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Structurally distinct plasma membrane regions give rise to extracellular membrane vesicles in normal and transformed lymphocytes

Marcia J. Armstrong [a], Judith Storch [b] and Nicholas Dainiak [a]

[a] *Departments of Medicine and Biomedical Research, St. Elizabeth's Hospital and Tufts University and* [b] *Department of Nutrition, Harvard School of Public Health, Boston MA (U.S.A.)*

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Shedding of extracellular membranes from the cell surface may be one of the means through which cells communicate with one another. In an attempt to elucidate whether cell surface exfoliation is a directed or random process, we investigated the membrane lipid and protein composition and membrane lipid order of shed extracellular membranes and of plasma membranes from which they arose in normal circulating lymphocytes and in the B-lymphoblastoid cell lines Raji, WI HF2 729 and the T-lymphoblastoid cell line Jurkat. Extracellular membranes derived from transformed cell lines were more rigid as assessed by steady state polarization of 1,6-diphenylhexatriene (DPH) and were highly enriched in cholesterol when compared with the corresponding plasma membrane. The extracellular membranes from normal lymphocytes, on the other hand, were more fluid and contained more polyunsaturated acyl chains than did the plasma membranes from these cells. Our results suggest that extracellular membranes are shed from specialized regions of the lymphocyte plasma membrane and that membrane exfoliation is likely to be a directed event.

Introduction

Cultured bone marrow cells may communicate with one another by different processes, including secretion of soluble factors via exocytosis, exfoliation of surface membrane components as shed vesicles (or extracellular membranes) and direct physical contact [1]. Work from this laboratory has shown that lymphocyte plasma membrane-derived vesicles that are shed into liquid culture medium contain an integral glycoprotein that stimulates human erythroid progenitor cell growth in vitro [2,3]. This activity appears to be released by both resting B cells of normal donors and Epstein-Barr virus transformed B-lymphoblasts [4]. Furthermore, mitogen-stimulated T cells exfoliate membrane derived vesicles that support the proliferation of hematopoietic progenitors of multiple lineages [5].

Little is known regarding the biochemical processes which drive exfoliation of membrane-associated hematopoietic growth factors and which regulate extracellular vesiculation in general. One body of evidence suggests that the lipid composition of membranes may be important for the expression of surface-associated molecules [6–8] and for modulation of plasma membrane enzymatic activities [9,10]. Here, we have explored the hypothesis that lipid and/or protein composition of membranes is a determinant of cell surface exfoliation. Furthermore, since tumorigenesis results in alterations in membrane composition [11–13], we questioned whether the relationship

Correspondence: M.J. Armstrong, Department of Biomedical Research, St. Elizabeth's Hospital, 736 Cambridge Street, Boston, MA 02135, U.S.A.

between plasma membranes and extracellular membranes is similar in normal and transformed lymphocytes. Our results indicate that shed membrane-derived vesicles and plasma membranes have distinct lipid composition, fluidity, and protein composition, and that vesicles are more fluid than parent membranes in normal circulating human lymphocytes, while in cultured human B- and T-lymphoblastoid cell lines, shed vesicles are more rigid than parent membranes. The data support the concept that exfoliation occurs from specific domains of the cell surface, and that the mechanism involved in shedding of surface components may be different in normal and transformed cells.

Materials and Methods

Cell lines. The B-lymphoblastoid lines Raji and WI HF2 729 and the T-lymphoblastoid line Jurkat were the generous gift of Dr. James Mier of Tufts University. The cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (Hyclone), penicillin, streptomycin and L-glutamine (GIBCO) at 37°C, 5% CO_2 in humidified air in a NAPCO incubator. The cells were split twice a week.

Preparation of conditioned medium and plasma membranes: lymphocytes. Plateletpheresis residues (obtained from the Dana Farber Cancer Institute, Boston, MA) were diluted in alpha medium (Gibco) and sedimented over Ficoll Paque to obtain lymphocytes as described [2,3]. Monocytes were removed by adherence to plastic tissue culture flasks at 37°C for 90 min. The nonadherent cells (>97% lymphocytes) were incubated in serum-free alpha medium at $5 \cdot 10^6$ cells/ml overnight at 37°C in humidified 5% CO_2/air. The cells were separated from the resultant conditioned medium by centrifugation at $400 \times g$ for 10 min. A vesicular fraction containing the extracellular membranes was obtained from the lymphocyte conditioned medium by centrifugation at $40\,000 \times g$ for 45 min. The vesicle-containing pellets were washed, suspended in phosphate-buffered saline (PBS) and stored at −20°C. For preparation of plasma membranes, the cells were washed twice in PBS, and purified plasma membranes were prepared according to a modification [2] of the method of Jett et al. [14]. Briefly, the cells were treated with glycerol, lysed with 20 strokes of a Dounce homogenizer, and membranes were isolated by differential centrifugation and subsequent purification over discontinuous sucrose density gradients of 20%–30%–40%–50% sucrose (w/v) in 5 mM Tes-2 mM $MgCl_2$. The purified membranes were harvested from the interface at 30–40% sucrose, washed once in PBS and stored at −20°C until analysis.

Preparation of conditioned medium and plasma membranes: cell lines. Cell lines were fed fresh serum-free medium 24 h prior to the preparation of conditioned medium. Plasma membranes were prepared in a manner analogous to that used for normal lymphocytes. The purity of the membrane fractions was assessed by determining the specific activity ratio (purified plasma membranes/homogenate) for alkaline phosphodiesterase, a plasma membrane marker [15]. The specific activity ratio was increased approx. 10-fold for both normal cells and transformed lines.

Lipid analyses. Total lipids were extracted from membrane preparations by the method of Folch et al. [16]. Total cholesterol content of the extract was estimated by a cholesterol oxidase/cholesterol esterase kit method (Sigma Diagnostics, St. Louis, MO) using ethanolic suspensions of the lipid extracts and standards. Total phospholipid content of the extracts was analyzed by the method of Bartlett [17]. Fatty acid methyl esters (FAMEs) were prepared with borontrifluoride (14% (w/v) in methanol) as catalyst [18]. The derivatives were analyzed on a Varian gas chromatograph equipped with a flame ionization detector and interfaced with an HP 3380A integrator. FAMEs were resolved on a 6 ft column (1/4 inch o.d., 2 mm i.d.) packed with 10% SP-2330 on 100/120 mesh Chromabsorb W AW (Supelco). The column temperature was 150°C for 10 min followed by an increase of 4°C/min to 250°C, and run at 250°C until 60 min. The injector temperature was 220°C. Sample peaks were identified by comparison with the retention times of authentic FAME standards (Sigma).

Fluorescence polarization studies. The lipid fluidity [19] of plasma membranes and conditioned medium pellets (extracellular membranes) was determined by assessing the steady-state fluorescence polarization of 1,6-diphenylhexatriene

(DPH) (Molecular Probes, Junction City, OR) as described previously [10]. Plasma membranes and extracellular membranes were suspended in PBS at a final concentration of 250–400 μg/ml and loaded with DPH at a 200:1 lipid to probe ratio for 45 min at 30°C. Measurements of the fluorescence polarization (P) were made at 24°C in an SLM 8000 spectrofluorimeter. Corrections for light scattering (membrane suspension minus probe) were made routinely and were always less than 3% of the total fluorescence intensity. Data are expressed as the anisotropy parameter $[(r_o/r) - 1]^{-1}$ where r_o is the maximum limiting anisotropy. For DPH, r_o is 0.39 [20]. The anisotropy, r, is derived from P, fluorescence polarization:

$$r = \frac{2P}{3 - P} \quad (1)$$

Analysis of membrane proteins. Protein contents of plasma membrane and extracellular membrane preparations were estimated by a micro Lowry [21] method as previously described [2]. Membrane proteins were examined by electrophoresis on $NaDodSO_4$ polyacrylamide gels run according to Laemmli [22]. Samples of plasma membranes and extracellular membranes were applied to 10% $NaDodSO_4$ polyacrylamide gels in the presence of denaturating agents. Proteins and glycoproteins were visualized by the dual silver-Coomassie blue staining procedure of Dzandu et al. [23].

Statistical analysis. Means ± S.E. of 3–5 measurements were calculated and the results were compared by Student's t-test for independent or paired means as appropriate.

Results

Lipid fluidity of plasma and extracellular membranes

The results of steady state fluorescence polarization studies performed on cell membranes loaded with DPH are presented as the anisotropy parameter $[(r_o/r) - 1]^{-1}$ in Table I. For normal circulating lymphocytes, values of the anisotropy parameter were 40% higher ($P < 0.05$) for plasma membranes when compared to extracellular membranes, suggesting that these cells shed vesicles that are less ordered than are the parent membranes. On the other hand, for each of the transformed cell lines, values of the anisotropy parameter for DPH polarization in plasma membranes were on average 43% lower ($P < 0.01$) than those obtained for the extracellular membranes, suggesting that exfoliated vesicles are more ordered than are the plasma membranes from which they are derived. Results also show that plasma membranes from normal lymphocytes were more ordered than those from transformed lymphocytes (1.69 ± 0.10 vs. 1.32 ± 0.03 (mean of transformed data), $P < 0.01$). Moreover, membrane vesicles shed from normal cells were less ordered than those shed from transformed cells (1.21 ± 0.01 vs. 2.03 ± 0.06 (mean of transformed data), $P < 0.01$).

TABLE I

ANISOTROPY PARAMETER IN NORMAL AND TRANSFORMED LYMPHOCYTE PLASMA AND EXTRACELLULAR MEMBRANES

Values are means ± S.E. Rows or columns with different superscripts are significantly different from one another by Student's t-test (a vs. b, $P < 0.05$; all others, $P < 0.01$).

Cell type	Anisotropy parameter $[(r_o/r) - 1]^{-1}$	
	plasma membrane	extracellular membrane
Normal lymphocyte	1.69 ± 0.10 [a]	1.21 ± 0.01 [b]
Transformed lymphocyte		
Jurkat	1.28 ± 0.04 [c]	2.28 ± 0.08 [d]
Raji	1.32 ± 0.0[illegible] [c]	2.25 ± 0.13 [d]
WI HF2 729	1.36 ± 0.08 [c]	2.38 ± 0.08 [d]

Cholesterol / phospholipid (C / PL) ratios

To determine whether alterations in membrane lipid composition correlate with the observed alterations in membrane fluidity, we examined the C/PL molar ratios in the various membrane preparations. As shown in Table II, C/PL ratios for the circulating lymphocyte plasma membranes tended to be higher, although not significantly so, than those obtained for the corresponding extracellular membranes. C/PL ratios were not significantly greater when comparing plasma membranes from circulating lymphocytes with those from transformed lymphocytes. However, vesicles shed from the surface of transformed lymphocytes exhibited markedly increased C/PL

TABLE II

CHOLESTEROL/PHOSPHOLIPID RATIOS IN NORMAL AND TRANSFORMED LYMPHOCYTE PLASMA AND EXTRACELLULAR MEMBRANES

Values are means ± S.E. Rows or columns with different superscripts are significantly different from one another by Student's *t*-test ($P < 0.01$).

Cell type	Cholesterol/phospholipid (mol/mol)	
	plasma membrane	extracellular membrane
Normal lymphocyte	0.82 ± 0.29^{a}	0.51 ± 0.12^{a}
Transformed lymphocyte		
Jurkat	0.50 ± 0.07^{a}	3.19 ± 0.32^{b}
Raji	0.43 ± 0.04^{a}	2.61 ± 0.35^{b}
WI HF2 729	0.48 ± 0.03^{a}	3.24 ± 0.42^{b}

ratios when compared with plasma membranes from the same cells (e.g., for Jurkat cells, 0.50 ± 0.07 vs. 3.19 ± 0.32, a 6-fold increase). Furthermore, C/PL ratios of these exfoliated vesicles were higher than those of vesicles shed from and plasma membranes of normal lymphocytes ($P < 0.01$).

Fatty acyl chain composition of membrane lipids in normal and transformed cells

Total lipid fatty acid methyl esters (FAMEs) were analyzed by gas chromatography (Fig. 1). For transformed cells, shed extracellular membranes had a higher percent of saturated fatty acyl chains than did corresponding plasma membranes ($P < 0.01$). Conversely, vesicles shed from normal cells had an insignificantly lower percentage of saturated fatty acyl chains than did the parent plasma membrane. The percentage of FAMEs consisting of saturated fatty acyl chains in vesicles shed from transformed lymphocytes was increased relative to that in vesicles shed from normal lymphocytes (62 ± 3 vs. 42 ± 2, $P < 0.05$).

In contrast, no differences in the percent of monounsaturated fatty acyl chains (palmitoleic acid, 16:1, and oleic acid, 18:1) were observed (see Fig. 1). However, for normal lymphocytes, the percent polyunsaturated fatty acyl chains was increased in the extracellular membranes when compared with plasma membranes (31 ± 3 vs. 15 ± 3, $P < 0.05$). In addition, when compared to

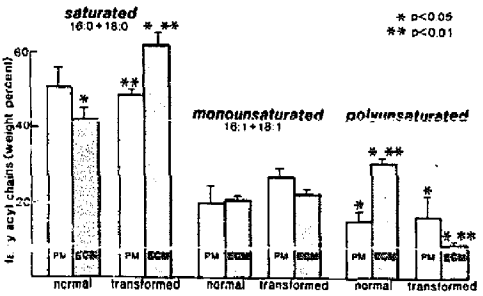

Fig. 1. Distribution of fatty acyl chain methyl esters in phospholipids in transformed and normal circulating lymphocytes. The bars on the left compare the saturated fatty acyl chains (16:0+18:0) in the plasma membrane (PM) and extracellular membranes (ECM) for normal (L) and transformed (R) lymphocytes. The bars in the middle depict the monounsaturated fatty acyl chains (16:1+18:1). The bars on the right depict the polyunsaturated fatty acyl chains (18:2+18:3+20:4+22:6). Symbols indicate significant differences; ** $P < 0.01$; * $P < 0.05$.

membrane vesicles shed from transformed lymphocytes, those from normal lymphocytes had an increased percent polyunsaturated fatty acyl chains (31 ± 3 vs. 9 ± 1, $P < 0.01$). The double bond index for normal lymphocyte plasma membranes was lower than that of the corresponding extracellular membranes (0.65 ± 0.02 vs. 1.26 ± 0.10, $P < 0.01$). The double bond index for plasma membranes of transformed cells was greater than that of the corresponding extracellular membranes (0.85 ± 0.07 vs 0.48 ± 0.04, $P < 0.01$).

Total lipid/protein ratios

Since membrane fluidity has been shown to increase as the ratio of lipid to membrane intrinsic proteins increases [33], we calculated the total lipid content relative to amount of protein present. The relationship between total lipid (cholesterol (μmol/ml) + phospholipid (μmol/ml)) content and membrane protein (mg/ml) content is shown in Table III. The ratio of total lipid to protein was increased by 56% in membrane vesicles exfoliated from transformed lymphocytes when compared to the corresponding plasma membranes (1.23 ± 0.04 (mean of data) vs. 0.79 ± 0.11, $P < 0.05$). In contrast, no difference in this ratio was observed for normal lymphocyte plasma membranes and shed extracellular membranes (see Table III).

TABLE III

TOTAL LIPID/PROTEIN RATIOS IN NORMAL AND TRANSFORMED LYMPHOCYTE PLASMA AND EXTRACELLULAR MEMBRANES

Values are means ± S.E. Total lipid is calculated as [cholesterol (μmol/ml) + phospholipid (μmol/ml)]/protein (mg/ml). Rows or columns with different superscripts are significantly different from one another by Student's *t*-test ($P < 0.05$).

Cell type	Total lipid/protein	
	plasma membrane	extracellular membrane
Normal lymphocyte	0.61 ± 0.06 [a]	0.70 ± 0.15 [a]
Transformed lymphocyte		
Jurkat	0.58 ± 0.12 [a]	1.30 ± 0.28 [b]
Raji	0.90 ± 0.21 [a]	1.23 ± 0.30 [b]
WI HF2 729	0.89 ± 0.52 [a]	1.15 ± 0.34 [b]

Characterization of membrane proteins

Membrane-associated proteins were separated by electrophoresis in 10% $NaDodSO_4$ polyacrylamide under reducing conditions. Proteins of shed extracellular membranes were distinct from those of plasma membranes (see Fig. 2). Several bands were present in membranes shed from transformed lymphocytes that were not present in corresponding plasma membranes. Moreover, those protein bands identified in both preparations were present in dissimilar quantitative relationships. For example, exfoliated vesicles contained several prominent low molecular weight glycoproteins that were present in diminished amounts in the parent plasma membranes. Interestingly, a prominent lipid band that ran before the dye front was observed in electrophoresed shed vesicles from transformed lymphocytes but not in plasma membrane polypeptides. This may be a reflection of the increased lipid/protein ratio (Table III).

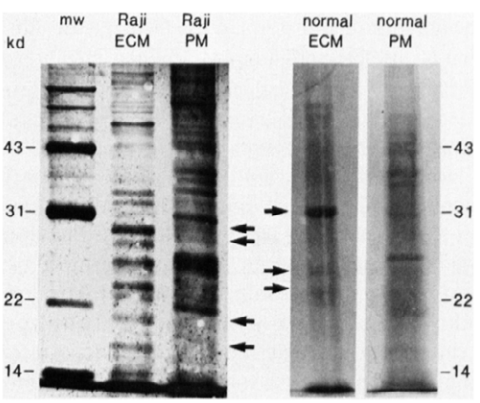

Fig. 2. SDS-polyacrylamide gel electrophoresis of extracellular membranes (ECM) and plasma membranes (PM) prepared from Raji B-lymphoblasts (L) and normal circulating lymphocytes (R). Similar amounts of protein were loaded on 10% polyacrylamide gels under reducing conditions. Gels were stained by the method of Dzandu et al. [22] for glycoproteins and proteins. Arrows indicate proteins present in extracellular membranes that are not present, or present in markedly reduced quantities, in plasma membranes.

Discussion

Spontaneous exfoliation of cell surface membrane vesicles in vitro and in vivo occurs widely in nature [6,12,24–26]. Extracellular vesiculation may be involved in diverse biological phenomena, including mediation of cell-cell interactions [2,6], determination of cell phenotype [27], initiation of an immune response [28,29] and maintenance of an appropriate concentration of plasma membrane components [30]. We have shown that shed plasma membrane-derived vesicles express surface-associated hematopoietic growth factors, one of which has been purified to apparent homogeneity [3]. In spite of the potential importance of cell surface exfoliation, little is known regarding physicochemical forces which drive this process. Results of studies with malignant cells and splenocytes indicate that fluctuations in incubation temperature may trigger extracellular vesiculation [31,32]. In this communication, we have asked whether shedding of membrane-derived vesicles is a random or directed process by examining the lipid and protein compositions and bulk lipid fluidty of plasma membranes and vesicles shed from the surface of normal human lymphocytes and transformed lymphoblasts. Our finding that the extracellular and plasma membranes of both normal and transformed lymphocytes have different lipid and protein compositions, as well as markedly different lipid order, suggests that shed extracellular membrane vesicles arise from distinct regions of the plasma membrane.

Not only are lipids, proteins and carbohydrates asymmetrically distributed between plasma membrane hemileaflets but also membrane components are apparently segregated into discrete lateral domains of varying size and composition within each leaflet [33]. It is possible that selected domains of lipids and/or intrinsic membrane proteins are shed from the cell surface. Our findings of significantly increased C/PL ratio, percent saturated fatty acyl chains, total lipid/protein ratio and altered protein composition of plasma membranes versus extracellular membranes in transformed cells strongly support this possibility. In contrast, only the protein composition and distribution of polyunsaturated fatty acyl chains in total membrane lipids were significantly different when comparing normal lymphocyte plasma membranes and shed vesicles (see Figs. 1 and 2). Together, these findings suggest that while extracellular vesiculation by human lymphoid cells occurs in a directed fashion, the biochemical mechanisms involved in shedding from transformed cells and from normal cells may be distinct.

Biochemical determinants of bilayer fluidity include the molar ratio of cholesterol/phospholipid (C/PL), the degree of saturation and side chain length of the phospholipid fatty acyl side chains, and the lipid/protein ratio [19]. Studies of DPH polarization with model phospholipid vesicles have demonstrated that increasing the mole fraction of cholesterol in such vesicles results in higher steady-state anisotropy due to ordering of the bilayer by cholesterol [34,35]. Here, we observed that decreased fluidity in vesicles shed from transformed cells was accompanied by an extremely elevated C/PL ratio and an increased proportion of saturated fatty acids. Interestingly, the ratio of lipid/protein, which generally reflects increased fluidity [19], was markedly increased in these vesicles. The increased lipid, however, was rich in both cholesterol and saturated fatty acyl chains. By contrast, increased bilayer fluidity in the normal lymphocyte was primarily associated with a higher percent unsaturated fatty acids. The data suggest that transformed cells shed cholesterol-enriched vesicles, an hypothesis that has been suggested by Coleman and co-workers [30]. Our results are of particular interest in relation to the expression of membrane-associated growth factors since proliferating cells synthesize cholesterol at a high rate in vivo [36], and because intermediates of cholesterogenesis may initiate DNA replication [36,37]. These findings here using established cell lines must be applied with caution to the case of leukemias since the characteristics of the cells may be altered during long term cultures.

In accordance with our results obtained with transformed human lymphocytes, murine leukemic GRSL cells have been found to shed extracellular membrane vesicles into ascites fluid that are rigid by DPH polarization and rich in cholesterol [12,24,26]. Normal rabbit thymocytes, on the other hand, have been shown to shed vesicles of essentially the same fluidity and lipid composition as the plasma membrane from which they were derived [12,24]. We have shown that normal circulating human lymphocytes shed vesicles that are more fluid and enriched in polyunsaturated fatty acyl chains than the plasma membrane. In studies with normal rabbit thymocytes, Roozemond and Urli [25] concluded, based on comparative fluidity and compositional analysis, that extracellular membrane vesicles arise from immature thymocytes. This finding of increased shedding of vesicles from the surface of less differentiated thymocytes may be analogous to vesicle shedding from the less differentiated lymphoblasts.

Several lines of evidence suggest that membrane composition and consequently, membrane fluidity, influence lymphocyte plasma membrane function. For example, activation of T-cells by concanavalin A, which results in changes in plasma membrane protein distribution, is accompanied by altered fatty acid composition of the phospholipids with an increased proportion of polyunsaturated fatty acids, and a decrease in the C/PL ratio [8]. Additionally, hydrogenation of fatty acids in lymphocyte plasma membranes, rendering them more rigid, selectively alters the expression of cell surface antigens, some of which may be involved in immunogenicity [6]. Finally, membrane rigidization by treatment with cholesteryl hemissuccinate alters the expression of histocompatibility (H2) determinants [38]. Whether the expression of membrane-associated hematopoietic growth factors is also altered by such treatments is unknown (work in progress).

In summary, we have presented data showing that membrane-derived, shed vesicles are structurally distinct from their parent membranes. Our findings are consistent with the hypothesis that in transformed lymphocytes, proteins are inserted in a directed fashion into rigid, cholesterol rich regions of the plasma membrane which may destabilize the membrane. These rigid areas may then be selectively shed from the cell surface.

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