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EXFOLIATION OF MEMBRANE ECTO-ENZYMES IN THE FORM OF MICRO-VESICLES

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Cultures from various normal and neoplastic cell lines exfoliated vesicles with 5'-nucleotidase activity which reflected the ecto-enzyme activity of the parent monolayer culture. The ratio of 5'-nucleotidase to ATPase activity in the microvesicles indicated that cellular ecto-ATPase was conserved in the exfoliative process. Phospholipids of the microvesicles contained significantly increased amounts of sphingomyelin and total polyunsaturated fatty acids. It was concluded that the shedded vesicles constituted a select portion of the plasma membrane. Examination by electron microscopy showed the vesicles had an average diameter of 500 to 1000 nm and often contained a second population of vesicles about 40 nm in diameter. As much as 70% of the plasma membrane ecto-5'-nucleotidase activity of a culture was released into the medium over a 24-h period. Phosphoesterhydrolases from C-6 glioma or N-18 neuroblastoma microvesicles dephosphorylated cell surface constituents when in contact with monolayer cultures. Exfoliated membrane vesicles may serve a physiologic function; it is proposed that they be referred to as exosomes.

Introduction

Plasma membrane ecto-ATPases and ecto-5'-nucleotidases have been found and characterized in a variety of eukaryotic cells and it is probable that each enzyme subserves more than one function on the cell surface. Both enzymes exhibit a broad specificity for the base moiety of nucleotide substrates [1] but it is not established that ATP or AMP are the predominant endogenous substrates. Ecto-ATPases have the properties of glycolipoproteins and are rather firmly bound to the plasma membrane, while ecto-5'-nucleotidases are composed of glycoprotein which appears to be collocated with sphingomyelin in situ and can be removed from the membrane matrix by fairly mild procedures [2]. During our investigations on the functional roles of these two ecto-enzymes we have observed that ATPase (EC 3.6.1.3) and 5'-nucleotidase (EC 3.1.3.5) were released into the superfusate media of cultured cell lines. We established that this release was not caused by cytolysis of moribund cells. The enzymes were released in the form of vesicles which are probably derived from specific domains of the plasma membrane. Whether or not the exfoliated microvesicles mediate physiologic processes in vivo has not been established.

Methods and Materials

Cell cultures. Cell lines employed in this study were established cultures (e.g. mouse neuroblastomas, N-18 and NB41A3; rat glioma, C-6; mouse melanoma, B-16), or derived from embryonic or neonatal tissue as primary cultures (rat aorta, RA-B; mouse astroblast, D-34) or grown from biopsy material (human melanoma, CL; human foreskin fibroblasts, KIN). Cells were grown in the appropriate medium as monolayers in 75 cm^2 plastic flasks (Falcon Plastics, Oxnard, CA) or on 530 cm^2 NUNC Bioassay dishes (A/S NUNC, Roskilde, Denmark). Passage numbers for a culture refer to the number of times the stock

cell line has been subcultured by trypsinization, dilution and explantation into maintenance or experimental culture vessels. In particular, we have used the term 'low passage' for the rat glioma cell line C-6 when the parent cell was obtained from the American Type Culture Collection (Rockville, MD) at the earliest available passage (P-38). During repeated passage of this line we have observed over a number of years that ecto-5′-nucleotidase activity decreased sharply after about 20 passages and that ecto-ATPase activity increased. The term low passage is used for the C-6 line for P-38 to P-55 and high passage for passages P-65 to P-160.

Enzyme assays. ATPase activity was assayed on intact monolayer cultures or on isolated vesicles by a modified method of Weil-Malherbe and Green [3] by addition of [γ-^{32}P]ATP (New England Nuclear Corp., Boston, MA) to a superfusate buffer or to the vesicle suspension. The activity of 5′-nucleotidase was determined in a similar manner with [^{32}P]AMP as substrate (New England Nuclear Corp.). Complete tissue culture growth media usually contain traces of ATPase and 5′-nucleotidase derived from the fetal calf serum component. Therefore, the cultures were washed prior to each experiment several times with a modified medium devoid of serum and routine incubations were performed in serum free media. We have used the term superfusate for modified media which were applied to confluent monolayer cultures in which enzyme accumulation was measured.

Lipid analyses. Phospholipid distribution in intact cells or extruded vesicles was estimated by two-dimensional TLC of a chloroform-methanol extract (2 : 1, v/v) according to Rouser et al. [4]. After development of the chromatogram, the TLC plates were charred with 50% $(NH_4)HSO_4$ and phosphate content of individual spots was determined by the method of Nelson [5]. For fatty acid analysis, aliquots of total lipid extracts were evaporated to dryness and methylated with BF_3 in methanol according to Morrison and Smith [6]. The fatty acid methyl esters were resolved and quantified on a Hewlett Packard 5840 gas chromatograph employing an SP 2330 column operated at 190°C.

Results

We have found that 5′-nucleotidase and ATPase were released into serum-free medium (superfusates) of monolayer cultures of normal and neoplastic cells. When a comparison was made between the ratio of ecto-5′-nucleotidase to ecto-ATPase activity in several cell lines and the activity of the two enzymes released into medium over a 24-h period, it was found that there was a proportionately larger release of 5′-nucleotidase (Table I). As we shall demonstrate below, the released enzymes had been derived from the corresponding plasma membrane ecto-enzymes. The relative preponderance of 5′-nucleotidase over ATPase in the microvesicles, compare ratios (1)/(2) to (3)/(4), indicated that either the ATPases were more labile, or that they had been conserved. When the decay of the catalytic activity of the released enzymes was measured by continued incubation in cell-free medium, it was found that 5′-nucleotidase lost from 3 to 20% of its activity in 24 h while the released ATPase averaged a catalytic loss of about 33% in the same period. Therefore, while the ATPases were somewhat more labile than the 5′-nucleotidases, the 2- to 13-fold enrichment of 5′-nucleotidase in the released microvesicles suggested a conservation of plasma membrane ecto-ATPases.

The release of 5′-nucleotidase activity into 24-h superfusates ranged from 2 to 70% of measured monolayer ecto-5′-nucleotidase activity and it was characteristic for a particular cell line and passage number. With increasing passage number, ecto-5′-nucleotidase/ecto-ATPase activity ratios changed in several cell lines and the amount of enzymes released into superfusates also changed. While duplication was satisfactory when measurements were made within a few days or within a few passages, comparisons made several months apart were not amenable to statistical treatment. The results diplayed in Table II on the release of 5′-nucleotidase from a variety of cell lines should be viewed as representative. Release of the enzyme was found to be low from the NB-41A3 mouse neuroblastoma clone and highest in a primary culture derived from neonatal mouse astroblasts (D-34). Only in superfusates from mouse melanoma B-16 was there no measurable enzyme activity released into superfusates, but there was also no detectable ecto-5′-nucleotidase in the monolayer cultures. The rate of enzyme accumulation in the superfusates was linear with time in low density cultures but increased somewhat when cell density was high as shown for two separate duplicate experiments on the

TABLE I

SPECIFIC ACTIVITIES OF MICROVESICLE 5′-NUCLEOTIDASE AND ATPASE COMPARED TO THE ECTO-ENZYME ACTIVITIES OF THE PARENT MONOLAYER CULTURES

Values expressed as nmol AMP or ATP hydrolyzed · min^{-1} · mg^{-1} protein of monolayer culture or isolated vesicles, presented as mean ± S.D. *n* is number of experiments performed in triplicate.

Cell line	Passage number	(*n*)	5′Nucleotidase		Ratio (1)/(2)	ATPase		Ratio (3)/(4)
			(1) Microvesicles	(2) Monolayer		(3) Microvesicles	(4) Monolayer	
Rat glioma (C-6)	40 and 41	(4)	611 ± 114	109 ± 16	5.6	5.7 ± 4.2	5.1 ± 0.6	1.1
Rat glioma (C-6)	157 and 158	(4)	7.6 ± 2.9	1.95 ± 0.2	3.9	2.5 ± 0.4	8.5 ± 0.5	0.3
Mouse neuroblastoma (NB-41A3)	63 and 64	(4)	31.7 ± 3.8	12.0 ± 1.5	2.6	30.7 ± 4.6	21.4 ± 5.1	1.4
Mouse neuroblastoma (N-18)	44 and 45	(3)	32.4 ± 3.3	4.4 ± 2.1	7.4	50.4 ± 9.1	25.5 ± 7.7	2.0

rat glioma cell line (Fig. 1). The rate of ATPase accumulation (not shown in Fig. 1) was very similar to that obtained with 5′-nucleotidase. The C-6 glioma culture generally exhibits a high ecto-5′-nucleotidase activity at low passage but the specific activity of the ecto-enzyme does not change substantially over a 30-h period (Fig. 1). The rate of enzyme liberation was not changed significantly by modification of fetal calf serum concentration in the medium (0 to 20%) or by the addition of 0.5% trypsin to the medium. The release of 5′-nucleotidase activity into superfusates was altered by several compounds; in C-6 glioma cultures the extrusion of enzyme was inhibited by 93 ± 3% in the presence of 10^{-6} M concanavalin A. With 10^{-5} M cycloheximide, inhibition was 32 ± 24% over a 24-h period. An increase of enzyme extrusion was found in the presence of 10^{-6} M colchicine (141 ± 35% over control) or when the medium contained 0.5 $\mu g \cdot ml^{-1}$ of cytochalasin B (95 ± 43% over control).

Filtration of superfusates showed that from 97 to 99% of 5′-nucleotidase activity was retained on 0.22 μm filters while about 80% passed through an 0.45 μm filter. The released enzyme activity was particulate and the particles could also be harvested by centrifugation. In Fig. 2, we show residual medium ATPase and 5′-nucleotidase after subjecting superfusate from glioma cultures (C-6) to increasing centrifugal forces. Cellular debris and unattached cells sedimented at or below $5 \cdot 10^3\ g \cdot h$ (Sorvall SS-34 rotor at $10^4 \times g$ for 0.5 h). The particulate enzymes contained in those supernates could be collected by centrifugation at high speeds. For routine collections of extruded enzyme, the Sorvall supernates were centrifuged for 90 min in a Spinco Ti-70 rotor at 310 000 × *g*. The small gelatinous pellet could be removed in toto or resuspended in buffer. ATPase activity sedimented at a faster rate than 5′-nucleotidase which indicated that the particle population was not homogeneous. Electronmicroscopy after fixation of the pellets in buffered glutaraldehyde revealed two populations of vesicles, one of which consisted of irregularly shaped vesicles approximately 500 to 1 000 nm in diameter. Contained within those vesicles was another population of smaller, spherical vesicles with an average size of about 40 nm (Fig. 3).

Conceivably, the vesicles were fragments from dying or lysed cells, but the liberation of as much as 70% of its 5′-nucleotidase activity from a healthy monolayer culture in 24 h would result in the accumulation of many other subcellular fragments if that were the case. Analysis of a representative high speed

TABLE II

EXTRUSION OF ECTO-5′-NUCLEOTIDASE FROM MONOLAYER CULTURES AS MEMBRANOUS VESICLES

The activity of monolayer cultures is expressed as nmol AMP hydrolyzed · min^{-1} · mg^{-1} protein. The activity of conditioned medium represents total enzyme of extruded vesicles accumulated in superfusate during 24 h from the respective monolayer culture; vesicle specific activity was calculated as nmol AMP hydrolyzed · min^{-1} · mg^{-1} protein, using the protein value of the cell monolayer.

Cell line	Passage number	Ecto-5′-nucleotidase of monolayer	5′-Nucleotidase of conditioned medium
Mouse neuroblastoma (N-18)	33	3.9	0.95
Mouse neuroblastoma (NB-41A3)	83	7.0	0.05
Rat glioma	40	88.7	11.0
(C-6)	73	29.6	1.02
Mouse astroblast	31	2.47	1.67
(D-34)	41	10.3	7.0
Human melanoma (CL)	4	16.0	1.1
Mouse melanoma (B-16)		0	0
Human fibroblast (KIN)	11	60.2	1.33
Human fibroblast (AG-1437)	9	162	65.5 *

* Vesicles extruded during 50 h.

pellet of 6.5 mg protein from rat glioma superfusates yielded 5′-nucleotidase activity of 1.003 μmol AMP hydrolyzed · min^{-1} · mg^{-1} protein, while marker enzymes for other subcellular particles were virtually absent. Activities of glucose-6-phosphatase (EC 3.1.3.9), cytochrome *c* oxidase (EC 1.9.3.1) and *N*-acetylhexosaminidase (EC 3.2.1.52) were nil and (Na^+, K^+)-ATPase (EC 3.6.1.3) was low (25 nmol · min^{-1} · mg^{-1} protein). The 5′-nucleotidase/LDH ratio in C-6 conditioned medium was several fold higher than in cell homogenates and there was no DNA detectable in sedimented vesicles. A comparison of the optimal requirements for divalent cations of the

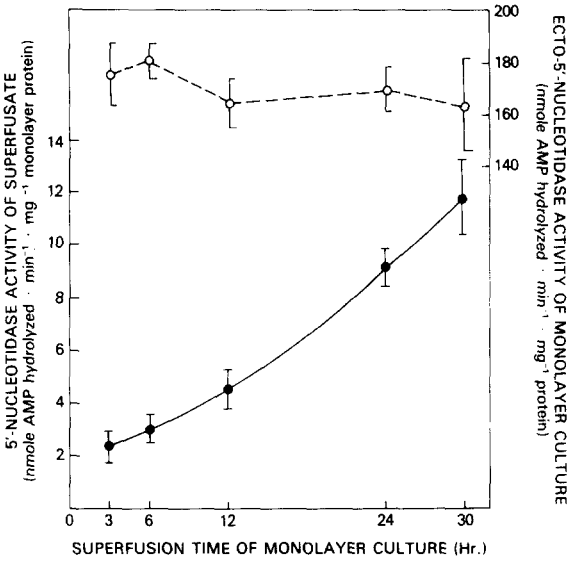

Fig. 1. Accumulation of microvesicular 5′-nucleotidase in superfusates of C-6 rat glioma monolayer cultures as a function of time (●——●) and specific ecto-5′-nucleotidase activity of parent culture (○- - - - -○). Average of two duplicate experiments; bars = S.D. Average doubling time of cultures was 27 h.

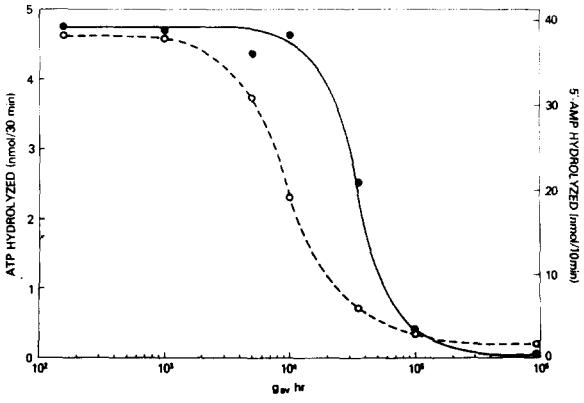

Fig. 2. Sedimentation pattern of particles obtained from confluent C-6 rat glioma cultures. Culture superfusate was subjected to centrifugation at increasing *g* forces and the resultant supernates assayed for remaining 5′-nucleotidase (●——●) and ATPase (○- - - - -○).

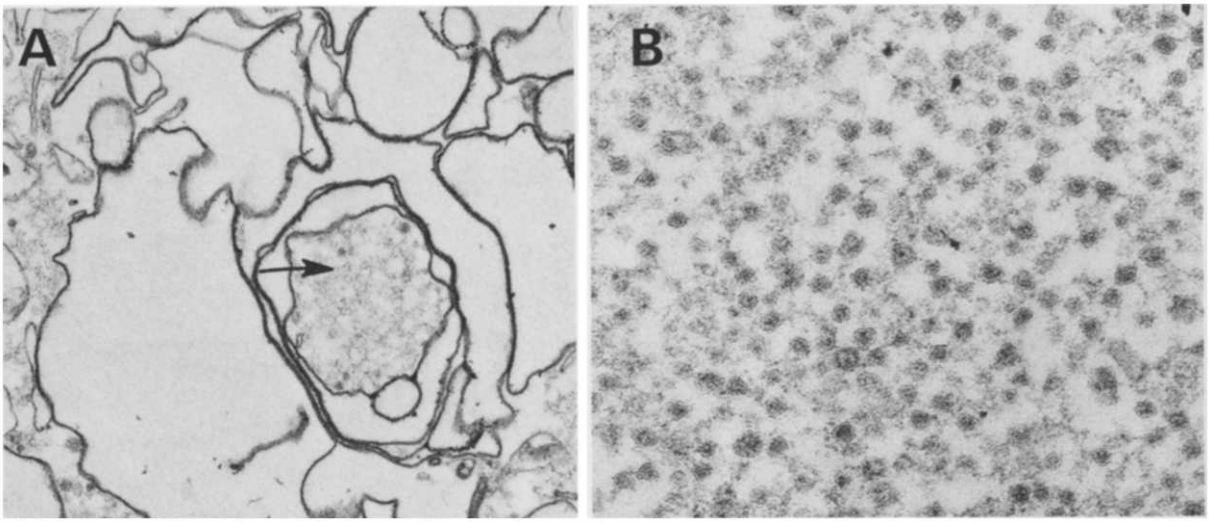

Fig. 3. A. Electron photomicrograph of vesicular particles sedimented from superfusate of C-6 rat glioma monolayer cultures. Particles in conditioned medium. (magnification X 33 600). Note smaller vesicles contained within the larger vesicles (arrow). B. Small vesicle population at greater magnification (glutaraldehyde fixed, magnification X 78 400).

released ATPase showed that stimulating and inhibitory concentrations of Mg^{2+}, Ca^{2+} and Mn^{2+} were identical with those required for the respective monolayer ecto-ATPase. Ecto-5′-nucleotidases have a high binding affinity for concanavalin A and about 70% of the nucleotidase activity of C-6 conditioned media was retained by a Sepharose-4G-Con A column, suggesting also a similarity between the ecto-enzyme and the released enzyme. Analysis of vesicle pellets from glioma superfusates disclosed an RNA content of about 5% and lipid content of 30 to 40%. Two-dimensional TLC of vesicle phospholipids [4] gave a pattern which was different from that of lipid extracts of whole cells and from plasma membrane preparations in which 5′-nucleotidase was enriched about 8-fold (Table III). The vesicles contained significantly increased amounts of sphingomyelin and decreased phosphatidylinositol. Comparison of total lipid fatty acid composition of whole cells with vesicles showed that the latter contained increased palmitic acid and total polyunsaturated fatty acids and decreased oleic acid. These compositional differences were further evidence that the exfoliated vesicles had not been derived from lysed cells.

That the vesicles had been derived from the plasma membrane of the respective monolayer cell lines was suggested by the observation that the specific activities of microvesicle and monolayer enzymes were roughly of the same order of magnitude (Table I). Both 5′-nucleotidase and ATPase are classical plasma membrane marker enzymes, but the conservation of ATPase in the exfoliative process strongly suggests that the microvesicles were derived from specific domains of the plasma membrane. Another plasma membrane marker G_{M1} (as measured by cholera toxin binding) was not conserved (Salem, N., Lauter, C.J. and Trams, E.G., unpublished results). This may indicate, that ecto-5′-nucleotidase and ecto-ATPase do not serve an interdependent function on the cell surface, as for instance in the catabolism of translocated cytoplasmic ATP [2].

The morphologic similarity of the extruded vesicles to synaptosomal preparations suggested a possible transport function for them. Cells transfer substances to target cells in order to support discrete functions and examples of trophic substances are fibroblast- or nerve growth-factors [7,8].

Our working hypothesis was that one or more of the ecto-phosphoester hydrolases might play a role in a recognition and/or transport process. For instance, the carbohydrate moiety of ecto-5′-nucleotidase might serve as an address which was recognized by a recipient cell and the catalytic moiety of the enzyme would serve to dephosphorylate a receptor constitu-

TABLE III

PHOSPHOLIPID AND TOTAL LIPID FATTY ACID DISTRIBUTION OF C-6 GLIOMA CELLS, PURIFIED MEMBRANE FRACTION AND EXTRUDED MICROVESICLES

Phospholipid composition as percentage of total phospholipid. Fatty acid distribution (from two experiments) as percentage of total peak area. Values are presented as mean ± S.D.

	Whole cells (n = 4)	Membranes (n = 2)	Microvesicles (n = 3)
Phospholipids			
Phosphatidylcholine	37.4 ± 4.2	28.4 ± 4.0	30.4 ± 0.9
Phosphatidylethanolamine	31.2 ± 2.4	37.3 ± 3.5	27.5 ± 4.3
Phosphatidylserine	7.3 ± 1.3	11.1 ± 0.3	10.7 ± 2.5
Phosphatidylinositol	7.9 ± 0.4	7.4 ± 1.6	2.4 ± 0.2
Sphingomyelin	11.5 ± 1.4	12.9 ± 0.7	26.1 ± 3.9
Cardiolipin	2.9 ± 1.1	2.1 ± 0.5	0.4
Phosphatidic acid	1.0 ± 0.6	0.3	0.3
Fatty acid composition of total lipid extracts			
14 : 0	1.12 ± 0.08	not determined	4.14 ± 2.94
16 : 0	15.96 ± 0.09		23.61 ± 1.03
16 : 1	8.0 ± 0.25		6.86 ± 0.11
18 : 0	12.56 ± 0.64		12.50 ± 0.75
18 : 1	31.61 ± 1.76		23.79 ± 4.16
20 : 4	3.36 ± 0.06		3.22 ± 0.13
20 : 5	0.58 ± 0.04		0.65 ± 0.44
22 : 1	0.93 ± 0		1.99 ± 0.37
22 : 4	0.87 ± 0.04		1.66 ± 0.35
22 : 5	2.55 ± 0.25		4.22 ± 1.58
22 : 6	0.72 ± 0.01		1.98 ± 1.36
22 : polyunsaturated	4.14 ± 0.21		7.86 ± 0.13
Others	21.75 ± 0.39		15.49 ± 0.08

ent and thereby facilitate a transfer mechanism between vesicle and cell. To test this hypothesis, mouse neuroblastoma cells (N-18) were incubated with $^{32}P_i$-containing medium with the intent to label cell surface phosphorous-containing compounds. After removal of the isotopic incubation medium, the N-18 cultures were first washed with unlabeled medium and then vesicle suspensions harvested from C-6 glioma conditioned medium were added; normal culture medium served as a control. There was a significant increase in ^{32}P release into the medium (over background ^{32}P diffusion from the cells) when glia-derived vesicles were in contact with the neuroblastoma monolayer cultures (Table IV). In another experiment, ^{32}P-prelabeled C-6 cultures were superfused with either C-6 or with N-18 vesicles. There was a larger release of ^{32}P when glioma cells were incubated with N-18 derived vesicles than when they were incubated with homologous vesicles which suggested that there were either quantitative or qualitative differences between the two experiments. We have no evidence at present to show that the increases of ^{32}P release in the presence of the vesicles was due only to dephosphorylation of cell surface constituents, but the experiments indicate that some interaction between the monolayer cells and the vesicles had taken place.

Because the release of microvesicles occurred in all cell lines which we have studied so far, we conducted some preliminary tests for their presence in the circulation. Plasma levels of 5′-nucleotidase may be elevated significantly in several diseases [9,10] and the enzyme might normally or pathologically be derived from plasma membranes. We assumed that the presence of such vesicles would be recognizable by their enzyme activity after filtration or centrifugation of blood plasma. We assayed heparinized blood from 16 randomly selected patients and found plasma 5′-nu-

TABLE IV

DEPHOSPHORYLATION OF ^{32}P-PRELABELED CELLS BY VESICLES OR CONDITIONED MEDIA

C-6 rat glioma cells (P 39) and N-18 mouse neuroblastoma (P 57) were incubated with $^{32}P_i$-enriched medium until cultures were confluent. $^{32}P_i$-medium was removed and cells incubated 60 min with DMEM containing 10% Fetal calf serum ('Control medium') to lower diffusable $^{32}P_i$ content. Medium was removed and cells once more were superfused with fresh medium for 10 min. Medium was removed and cells (in 9.6 cm^2 Linbro trays) were superfused for 0.5 min with the respective media shown above. Vesicles had been prepared by sedimentation and washing with medium. Values are means of three experiments

Medium superfused onto monolayers	Inorganic ^{32}P released into superfusate (cpm/ml ± S.D.)
	From N-18 neuroblastoma monolayers
Control medium	5 012 ± 451
C-6 vesicles suspended in control medium	8 433 ± 630
	From C-6 glioma monolayers
Medium conditioned on C-6 cells	2 766 ± 145
Medium conditioned on N-18 cells	14 062 ± 1200

cleotidase activities ranging from 3.4 to 26 nmol AMP hydrolyzed · min^{-1} · ml^{-1} plasma. Only a minor fraction of that activity was sedimentable, however, or retained on Millipore filters and there is at present no firm evidence that plasma membrane derived microvesicles are present in the circulation.

Discussion

Our observations suggest that exfoliation of membranous vesicles might occur in many different normal and neoplastic cells. The accumulation of as much as 70% of plasma membrane 5'-nucleotidase in microvesicular form in the medium over a 24-h period suggests a fairly high membrane turnover. This is not extraordinary, because it has been calculated that macrophages and L-cells were capable of interiorizing the equivalent of their cell surface every 33 and 125 min, respectively [11]. Replacement of apical plasma membrane in the lactating mammary gland requires formidable capapcity for membrane synthesis [12] and replacement of exfoliated membrane is a requirement that presumably is easily met by most cells. We have presented evidence that the microvesicles harvested from tissue culture superfusates were not mere fragments from the cytolysis of moribund cells. The preferential release of plasma membrane ecto-5'-nucleotidase over ecto-ATPase furthermore suggests that the exfoliative process was selective and that the microvesicles consisted of specific domains of the plasma membrane. The substantial enrichment of sphingomyelin in the microvesicular fraction supports this contention. A similar finding of increased sphingomyelin in extracellular membranous vesicles associated with a murine ascitic leukemia was reported by Van Blitterswijk et al. [13]. Microvillous membrane accumulation in media of cultured chick embryo intestines was observed recently by Black et al. [14] and extracellular membrane-invested vesicles have been described by Anderson [15]. The latter particles appear to play a role in mineralization processes and they have been referred to as matrix vesicles. Their size ranged from 300 to 1 000 nm and it was postulated that they were derived from the plasma membrane of chondrocytes by budding [15]. Their lipid composition was very similar to that of chondrocyte plasma membrane [16] and similar to the lipid composition of the vesicles which we have collected from rat glioma cultures. The electronmicroscopic images of the particles from our rat glioma culture superfusates suggest that the larger membranes were of plasmalemma origin. The smaller population has some similarities to vesicles purified from pig brain [17] or from calf, rat and rabbit brain [18], while some of the more densely shadowed small vesicles resemble C-type virus particles (Todaro, G., personal communication).

The dephosphorylation, presumably of monolayer cell surface components by microvesicle ecto-phosphoesterhydrolases, suggested an interaction between vesicles and cells. We also have recently found that isotopically labeled constituents of the microvesicles can be transfered to recipient cells (Trams, E.G., Lauter, C.J. and Salem, N., unpublished results) and the question must be asked if the shedding of microvesicles and their interaction with a target cell or target organ represents a physiologic phenomenon that takes place in vivo? Inter-cellular transfer of a quantum of material by means of vesicles has been

recognized in neurochemical transmission and there is evidence that metabolic cooperation by packaged transfer of substances may occur elsewhere, such as the transport of macromolecules between glia and neurons [19–21]. It is also conceivable that the vesicle in part or in toto can be incorporated into a recipient cell, thereby producing a modification of the host cell. Such an effect was observed when exfoliated vesicles from a B-16 mouse melanoma subline were fused experimentally with cells from another B-16 subline [22]. Attempts are made currently in several laboratories to design packaged substances for targeted therapeutic use. As an example, liposomes are provided with an organ-specific address [23] and it is hoped that such models will find application, for instance in the treatment of metabolic dystrophies by enzyme replacement. Conceivably, the physiologic distribution of some cellular products between cells or organs is achieved in a similar way, i.e. they are packaged and provided with an address, rather than simply diffused through extracellular fluid compartments. The inter-cellular transport of some trophic substances or nutrients might involve such vehicles as the microvesicles which have been harvested from cell culture superfusates. In a preliminary report we have suggested that such plasma membrane derived vesicles could be referred to generically as exosomes [24].

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