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Human Platelet Cytoskeletons: Specific Content of Glycolipids and Phospholipids

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The lipid composition of platelet cytoskeletons was analyzed. Triton X-100 (0.5%) was used to prepare cytoskeletons from thrombin-treated platelets. The lipid/protein ratio of platelet cytoskeletons was 0.260 and the phospholipid/protein ratio was 0.177, which were comparable to the ratios present in platelets. However, there was a selective enrichment of platelet lipids in platelet cytoskeletons. Only 2 of the 5 major platelet phospholipids were detected. About 14% platelet sphingomyelin and 2% platelet phosphatidylcholine were present in platelet cytoskeletons. Only 1 of the 4 platelet neutral glycolipids, trihexosyl ceramide, was detected and was about 7% of that in intact platelets. Two percent of platelet hematoside, the predominant ganglioside in platelets, was found in cytoskeletons. Six percent of platelet cholesterol was present in platelet cytoskeletons, while no other neutral lipid could be detected. The study demonstrates that the lipid/protein ratio of platelet cytoskeletons is similar to that in platelets, but the composition of cytoskeleton lipids is specific and distinctly different from that in platelets. The selective glycolipid and phospholipid composition of cytoskeletons may be important for cytoskeleton and platelet function.

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CELL CYTOSKELETONS are cytoplasmic structures that are thought to be important for maintaining cell integrity,[1] providing structural support for the plasma membrane,[2] and stabilizing plasma membrane receptors.[3] Cytoskeletons are composed of filamentous structures that include microtubules, and thin, intermediate, and thick filaments.[2] Triton extraction of cells results in the precipitation of major portions of the filamentous systems[4] and is the best available model for the study of cytoskeletons.[1]

Thrombin increases the amount and organization of filamentous actin in platelets.[5] Analysis of Triton-insoluble platelet cytoskeletons revealed F-actin, myosin, and filamin.[5,6] In addition, plasma membrane components that serve as receptors for platelet activities, glycoproteins IIb and IIIa,[7,8] factor V,[9] fibrinogen, and/or fibrin[10] have been detected in platelet cytoskeletons.

There appears to be a transmembrane association of membrane proteins with cytoskeletons. A major component of membranes is lipids, and conceivably, lipids are present and important for cytoskeleton structure and function. The lipid composition of platelet cytoskeletons has not been analyzed prior to this study. Our study demonstrates that the lipid/protein ratio in cytoskeletons is similar to that in platelets, but the composition of lipids is specific and distinctly different from that in platelets.

MATERIALS AND METHODS

Platelets from a freshly collected unit of blood were washed and isolated by differential centrifugation using Tris-buffered saline and EDTA as previously described.[11] In a few experiments, platelets were prepared by gel-filtration using a Sepharose 2B column equilibrated in Hepes-buffered Tyrode's solution.[9] Cytoskeletons were produced from platelets that had been exposed to 1 U/ml thrombin, kindly provided by Dr. J. F. Fenton, New York State Department of Health, for 3 min at 37°C.[9] Platelet aggregates were not seen. During incubation of platelets with thrombin and treatment with Triton X-100, Tris-buffered saline served as the suspension medium for platelets washed by differential centrifugation and Hepes-Tyrode's solution was used with gel-filtered platelets. Neither EDTA, EGTA, nor calcium had been added to these media. Reaction in thrombin-incubations was stopped by the addition of Hirudin (5 U/ml) and diisopropylfluorophosphate (1 m*M*). Both reagents were obtained from Sigma, St. Louis, Mo. Triton X-100 (0.5%) was used to prepare precipitates that were centrifuged for 2 min at 12,000 *g*. The pellet was extensively washed with (0.5%) Triton-Hepes buffer prior to lipid extraction to remove nonincorporated membrane contaminants.[9] Cytoskeletons produced under these conditions contain about 5% of the original platelet protein that has previously been shown to contain significant amounts of actin, myosin, actin-binding protein, and other proteins[9] characteristic of platelet cytoskeletons.[7-9] Platelet counts were done with a Baker MK-4/HC platelet counter (Baker Co., Bethlehem, Pa.). Protein content was analyzed by the Lowry method.[12]

Lipid Extraction and Analysis of Phospholipids and Cholesterol

Lipids were extracted from cytoskeletons and whole platelet controls in each experiment. For the analysis of phospholipids, lipids were extracted, separated by thin-layer chromatography, and assessed for lipid-phosphorus.[13] Cholesterol was quantitated in aliquots of the lipid extract by gas–liquid chromatography.[14]

Isolation of Neutral Glycolipids and Gangliosides

Cytoskeleton and platelet samples were suspended in 2.4 ml isotonic saline buffered at pH 7.4 with Tris buffer, and their lipid content was extracted by the addition of 6 ml methanol and then 3

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ml chloroform. Subsequently, an additional 3 ml chloroform and 3 ml HOH were added. Precipitates were reextracted, combined extracts were dried under nitrogen, resuspended in solvent no. 1, and passed through a coarse Sephadex G-25 column that served to desalt and separate neutral glycolipids, gangliosides, and other lipids.[11,15] Three eluting solvent systems were used with the Sephadex column to obtain 3 fractions.[15] Neutral glycolipids along with phospholipids and neutral lipids were collected in the first fraction eluted with solvent no. 1, and hematoside and minor platelet gangliosides were isolated in the second and third fractions eluted with solvents nos. 2 and 3, respectively. Neutral glycolipids eluted with solvent no. 1 were evaporated to dryness and then suspended in chloroform and separated from other lipids with a Unisil column as previously described.[11] One advantage of using the Sephadex column is that it avoids partitioning the lipid extract in which hematoside is present in both phases. Thus, total platelet and cytoskeleton hematoside could be collected from fractions 2 and 3 of the Sephadex column.

Analysis of Neutral Glycolipids and Gangliosides

Neutral glycolipids isolated by the Unisil column and gangliosides in fraction 2 from the Sephadex column were separated by thin-layer chromatography.[11] Neutral glycolipids were eluted from the thin-layer chromatoplate using the Bligh-Dyer extraction method[16] with over 95% recovery. Neutral glycolipids were analyzed by the anthrone reaction,[17,18] which quantitates glycolipid hexoses. Eluted neutral glycolipids were dried under nitrogen and resuspended in 0.3 ml distilled water and 0.7 ml 0.2% Anthrone in 95% sulfuric acid. Tubes containing the mixtures were shaken vigorously, heated at 100°C for exactly 7.5 min, cooled rapidly, and read for absorbance in microcuvettes at 630 nm. Standards used were combinations of galactose and glucose in molar ratio comparable to that in platelet trihexosyl ceramide (2/1), lactosyl ceramide (1/1), and globoside (2/1). Commercial globoside and trihexosyl ceramide (Sigma, St. Louis, Mo.) were also used as standards. Trihexosyl ceramide, but not globoside, gave comparable absorbance to that of hexose mixtures. This most likely is due to factors previously described.[18] Therefore, only commercial globoside served as a valid standard for platelet globoside. Gangliosides were quantitated by assay of sialic acid by the Aminoff methods.[19] The sensitivity was enhanced by drying the gangliosides under nitrogen, resuspending them in 0.4 ml 0.1*N* sulfuric acid, and then using the entire 0.4 ml in the analysis. Thin-layer chromatography showed that platelet hematoside was eluted in the second and third fraction of the Sephadex column and that hematoside was the only ganglioside present in platelet cytoskeletons. Therefore, hematoside assessments were made directly in eluates of fractions 2 and 3 from the Sephadex column.

The results of the assay of platelet neutral glycolipids and gangliosides by the modifications of the spectrometric methods described above were consistent with those that were previously reported using sphingosine quantitation by gas-liquid chromatography.[11]

Table 1. Relative Amounts of Lipids and Proteins in Cytoskeletons (CSK)

	CSK	Platelets
Protein (mg)	0.05	1.00
Lipids (mg)	0.013	0.321
Phospholipid (mg)	0.0083	0.253
Phospholipid/protein ratio	0.177	0.253
Lipid/protein ratio	0.260	0.321

Cytoskeletons were prepared from thrombin-treated platelets by using Triton X-100. Lipid analysis was carried out as described in Materials and Methods.

RESULTS

The study showed that there is a considerable amount of lipid in platelet cytoskeletons. The lipid/protein ratio of cytoskeletons when calculated weight per weight is 0.260, which is similar to that in platelets, as shown in Table 1. The phospholipid/protein ratio of platelet cytoskeletons is 0.177.

The analysis of individual lipids in platelet cytoskeletons is shown in Table 2. The neutral lipid content of platelet cytoskeletons was shown to be 6% of platelet cholesterol, while no other neutral lipid was present in significant quantities. Only two of the five major platelet phospholipids were detected. About 14% platelet sphingomyelin and 2% platelet phosphatidylcholine were detected in cytoskeletons. Only one of the four platelet neutral glycolipids, trihexosyl ceramide, was detected and was about 7% of that in intact platelets. Two percent of platelet hematoside, the predominant ganglioside in platelets, was detected in cytoskeletons. Lipids other than the ones mentioned above were not visualized on thin-layer chromatoplates. However, our assays were not sufficiently sensitive to exclude the presence of trace amounts of these lipids in cytoskeletons.

It was important to rule out the possibility that cholesterol was not a component of cytoskeletons but was extracted from platelets by Triton and then trapped during the precipitation of cytoskeletons. This was achieved by adding 2 μCi ^{3}H-cholesterol (0.12 pmole) (Amersham, Arlington Heights, Ill., TRK-122) to the Triton at the time of the preparation of cytoskeletons. When this was done, less than 0.1% of

Table 2. Lipid Composition of Platelet Cytoskeletons (CSK)

	Lipids in CSK From 10^9 Platelets (μg)	Lipids per 10^9 Platelets (μg)	Percent Platelet Lipids in CSK
Cholesterol	4.7 ± 1.0 SD (N = 5)	78.5	6%
Phosphatidylcholine	2.5 ± 1.5 SD (N = 4)	115.0	2%
Sphingomyelin	7.27 ± 2.25 SD (N = 4)	53.0	14%
Trihexosylceramide	0.063 ± 0.03 SD (N = 5)	0.88	7%
Hematoside	0.014 ± 0.008 SD (N = 4)	0.72	2%

Cytoskeletons were prepared from thrombin-treated platelets by using Triton X-100 (0.5%). Lipid analysis was carried out as described in Materials and Methods.

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the radioactivity was detected in platelet cytoskeleton cholesterol as compared to 7% of endogenous platelet cholesterol. Lipids present in cytoskeletons prepared from gel-filtered platelets were similar to those in platelets prepared by centrifugation of platelets, which were used in the experiments shown in Tables 1 and 2.

DISCUSSION

The lipid content of platelet cytoskeletons is comparable to that of platelets in that the lipid/protein ratio of the former is 0.260 and the latter is 0.321. The phospholipid/protein ratio of platelet cytoskeletons is 0.177 as compared to 0.253 in platelets. It is thought that phospholipid/protein is indicative of a metabolically active membrane. Intestinal villi membranes have a phospholipid/protein ratio of 0.11 mg/mg protein, while brain myelin, which is less metabolically active, has a ratio of 0.92.[20] This suggests that platelet cytoskeletons are active membraneous structures.

There is a selective enrichment of platelet lipids in platelet cytoskeletons. Only two phospholipids, sphingomyelin and phosphatidylcholine, are present in platelet cytoskeletons. These lipids are thought to be important for membrane structural integrity and both are located in substantial amounts in the outer layer of platelet plasma membranes.[21,22] However, we cannot conclude that the outer layer is the exclusive source of cytoskeleton, since about 20% of sphingomyelin and 60% phosphatidylcholine most likely are present in the inner plasma membrane layer.[22] Phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol are located primarily in the inner portion of the platelet plasma membrane[13,22] and have been implicated in platelet physiologic activities.[23] However, this study demonstrates that these lipids are not present in platelet cytoskeletons. Only one neutral lipid, cholesterol, was present in platelet cytoskeletons and represented about 6% of total platelet cholesterol.

Two of the five major platelet glycolipids were detected in platelet cytoskeletons. About 2% of platelet hematoside and 7% trihexosylceramide were found. Hematoside represents about 92% of platelet gangliosides and has been proposed as a receptor for serotonin in platelets.[24] There is evidence that there is an increase in the quantity of hematoside in thrombin-treated platelets[25,26] that is present on the platelet surface.[11] Little is known about the role of trihexosyl ceramide in platelets. Neither lactosyl ceramide, which is the predominant glycolipid in platelets, nor globoside were detected in platelet cytoskeletons. The presence of glycolipids in cytoskeletons is of considerable interest. They have been proposed as mediators of cellular adhesion and can serve as receptors.[21,27]

The erythrocyte cytoskeleton lipid content has been analyzed. It was found that only small amounts of sphingomyelin and phosphatidylcholine were present, but cholesterol and glycolipids were not analyzed.[28] It has been reported that with higher concentrations of Triton used in the preparation of erythrocyte cytoskeletons, phospholipids could no longer be detected. This was interpreted to indicate that phospholipids are not integral components of cytoskeletons.[1] This conclusion does not appear warranted, since greater than 1% Triton most likely leads to artifactual results.

The plasma membrane is most likely the source of cytoskeleton lipids. One possibility is that the plasma membrane and its lipid moieties serve as an anchor for cytoskeletons and a portion of the plasma membrane becomes an integral component of cytoskeletons. It has been reported that an erythrocyte plasma membrane protein, ankyrin, serves as an anchor for erythrocyte cytoskeletons.[1] An alternate role for lipids in cytoskeletons is to provide structural support for cytoskeletons or possibly to directly mediate cytoskeleton activities. The selective inclusion of lipids in platelet cytoskeletons indicates that specific domains of platelet plasma membrane lipids and proteins are intimately associated with cytoskeletons.

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