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EFFECTS OF IN VITRO INCORPORATION OF CHOLESTEROL AND CHOLESTEROL ANALOGUES INTO RAT PLATELETS

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Cholesterol and analogues of cholesterol bearing shorter side chains were incorporated into rat platelet membranes by incubation with sterol-rich liposomes in vitro. Cholesterol-enriched platelets showed increased aggregability to collagen compared with controls. Platelets containing the cholesterol analogues pregn-5-en-3β-ol and chol-5-en-3β-ol were even more sensitive to aggregation and could aggregate spontaneously on stirring. The size of the platelets containing pregn-5-en-3β-ol was markedly reduced when compared with controls in the scanning electron microscope. The results suggest that the sterol content and structure of the platelet membrane can have a critical role in maintaining the normal function of the cell.

Introduction

Cholesterol is an important component of mammalian cell membranes and is found in unesterified form to varying extents in plasma and subcellular membranes. Its presence can be critical in the maintenance of the integrity of the membrane as, for example, in the red blood cell where the unesterified cholesterol content is thought to be tightly regulated [1,2]. Deviations from the normal cholesterol content of red blood cell membranes induced in vitro or found in vivo result in a change of shape of the cells with a reduction in the integrity of the membrane. Similar effects have been observed in human platelets both in vitro and in vivo, with changes in the cholesterol content of the cells being correlated with modified aggregability of the cells in response to several important stimulators of aggregation [3,4].

Since aggregation requires substantial changes in cell shape, these events must be closely dependent on the structure and physical properties of the platelet membrane. Cholesterol is known to modify the rigidity of biological membranes, and the structural requirements within the sterol molecule on which this depends have been largely worked out [5,6]. We have found, for example, in model membrane studies, that the sterol side chain is critical in the achievement of the maximal interaction between cholesterol and phospholipid [6–8]. In this work, analogues of cholesterol bearing lengthened or shortened side chains were used, and these compounds are useful probes of the role of cholesterol in membranes or systems derived from membranes [9,10]. As part of a larger study involving a comparison of platelets from several different species, we report here on the effect of the in vitro incorporation of cholesterol analogues and cholesterol on the properties of rat platelets. The structure of the sterol and the amount of the sterol in the cell membrane were foun to be critical in the maintenance of the function of the platelet.

Materials and Methods

Cholesterol and dipalmitoylphosphatidylcholine were obtained from Sigma London, Poole, Dorset, U.K. The analogues of cholesterol used were those described previously [6]. They are referred to by the number of carbon atoms in the sterol. Thus C_{21} is pregn-5-en-3β-ol, C_{24} is chol-5-en-3β-ol and C_{26} is 27-norcholest-en-3β-ol. Scanning electron micrographs were kindly provided by the Teaching and Research centre, Western General Hospital, Edinburgh. Phosphatidyl[^{14}C]choline was prepared following Waite and van Deenen [11].

Isolation of rat platelets. Rats of the Wistar strain of either sex (Centre for Laboratory Animals, The Bush, Penicuik, Midlothian) weighing about 150 g were anaesthetised with ether. Blood was withdrawn by cardiac puncture into a plastic 10 ml syringe rinsed with 3.8% w/v trisodium citrate. 9 vol. of blood were mixed in a 10 ml plastic blood sample tube with 1 vol. of the trisodium citrate. The blood samples were centrifuged at $100 \times g$ for 10 min to sediment red cells and the supernatant platelet-rich plasma was removed. Platelet-rich plasma from several animals was pooled and used in the experiments.

Preparation of liposomes. Liposomes were prepared from dipalmitoylphosphatidylcholine and the appropriate sterol according to Shattil et al. [3]. The composition of the liposomes used is shown in Table I. The liposomes were analysed for sterol and phospholipid as described previously [6].

Modification of the sterol content of platelets. Equal volumes of platelet-rich plasma and the required liposomes were mixed and incubated for 3 h at 37°C with mixing by inversion every 30 min. An incubation time of 3 h was found to be sufficient for incorporation of cholesterol and cholesterol analogues into the rat platelets. The mixture was then centrifuged at $4800 \times g$ for 15 min, the supernatant discarded and the pellet resuspended in the same volume of phosphate-free platelet washing buffer (0.15 M NaCl, 10 mM tris, 0.2 mM EDTA, pH 7.4). The washing was repeated twice. The platelet pellet from 2 ml platelet-rich plasma was finally suspended in 2 ml washing buffer and aliquots removed for assay of protein, sterol and phospholipid as described previously [6].

TABLE I

COMPOSITION OF LIPOSOMES USED TO MODIFY PLATELET STEROL CONTENT

Lipids were dissolved in a round-bottomed flask and the solvent evaporated to leave a thin lipid film. After 1 h in vacuo 10 ml modified Tyrode's buffer was added [3] and the mixture sonicated under nitrogen for 3 h at 55–60°C. The mixture was centrifuged at $22000 \times g$ for 30 min. Glucose and bovine serum albumin were added to a final concentration of 5 mM and 3.5 mg/ml, respectively.

Sterol	Mixture for liposome preparation		Analysis of liposomes (molar ratio sterol/phospholipid)
	Phospholipid (mg)	Sterol (mg)	
Cholesterol-normal	40	12.5	0.62
Cholesterol-rich	40	30	1.36
C_{21}	40	30	0.80
C_{24}	40	40	0.88
C_{26}	40	40	1.16

Aggregation of platelets. Platelet aggregation was followed by the method of Born and Cross [12]. A platelet-poor plasma and liposome mixture was prepared by centrifugation of 1 ml of each incubation mixture at $1000 \times g$ for 15 min. With this mixture in the light path of the aggregometer (Payton Associates, Scarborough, Ontario, Canada) the recorder pen deflection was set to 100%. The absorbance changes of the platelet-rich plasma and liposome samples on addition of collagen (Hormon Chemie, Munich, F.R.G.) were then followed in the aggregometer. Examples of the aggregometer readings are shown in Fig. 1.

Results

Incorporation of cholesterol and cholesterol analogues into rat platelets

The sterol content of platelets was modified by incubation of platelet-rich plasma with liposomes loaded with the appropriate sterol. A substantial change in the sterol present in the platelets was found to have taken place after 3 h incubation at

37°C. Incubations were carried out with cholesterol-normal liposomes (Table I) to maintain the cholesterol content of the platelets at the endogenous level, and with the appropriate sterol-rich liposomes. The composition of control and cholesterol-rich platelets prepared in this way is shown in Table II. An increase in the cholesterol content of 20% was achieved, showing that in rat platelets there is a capacity for uptake of cholesterol, as has been observed for human platelets [3,4]. No uptake of phospholipid from the liposomes into the platelets was detected by analysis and this was confirmed by the absence of transfer of phosphatidyl[^{14}C]choline from the liposomes to the platelets.

In a similar way, platelets could be enriched with the three analogues of cholesterol (Table III). There was both a net uptake of the analogue and exchange with cholesterol resulting in between 23% and 60% of the total sterol in the platelet being the analogue, depending on which was used. Higher centrifugation rates were required to pellet the platelets after incubation with the liposomes containing the C_{21} analogue. This suggested that some major change in the structure of the cells had occurred.

Aggregation behaviour of platelets with modified sterol content

Typical aggregation curves for normal and sterol-enriched platelets are shown in Fig. 1. The presence of liposomes in the platelet-rich plasma was shown not to interfere with the aggregation process. The normal response of platelets after incubation with cholesterol-normal liposomes for 3 h to a submaximal concentration of collagen (5 μg/ml, which is not enough to stimulate the full aggregation response) is shown by curve A. It is characterised by an initial lag period of about 1 min followed by a change in the shape of the cells and rapid aggregation. Dissociation of the aggregates occurs at this concentration of colllagen once the maximum has been reached.

Cholesterol-enriched platelets clearly show enhanced aggregability (curve B) compared with control platelets (curve A). In the cholesterol-enriched samples the extent of aggregation was increased and the lag time was decreased. Irreversible aggregation also occurred in this case. This result indicates that rat platelets can also be rendered hypersensitive to aggregating agents, as is already known for human platelets [3,4].

In curve C the behaviour of the platelets enriched with the C_{21} sterol is shown. No stimulator of aggregation was necessary for all the C_{21} sterol-enriched platelets because aggregation invariably occurred spontaneously with the commencement of stirring in the aggregometer. The behaviour of the C_{24} sterol-enriched platelets was similar to that of the C_{21} sterol-enriched platelets and aggregation was also found to be spontaneous (curve D). The platelets enriched with the C_{26} sterol were in all respects similar to cholesterol-rich platelets.

At the end of the incubation period for sterol enrichment the mixtures were taken for platelet counting (kindly carried out by the Coagulation

TABLE II

ENRICHMENT OF RAT PLATELETS WITH CHOLESTEROL IN VITRO

Results are the means of five experiments ± S.E.

	Molar ratio sterol/phospholipid	Cholesterol/protein (mg/mg)
Control (cholesterol-normal) liposomes)	0.604 ± 0.018	0.053 ± 0.0002
Cholesterol-enriched	0.716 ± 0.023 ($P < 0.01$)	0.064 ± 0.003 ($P < 0.05$)

TABLE III

INCORPORATION OF CHOLESTEROL ANALOGUES INTO RAT PLATELETS

Results are the means of 1–3 experiments.

Analogue	Sterol/phospholipid molar ratio	% total sterol as analogue	Sterol/protein (mg/mg)
C_{21} [a]	0.900	59	0.068
C_{21} [b]	0.903	83	0.094
C_{24} [a]	0.782	61	0.068
C_{26} [a]	0.684	25	0.070

[a] 3 h incubation at 37°C.

[b] 5 h incubation at 37°C. This latter preparation was used in the scanning electron microscopy.

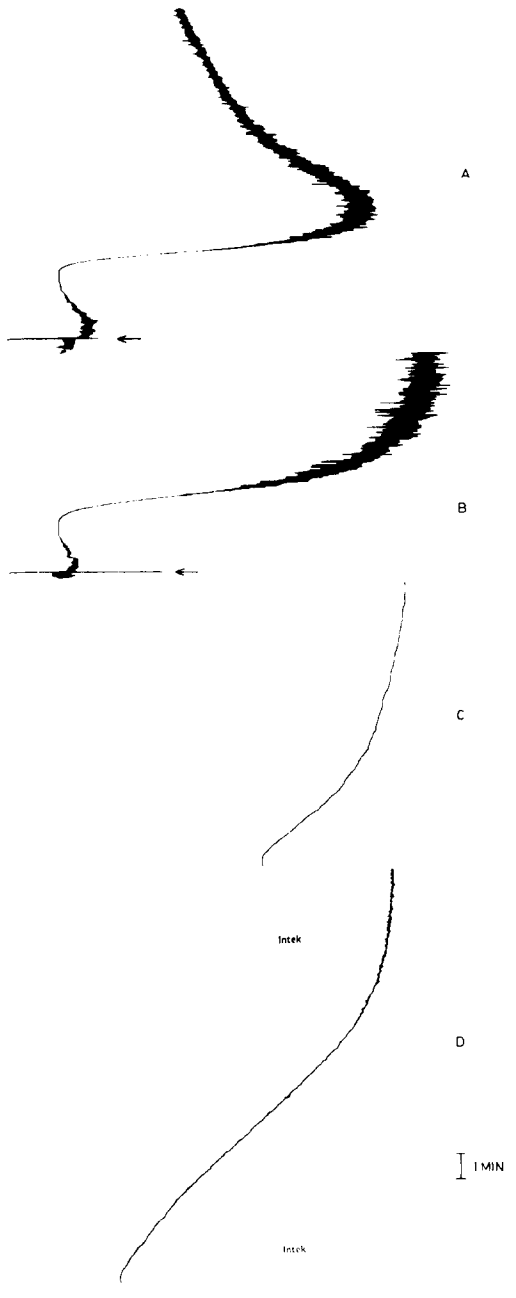

Fig. 1. Typical aggregation curves for rat platelets after incubation with liposomes. (A) Platelets incubated with cholesterol-normal liposomes for 3 h. (B) Platelets incubated with cholesterol-rich liposomes for 3 h. Both A and B were stimulated at the point marked with collagen (final concentration 5 μg/ml). (C) Platelets enriched with the C_{21} analogue. (D) Platelets enriched with the C_{24} analogue. No collagen was added to C or D: aggregation commenced with stirring.

Unit, Royal Infirmary of Edinburgh). The cholesterol-normal and cholesterol-rich platelets invariably contained the same number of cells in any one experiment. The differences in the aggregation responses to collagen could not therefore be due to a difference in the platelet count. In the experiments reported here the platelet counts ranged from 378 to 542 cells/nl. However the platelet counts of the C_{21} sterol incubations were consistently lower, ranging from 59 to 143 cells/nl, although these incubations contained the same amounts of platelet-rich plasma as the cholesterol-normal and cholesterol-enriching incubations. This difference could be explained by a substantial change having occurred in the size of the platelets during enrichment with the C_{21} sterol such that many of the cells became too small to be detected by the thrombocytometer.

This apparent change in cell size was consistent with the observed aggregation behaviour and the lower rate of sedimentation of the C_{21} sterol-enriched platelets and this interpretation was confirmed by scanning electron microscopy. Cholesterol-normal and cholesterol-enriched cells both appeared as normal platelets with partially extended pseudopodia characteristic of platelets several hours after isolation from the blood. In marked contrast, the C_{21} analogue-enriched platelets were about a quarter to half the size of the normal cells and showed no pseudopodia. Some of the larger cells appeared to be budding off smaller fragments. The size of these platelets enriched with the C_{21} sterol observed in the scanning electron microscope is consistent with their behaviour in the counter, the aggregometer and the centrifuge. The scanning electron micrographs were all obtained from cells fixed after 5 h incubation with the liposome preparations. C_{21} sterol-enriched cells obtained after 5 h incubation showed the same differences in sedimentation and platelet counts as were observed after 3 h in the samples which were used for analysis and for aggregometry.

Discussion

The results presented here show that rat platelets can be enriched in cholesterol in vitro to give cells with enhanced aggregability. The response in the rat platelets to additional cholesterol appears

to be similar to that found in human platelets, although there are some differences in detail. The cholesterol-rich rat platelets appear very similar in shape to control platelets in which the cholesterol content has been maintained at a normal level. However, a substantial increase in aggregability to collagen was observed in the cholesterol-rich cells. The C_{26} analogue caused a similar increase in aggregability.

Less C_{26} analogue than the others was taken up by the platelets (Table II). It is possible that incorporation of higher amounts of this compound into the membrane could have caused changes in aggregation behaviour similar to those caused by the C_{21} and C_{24} sterols. However, earlier work has clearly shown that the C_{26} analogue does not alter the physical state of model membranes when compared with cholesterol [6–8].

When the sterols with shorter side chains than cholesterol (C_{21} and C_{24}) were incorporated into platelets, a marked change in behaviour was observed. Both systems tended to aggregate spontaneously and in addition the C_{21} sterol-enriched cells appeared to fragment, whilst still retaining aggregability. These sterols are incorporated into membranes in a similar way to cholesterol and replacement of cholesterol by such compounds leads to a decrease in the membrane properties related to rigidity [6–8]. It is likely that the platelets containing the C_{21} and C_{24} sterols are bounded by membranes that are significantly more fluid towards the central region of the phospholipid bilayer of which the membrane is formed. Such a change in the physical state of the membrane may lead to the greater sensitivity of the cells towards aggregation which is described here, a sensitivity which is even greater than that found in cells which are enriched in cholesterol.

It has recently been suggested that the transmembrane movement of cholesterol in the red blood cell is important in allowing the cell to change shape without damage to the membrane [13]. Such a redistribution of sterol between the two halves of the phospholipid bilayer could be equally important in the platelet, where changes in shape have a much greater functional importance as the first observable change in the aggregometer after stimulation. The dramatic effects of the short-chain sterols may be related to the extent to which they are unable to imitate the behaviour of cholesterol in crossing from one half of the membrane bilayer to the other. Whatever the mechanism of the observed differences in behaviour between platelets enriched with these sterols it is apparent that the cholesterol content of the platelet membrane must be maintained within quite narrow limits to preserve the normal function of the cell.

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