treated plasma indicated (in terms of fractional esterification rate, percent/h, not shown) more efficient and uniform utilization of high-density lipoproteincholesterol (HDL-ch) by the enzyme than in whole plasma.

Platelet function was monitored in the PRP obtained from both groups. The uptake of [^{14}C]serotonin in SD and WF platelets ranged from 78 to 92%. The shapes of the dose-response curves in SD and WF showed similar patterns (Fig. 1). The percent of [^{14}C]serotonin released from control and WF platelets was proportional to the concentration of collagen used.

Steady-state fluroscence anisotropy V_s^0, was measured in DPH-labelled platelets from SD and WF rats. The anisotropy data (Table V) indicate that V_s^0 was significantly higher in WF platelets, compared with the control group, suggesting decreased platelet membrane fluidity in the WF rats.

Discussion

It has been well established [3,4] that abnormal membrane organization in megakaryocytes and platelets is directly associated with the macrothrombocytopenia. Platelets are produced from megakaryocytes in the marrow, and the regulation of platelet membrane synthesis by megakaryocytes is mainly dependent on the lipid biosynthetic pathways [15]. Megakaryocytes and platelets synthesize phospholipids from acetate. Our data suggest that the phospholipids retained a substantial portion of the lipid radioactivity and that the pathways of phospholipid synthesis in the two cells are similar. Phosphatidylcholine appears to be the major phospholipid synthesized in both cells. Although the uptake of radioactivity in SD and WF megakaryocytes showed slight variation, the production of phosphatidylcholine in WF megakaryocytes as indicated by the distribution of the radioactivity, was significantly reduced. Changes in platelet mass and volume are thought to be associated with the abnormal lipid metabolism [16]. However, the altered phospholipid synthesis in WF megakaryoctyes that we have seen and its effects on the membrane organization remains unclear.

In terms of quality, the phospholipid composition of SD platelets is in good agreement with that of rabbit [17] and human [18] platelets. However, certain quantitative differences in the levels of individual PL were observed. The value for platelet PC in this study was higher and those for PS and PE were lower than the values reported elsewhere [17,18]. Total lipid level of larger sized WF platelets did not significantly differ from that of normal sized SD platelets. The reason for this unexpected finding remains unclear. Our data revealed that the distribution of radioactivity from acetate in platelet phospholipids does not coincide with the concentration of the phospholipids quantitated in the platelets. This variation suggests that exogenous fatty acids must have been extensively utilized in the synthesis of platelet phospholipids [15]. In WF platelets, LPC and UL were high not only as a percent of total phosphorus but also in absolute amounts. Simultaneously the levels of PC and PI were considerably reduced. Among NL, there was pronounced to moderate increases in the levels of CE, ceramides and TG. Our previous study [3] along with others [4] have shown that the mean platelet volume (mpv) of adult WF rat was twice that of their counterparts. Our present study revealed a 3-fold increase of the total protein in the WF platelets. The effect of such a massive accumulation of protein on the fluidity of the platelet membrane remains unclear. Cook et. al. [19] reported that when platelets incubated with a large quantity of fluid liposomes, intrinsic membrane proteins or enzymes are altered to a degree that could be detrimental to the membrane functionality.

Since significant reduction in the biosynthesis of WF platelet PC was observed, it became necessary to evaluate the fatty acid composition of platelet PC. In both

TABLE V

Steady-state fluorescene anisotropy (V_s^0) of DPH-labelled rat platelets

Mean ± S.E. of nine determinations per group

	V_s^0
Sprague-Dawley	0.196 ± 0.008
Wistar Furth	0.215 ± 0.004 [a]

[a] Significantly different from control: $P < 0.025$.

quality and quantity the fatty acid composition of SD platelet PC was comparable to that reported by Galli et al. [17] for the rabbit but differed in concentrations from the human [18] platelet PC fatty acids. Nearly 51% of the total fatty acid was represented by the mono and polyunsaturated fatty acids in the SD platelet PC. There were major changes in the fatty acid composition of platelet-PC from WF rats, compared with the control group. However, these quantitative changes did not alter the unsaturation index. Our findings in general concur with those observed by Rand et. al. [13], suggesting no correlation between increased membrane fluidity and changes in the fatty composition of diet-rabbit platelet phospholipids. The exact role of these quantitative changes in fatty acid composition in altering membrane fluidity remains uncertain, perhaps because of the difficulty in correlating membrane fluidity with the unsaturation index, that is derived by means of a simplified formulae [13]. It has been established both in animals and human beings that the size and number [16] of platelets, along with their lipid composition [6] plays a significant role in determining platelet reactivity. In this study, in spite of marked changes in the WF platelet lipid synthesis, no alteration in the collagen induced aggregation/release activity was seen. This observation is in good agreement with the finding of Jackson [20]. The LCAT enzyme, that catalyzes the transfer of 18:2 from PC to free cholesterol to form LPC and CE, measured in whole plasma and VLDL/LDL free plasma of SD and WF rats, showed no major variation, despite significant accumulation of LPC and CE in WF-platelet. Although it is clear that LCAT is responsible for generating most of the esterified cholesterol in plasma, the accumulation of both CE and LPC in WF platelets may be independent of plasma LCAT activity. *In vitro* study [21] has shown that platelet can take up LPC from the suspending medium. In view of this it is reasonable to assume that the ability of larger sized platelet to direct exchange and or uptake of endogenous LPC could have been altered.

The differences in fluidity of platelet membranes observed in this study, although statistically significant, were rather small. This altered membrane fluidity in WF platelets was not associated with any changes in the FC, PL content or in the molar ratio of cholesterol to phospholipid. At the same time there were marked changes in the fatty acid composition of platelet PC compared with the control platelets. However, this did not result in the significant changes in the unsaturation index. The fact that in this study we were unable to establish a firm association between alteration in membrane fluidity, membrane lipid and fatty acid (unsaturation index) composition, despite a proven direct dependence of fluidity upon the actual mass of unsaturated FFA in the platelet-PL [22], does not necessarily imply that such an association can not exist. It should be noted that DPH attaches well into both solid and fluid domains of the membrane, thereby, giving a measure of average membrane fluidity and since, overall membrane lipid composition was determined, it seems that few of the biochemical determinants of fluidity that remained undetected, must have been altered. Membrane fluidity is an important property of many, if not all, biological systems. Fluidity is modified or controlled in several ways. For example, changes in platelet membrane fluidity associated with Alzheimer's disease or patient with abetalipoproteinemia, may have resulted from a dysregulation of cell membrane biogenesis, turnover or mass of unsaturated membrane lipid or by cholesterol content [23,24]. These types of control are also known to influence membrane protein or enzyme conformation and vice versa [13,14].

The fluidity differences in the present work are not related to the masses of unsaturated membrane lipids or platelet aggregation. This does not necessarily contradict the cited studies as many distinguishing factors, such as species differences in the activities of human and animal platelets and the functional implications of *in vitro* and *in vivo* alterations of membrane fluidity, may be different. In addition, increased levels of LPC and ceramides enhance cell adhesion which consequently could alter platelet membrane function by making it more rigid [6,7].

In conclusion, the present study, designed to test whether ultrastructural abnormalities in platelets or megakaryocytes are associated with their functional and biochemical behavior, has shown abnormal lipid biosynthesis in both megakaryocytes and platelets together with the decreased platelet membrane fluidity. The relationship of these abnormalities to the process of megakaryocytopoiesis, platelet size and number is not clear. However, the Wistar Furth rat, can serve as an appropriate model, since abnormalities of lipids along with the changes in the platelet production from megakaryocytes have been shown to be associated with the development of atherosclerosis and dysthromobopoiesis.

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