Stimulus Dependent Redistribution of Membrane Raft Cholesterol in Human Platelets

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Summary: Cell membranes provide a requisite dynamic interface to facilitate communication between the extracellular environment and the intracellular milieu. These membranes contain proteins that span and/or are loosely associated with the lipid bilayer. The organization of lipids and proteins components into membrane micro-domains provides a temporal and spatial signaling platform for communication. Recently, cholesterol and sphingomyelin enriched membrane micro-domains known as lipid rafts have been implicated in cell signaling events. In these studies we have advanced our hypothesis that stimulus dependent rearrangement of cholesterol into and out of membrane rafts provides a unique lipid–mediated regulatory mechanism. Using fluorescent derivatives of cholesterol, we have shown that membrane raft associated cholesterol was altered in response to collagen-induced platelet aggregatory stimulation. Collagen stimulation resulted in a rapid redistribution of cholesterol from the outer to the inner membrane monolayer. The reorganization of the outer membrane monolayer resulted in a concomitant increase in outer monolayer fluidity. These studies are the first to show that membrane cholesterol was released from the exchangeable membrane raft pool in response to physiological stimuli.

Keywords: cholesterol; fluorescent probes; membrane; membrane raft; phospholipids

Introduction

Platelets are hematopoeitic cells essential in the maintenance of the vascular system. Through a series of sequential and coordinated mechanisms they are activated in response to vascular injury. In hypercholesterolemic individuals this activation process is attenuated due in part to the presence of high levels of serum cholesterol. The platelet plasma membrane is the site of interactions between extra cellular stimuli and the organized biochemical events necessary to support aggregation. It provides the charged phosphatidylserine rich surface for the activation of the prothrombinase complex as well as membrane raft signaling platforms. These cholesterol enriched platelet rafts were specifically enriched in the membrane glycoprotein CD36 (a

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member of the tetraspanin protein family) and the surface protein GPIb, as well as Src p60 $^{c\text{-}src}$ and the Src-related kinases lyn; p53/56 ^{lyn} (1). This initial work suggested that platelet rafts were involved in platelet signal transduction. Recent work has provided more compelling data to support this hypothesis and moreover has met essential criteria in the characterization of detergent resistant membranes (DRMs) as membrane rafts which includes a detailed description of the lipid composition of the platelet DRMs (2). The phosphatidylinositol (PI) pool of phospholipids was the most significantly affected in DRMs isolated from thrombin-stimulated platelets. The change in DRM morphology in the thrombin stimulated platelets correlated with an increase in phosphatidic acid and products of the phosphoinositide-3 kinase reaction.

Although membrane rafts have been well characterized, a considerable amount of debate has focused on their relative distribution between the inner and outer membrane monolayer. We have previously documented a stimulus dependent redistribution of platelet cholesterol in response to the strong aggregatory stimuli, collagen. In these studies we provide evidence suggesting that this stimulus dependent reorganization is a translocation of cholesterol from cholesterol enriched outer monolayer raft to an inaccessible inner monolayer cholesterol pool. We hypothesize that this intracellular pool forms inner monolayer rafts and is required for platelet aggregation.

Materials and Methods

Materials. Triton X-100 was purchased from Sigma (St. Louis, MO). Octylglucopyranoside (OG) was purchased from Calbiochem. Cholesterol and phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Birmingham, Alabama).

Platelet Isolation and Additional Assays

Platelets were isolated from platelet rich plasma obtained from the American Red Cross. Erythrocytes were removed from the platelet-rich plasma by centrifugation at 750 rpm, the platelets were collected by centrifugation at 2500 rpms for 20 min. and the platelets resuspended in platelet buffer; 145mM NaCl, 5 mM KCl, 1mM MgSO4, and 10 mM Hepes, pH 7.4. This platelet suspension was filtered on a column of 40 ml Sepharose 4B (Pharmacia) to remove remaining plasma (3). Eluted platelets were centrifuged at 750 rpm to remove residual

erythrocytes and suspended in platelet buffer to a final volume of 1-3 mls. Aliquots of the platelet suspension were removed for determination of cholesterol (4) and phospholipid-phosphate (5).

Incorporation of Fluorescent Probes

22-(N-(7-nitrobenz-2-oxa-1,3- diazol-4-yl)amino)-23,24-bisnor-5- cholen-3-ol (NBD-CHOL) and cholestatrienol (C-3) were incorporated into platelets using small unilamellar vesicles (SUVs) as a donor (6). Briefly, phosphatidycholine, cholesterol and NBD-cholesterol or cholestatrienol (probes added at 2 mole% relative to total lipid) were dissolved in 2:1 chloroform: methanol, dried under nitrogen, lyophilized to remove trace amounts of organic solvents, resuspended in platelet buffer and sonicated to clarity (Branson 500 Sonicator). Large unilamellar species were removed by centrifugation at 45,000 rev min-1 for 30 min (7). The SUV exchange vesicles contained 40 mole % cholesterol so as not to alter the endogenous cholesterol content of the platelets. Platelets were incubated with probe containing exchange vesicles for 3 h at 37°C (8) and recovered by centrifugation at 2500 rpms (15min). The fluorescently labeled platelet pellet was resuspended in 2-5mls platelet buffer and used immediately for membrane raft isolation as described below. For membrane fluidity studies platelets were labeled with cis-parinaric acid as described (Sklar, et al., 1970).

Fluorescence Measurements

All fluorescence measurements were performed on a Perkin-Elmer LS50B spectrofluorimeter as described (6). Prior to the start of each experiment $CaCl_2$ was added to the platelet suspension to a final concentration of 0.10 mM, platelets were transferred to cuvettes placed into a water-jacketed turret at 37°C. The intra-membrane distribution of NBD-cholesterol was determined using dithionate dependent fluorescence quenching of this probe as described (9) and of C-3 using fluorescence resonance energy transfer upon the addition of 2,4,6-trinitrobenznesulfonic acid as described (6).

Isolation of Triton X-100 Resistant Membrane Rafts

Triton X-100 resistant membrane fractions were prepared from platelet membranes essentially as described (10). Freshly isolated platelet membranes were suspended in MOPS buffer to a

final protein concentration of 8-10 mg/ml. Triton X-100 (2% w/v) was added to this suspension to a final concentration of 1% w/v Triton X-100 and the suspensions were homogenized with three passes of a glass pestle through glass Tenboeck Tissue Grinder. Homogenates were prepared in the light or in the dark. The homogenate was mixed with 1.23 ml of 2.4 M sucrose to yield a final sucrose concentration of 0.9 M and transferred to a SW-41 centrifuge tube. The sample was overlaid with sucrose solutions in MOPS buffer of decreasing concentrations of 0.8M, 0.7M, 0.6M and 0.5M sucrose and centrifuged at 46,000 rpm for 20 hours at 4°C. The fractions were collected and analyzed for cholesterol, phosphate and protein. In control experiments equal volumes of platelets were treated with 2% octylglucopyranoside (OG) as described (11). The isolated rafts were analyzed for total ganglioside levels by quantitative immunoblotting using anti-cholera toxin antibody as described (12).

Cholesterol Oxidase Treatment

Platelets were incubated with cholesterol oxidase (*Nocardia.sp*, Calbiochem, La Jolla, CA) at a concentration of 5 units/ml platelets. The platelets were treated with collagen and cholesterol oxidase simultaneously, and aliquots removed at the indicated times. The reaction was terminated with the addition of ice-cold methanol, and lipids immediately extracted into chloroform (13). The extracted lipids were analyzed for cholesterol and cholestanone content by HPLC as described (6).

Results

Cholestatrienol Associates with Platelet Membrane Rafts

Cholesterol enriched platelet rafts provide a signaling matrix necessary to promote aggregatory responses. Although rafts have been rigidly defined based on phase behavior as lipids in the liquid ordered phase, in our studies, rafts are more loosely defined as low buoyant density detergent resistant membrane fractions. In these studies we sought to identify a fluorescent cholesterol marker that would reflect membrane raft cholesterol behavior. We focused on two well-characterized cholesterol derivatives, NBD-cholesterol and cholestatrienol. Cholestatrienol is structurally most similar to cholesterol and is efficiently incorporated into both cell membranes and unilamellar vesicles. It distributes into the bilayer and has exchange

rates similar to those obtained using [^{3}H] cholesterol (14). In contrast NBD-cholesterol has a larger bulky NBD moiety, which led us to hypothesize that this probe most likely reflects the behavior of cholesterol-poor non-raft domains. Platelets were isolated and labeled with one of these two probes as described above. Labeled platelets were treated with ice-cold Triton X-100 and membrane rafts isolated. As shown in Figure 1, fractions 3-5 contained a low buoyant density membrane species enriched in cholesterol and ganglioside 1 (GM1), indicative of a membrane raft. In contrast, the Triton X-100 soluble species were isolated in fractions 12-14 as expected based on previous studies (15); (2). In order to determine if the cholesterol probes were reversibly associated with the raft fraction all fractions were analyzed for NBD-cholesterol and cholestratrienol. Cholestatrienol was preferentially associated with the low buoyant density membrane raft fraction (fractions 3-5). In contrast NBD-cholesterol was found in the non-raft Triton X-100 soluble fractions (fractions 12-14). These studies suggest that cholestatrienol can be used as a marker of membrane raft cholesterol behavior and NBD-cholesterol as a marker for non-raft cholesterol. Moreover, cholestatrienol can be used to probe the dynamic properties of platelet membrane raft cholesterol pools.

Cholestatrienol Accessibility to Quenching Decreases upon Collagen Stimulation of Platelets

We have previously shown that platelet membrane cholesterol becomes inaccessible to outer monolayer quenching reagents upon stimulation with collagen (6). Those early studies suggested that cholesterol translocates from the outer to the inner membrane monolayer. In the next part of this study we wanted to determine if cholesterol translocates into or out of an outer monolayer membrane raft. Using C-3 and NBD-cholesterol to follow movement of membrane cholesterol in raft and non-raft micro-domains we asked which of these two probes becomes less accessible to outer monolayer quenching upon stimulation with collagen. Platelets were labeled with either C-3 or NBD-cholesterol and outer monolayer associated probe quantitated using 2,4,6-trinitrobenezenesulfonic acid (TNBS) or dithionate, respectively (6). We have previously shown that these probes incorporate into the platelet plasma membrane with a $t_{1/2} = 39$ min and -k= -0.0175 fluorescence units/ min. As seen in Figure 2, when C-3 labeled platelets were stimulated with collagen (10ug/ml), a decrease in C-3 accessibility was observed

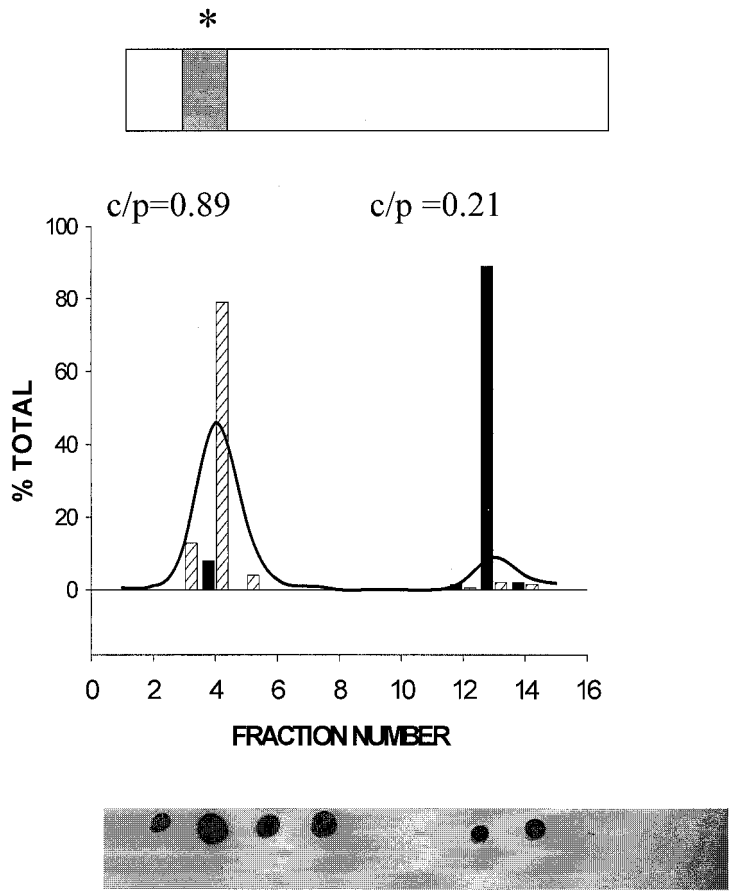

Fig. 1. Cholestatrienol (C-3) preferentially partitions into platelet membrane rafts. Freshly isolated human platelets labeled with either C-3 or NBD-cholesterol were treated with ice-cold Triton X-100 and the detergent resistant membrane fractions isolated (10). The low buoyant density fraction is indicted in gray and the % of the total membrane cholesterol is indicated on the ordinate and the GM1 levels shown on the dot blot. The cholesterol to phospholipid mole ratio (c/p) of the two major fractions; the membrane raft, fractions 3-5, and the Triton soluble fractions, 12-14 are indicated in the upper panel. The amount of NDB-cholesterol and cholestatrienol in each fraction is indicated.

within the first 5 minutes; no detectable change in NBD-cholesterol accessibility to probe was observed over this same time frame. Over the course of 30 minutes, the amount of C-3 accessible to quenching decreased from 60% to approximately 40%, with the most rapid translocation occurring within the first 5 minutes (Figure 3). In addition, there is no apparent redistribution of C-3 (from inner to outer monolayer) once it becomes inaccessible, as indicated by no change in accessibility for up to 30 minutes. Since C-3 preferentially labeled the membrane raft associated pool these results suggest that C-3 translocates out of a membrane raft upon collagen stimulation. We correlated these changes in C-3 accessibility with the translocation of phosphatidylserine from the inner to the outer monolayer. When NBD-PS labeled platelets were treated with collagen, there was a redistribution of PS, such that within the first five minutes there was a 50% increase in outer monolayer accessible PS (data not shown). Thus suggesting that cholesterol redistribution occurs on the same time scale as PS translocation; a necessary step in the platelet aggregator response.

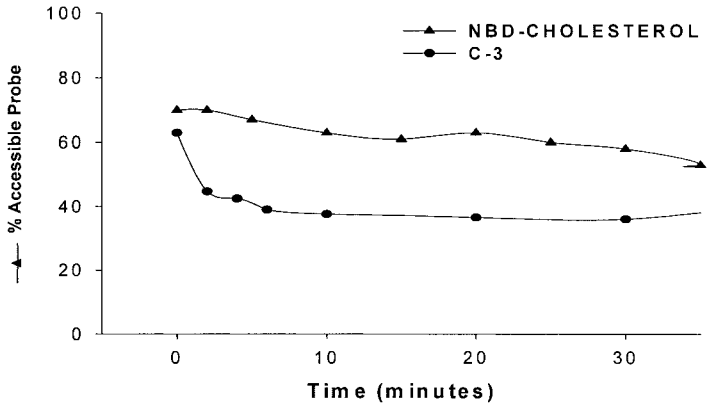

Fig. 2. C-3 Redistributes within the platelet plasma membrane upon collagen stimulation. Platelets labeled with either C-3 or NBD –cholesterol were stimulated with collagen (10 ug/ml) and aliquots removed at the times indicted. The amount of C-3 or NBD-cholesterol accessible to quenching by TNBS or dithionite respectively is indicated on the ordinate.

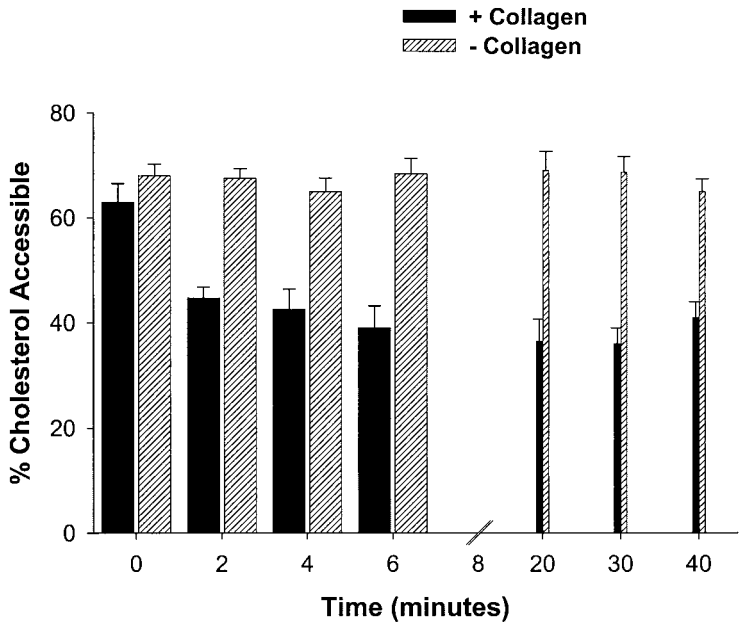

Fig. 3. Collagen decreases the accessibility of cholestatrieonol to TNBS quenching within the first five minutes. Platelets were labeled with C-3 and incubated in the presence [filled column] or absence [striped column] of collagen 10ug/ml for the times indicated on the abscissa. The amount of C-3 in the outer leaflet is determined as the decrease in cell-associated fluorescence after quenching with TNBS and calculated as described (Boesze-Battaglia, et al. 1996). Each value presented is the mean and standard error of four replicate experiments for platelets incubated in the absence of collagen and six experiments for platelets incubated with collagen. Each experiment was performed on an individual platelet preparation.

Stimulation With Collagen Alters The Distribution of Endogenous Platelet Membrane Cholesterol and Outer Monolayer Fluidity

The decrease in accessibility of C-3 to quenching upon collagen stimulation suggests that this probe translocates from the outer to the inner membrane monolayer. While the use of fluorescent sterol probes to study membrane cholesterol distribution has been well documented (16); (17), a caveat in the present studies is that the C-3 experiments do not directly study the

distribution of endogenous cholesterol. Outer monolayer cholesterol is susceptible to oxidation by cholesterol oxidase (18);(19);(20). A product of this oxidation reaction, cholest-4-en-3-one (cholestanone), is readily quantitated by HPLC (6). In this study we asked if endogenous cholesterol was more or less susceptible to oxidation by cholesterol oxidase upon stimulation with collagen. Platelets were treated with collagen and subsequently with cholesterol oxidase as described in methods. If endogenous cholesterol redistributes from the outer to the inner membrane monolayer we anticipate a decrease in oxdizable cholesterol reflected as a decrease in cholestanone. As seen in Figure 4, the amount of oxidizable cholesterol decreased by almost half upon stimulation of platelets with collagen.

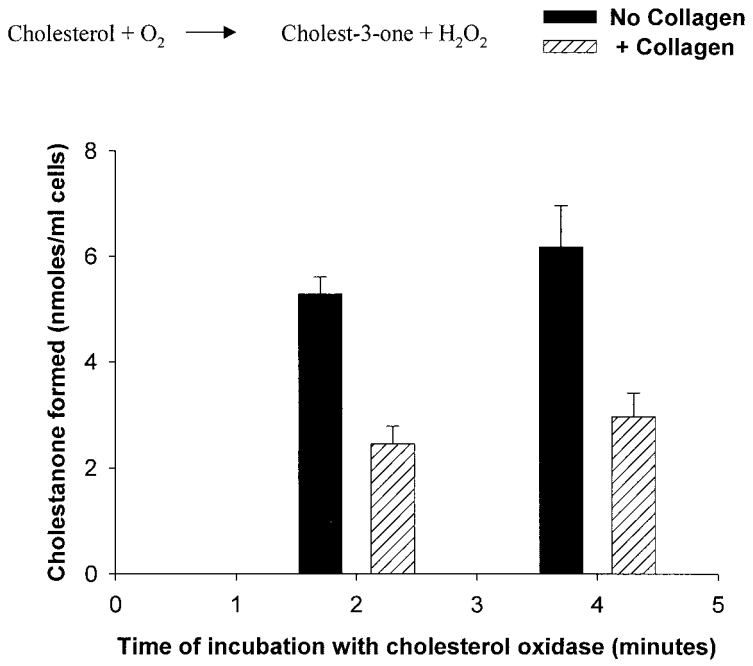

Fig. 4. Collagen treatment decreases the enzymatic conversion of endogenous cholesterol to cholestenone. Platelets were incubated in the presence or absence of collagen (10ug/ml) and cholesterol oxidase (5U/ml platelets) for the times indicated. The reaction was terminated and the cholestenone content determined by HPLC. Each value presented is the mean and standard error of eight observations.

The time course of this decrease; changes occurred within the first 5 minutes was identical to that observed with C-3 (Figure 3). We have previously shown that the redistribution of endogenous cholesterol is not directly dependent on ATP since pre-incubation of platelets with iodoacetamide, resulting in a decrease in ATP by 50 %, had no effect on cholesterol oxidase in stimulated platelets as described in Methods (6).

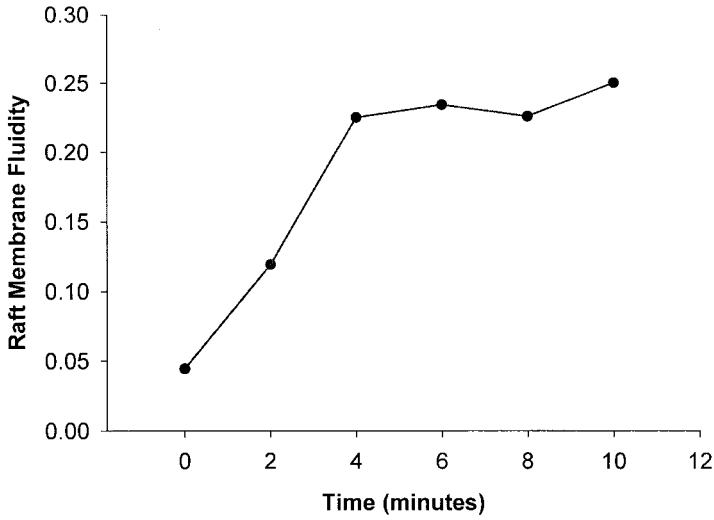

Fig. 5. Collagen treatment increases the fluidity of the outer membrane monolayer. Platelets were labeled with cis-parinaric acid treated with collagen (10ug/ml) for the times indicated and the fluorescence polarization of the cis-parinaric acid determined at 37°C. The results are representative of a series of five individual platelet preparations.

Lastly, to test the hypothesis that the movement of C-3 and endogenous cholesterol was from the outer to the inner membrane monolayer as opposed to a lateral diffusion within the plane of the outer monolayer, we measured the dynamic properties of the outer membrane monolayer. The fluorescent probe cis-parinaric acid has been used to measure outer membrane monolayer fluidity. Platelet membranes were labeled with cis-parinaric acid, and the change in outer monolayer fluidity measured as fluorescence polarization was monitored over time after collagen stimulation. As seen in figure 5, there was a rapid increase from 0.05 to 0.25 polarization units over the first 5 minute. This is the same time frame in which a

redistribution of both C-3 and endogenous cholesterol was observed. Collectively, these results suggest that collagen stimulates a translocation of cholesterol from an accessible (outer monolayer) to an inaccessible (inner monolayer) pool. Moreover, movement is most likely out of a cholesterol enriched outer monolayer raft and directly alters the fluidity of the outer leaflet.

Discussion

We have previously documented an intramembrane redistribution of cholesterol upon stimulation of human platelets with collagen. In this study we employed fluorescent sterol analogs to probe this redistribution of cholesterol as raft or non-raft associated in response to platelet stimulation by collagen. We have shown that C-3, a membrane raft specific cholesterol probe, as well as endogenous cholesterol, redistributes in platelet membranes upon collagen stimulation. Although the present studies do not directly distinguish between a lateral redistribution or the movement of cholesterol from the outer to the inner membrane monolayer, indirect evidence for the later comes from a change in membrane fluidity as the cholesterol translocates. Using cis-parinaric acid to probe the change in outer membrane monolayer dynamics (Figure 5), we showed that upon collagen stimulation and cholesterol movement the fluidity of the outer membrane monolayer increased. It is well documented that such increases in fluidity are often due to a decrease in localized cholesterol levels. Thus, the fluidity results are consistent with redistribution of cholesterol to the inner monolayer.

Although we favor the view that cholesterol translocates out of the membrane raft pool, it is not inconceivable to hypothesize that cholesterol redistributes to a collagen-membrane interaction zone that is inaccessible to quenching and cholesterol oxidase treatment. Although a viable hypothesis, one consistent with a lateral reorganization of cholesterol in rafts as proposed by Brodin, et al., (23), a number of experimental observations argue against this interpretation. The redistribution of cholesterol in response to stimulation is not unique to collagen as described here but is also seen in response to stimulation by ADP (21). Since collagen and ADP act through two different receptor classes; G-protein coupled receptors and the GpIIb/IIIa family of integrin like receptors we propose that the translocation of cholesterol is not due to the formation of a collagen-membrane reaction zone which is inaccessible to quenching reagents. In addition, TNBS is a small water soluble quenching agent and is not predicted to have limited

access to C-3. The most compelling support for a translocation of cholesterol out of the membrane raft comes from cholesterol enrichment studies. In those studies when platelet membrane cholesterol is enhanced the C-3 accessibility profile is virtually identical to that observed upon collagen treatment, suggesting that the added cholesterol is incorporated into an inaccessible possibly inner membrane raft pool (unpublished observations, Boesze-Battaglia and Schimmel).

Cholesterol alters platelet response to aggregatory stimuli in vitro (22); (23) and *in vivo* hypercholesterolemia results in abnormal clotting and the formation of atherosclerotic plaque. The precise role of cholesterol in the aggregatory response is under intense investigation. A number of signaling proteins and lipid second messengers have been found in membrane rafts of both resting and stimulated platelets (23). In recent studies, Gousset, et al., (15), have suggested that raft formation is a dynamic reversible event triggered by activation, they however provide little evidence to confirm the reversible nature of this domain formation. We propose a variation on this theme; that upon stimulation, cholesterol redistributes out of an outer monolayer membrane raft into an inaccessible- inner monolayer pool of cholesterol. We propose that this monolayer pool is a transient raft complex involved in organizing lipid mediators to further platelet aggregation and commit the platelet to an irreversible aggergatory event. Because it has been well documented that some platelet agonists mediate reversible platelet aggregation while stronger agonist mediate irreversible aggregation and rapid phospholipid translocation, it will be intriguing to determine how membrane raft formation is correlated with platelet phospholipid translocation and the reversible versus irreversible processes. Collectively this very new and growing body of work coupled with the stimulus dependent redistribution of PS, PE and cholesterol provides an ideal model system in which to study membrane raft formation and dissolution in response to stimuli. The dynamic redistribution of cholesterol might represent a critical mechanism for the early steps of platelet activation under physiological conditions (2); (24). In a manner analogous to hematopoietic cells, small lipid rafts may cluster into larger raft signaling platforms- traversing both monolayers of the plasma membrane bilayer. Moreover, the stimulus dependent reorganization of cholesterol provides an attractive mechanism to explain the membrane structural aspects of shape change. Shape change and the formation of filipodia (in platelets) and matrix vesicles (in

chondrocytes and osteoblasts) requires areas with small radii of curvature, a structure more readily achieved in a cholesterol-poor region of the membrane.

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