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Characterization of vesicles, containing an acylated oligopeptide, released by human colon adenocarcinoma cells

NMR and biochemical studies

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RNA-containing vesicles, recovered from the supernatant of high-density cell samples of human colon carcinoma, produce a high-resolution ^1H NMR spectrum of lipids characterized by isotropic tumbling; these vesicles contain large amounts of triglycerides and cholesterol esters. Both findings have strict analogies to what is displayed by the proteolipid complexes isolated from the sera of tumor-bearing patients [(1985) Proc. Natl. Acad. Sci. USA 82, 3455–3459; (1986) FEBS Lett. 203, 164–168]. Lipid analysis and enzymatic tests indicate that these vesicles are selected micromaps of plasma membranes, analogous to those that can be recovered from culture media in which tumor cells are grown [(1985) Dev. Biol. 3, 33–57]. Peculiar lipids, an acylated oligopeptide and a modified phospholipid, are also present in the vesicles.

Vesicle shedding; Lipid analysis; NMR; (Tumor cell)

1. INTRODUCTION

The well-assessed phenomenon of vesicle shedding from tumor cells [1] deserves much attention mainly in relation to the observation that similar membrane vesicles [2] or RNA-proteolipid complexes [3–5] circulate in the blood of patients with cancer. Our previous work [6–8] has demonstrated that RNA is present in vesicles released from the plasma membrane of human colon carcinoma cells, namely HCT-8R [9]. Preliminary experiments [10] indicate that the same structures are released from LS-174T cells derived from the same tumor [11].

Here, the lipid composition of the structures released by HCT-8R cells has been analyzed in great detail with the aim of their further characterization. According to current knowledge on vesicle shedding [12–14], they consist of selected micromaps of plasma membrane; furthermore, they contain large amounts of triglycerides and cholesterol esters. Two uncommon lipids have also been found. The first is an acylated oligopeptide, not related to the known classes of acylated proteins of eukaryotic cells. The second is a phospholipid metabolically related to the former. These findings are discussed in view of the analogies with the complexes found in the sera [2–5] and those isolated from the culture media of cells [1].

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Abbreviations: PL, phospholipids; PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; $\overline{PS}$, modified phosphatidylserine; PE, phosphatidylethanolamine; TG, triglycerides; LPC, lysophosphatidylcholine; VC, vesicles from cells; VM, vesicles from cytoplasm-deprived cells; VDC, vesicles from exhausted cells; VRC, vesicles from partially restored cells; HPTLC, high-performance thin-layer chromatography; TLC/GLC, thin-layer chromatography/gas-liquid chromatography

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2. MATERIALS AND METHODS

HCT-8R cells, from human adenocarcinoma [9], were grown as described [6,7]. Confluent cells (3×10^8 per experiment) were detached with 3 mM EDTA, washed and suspended in 1 ml of 150 mM NaCl to achieve vesicle release [7].

Cytoplasm-deprived cells were prepared from confluent cells upon freezing at −80°C for 60 min. The lysed cells were then centrifuged at $800 \times g$, extensively washed with 150 mM NaCl and finally resuspended in 150 mM NaCl for vesicle release.

Plasma membranes were isolated according to Lerner et al. [13] with minor modifications.

Enzymatic tests and protein determination were performed as in [7].

Lipids were extracted from lyophilized supernatants using chloroform/methanol (2:1, v/v) [15]. The fatty acid composition of the major phospholipid classes was determined by TLC/GLC [16]. Methyl esters of all the phospholipid classes were prepared by acid-catalyzed transesterification as follows: 1 ml of 3 N methanolic HCl (Supelco, Bellafonte, PA) was added and the tubes were left to stand for 18 h at 50°C, then 1 ml of water and the fatty acid methyl esters were extracted twice with 3 ml hexane/diethyl ether (1:1, v/v).

Quantitative determination of phospholipid classes was performed by densitometric scanning of HPTLC plates [17], based on molybdenum reagent for lipid phosphorus. The identity of the bands individuated by one-dimensional TLC has been confirmed by two-dimensional TLC with specific reagents for phosphorus, amino groups, carbohydrates and sialic acid residues, as described by Christie [18].

Cholesterol present in the lipid extracts was recovered from TLC scrapings of the band moving with the solvent front. Its quantitative determination was performed on an isopropanol solution by using a commercial enzymatic kit (Boehringer, Biochemio Robin, Milan).

The fatty acid composition and quantitative estimation of triacylglycerols were determined by TLC-GLC, as described by Christie [19].

The new lipid compound, not containing phosphorus, was quantitated on the basis of its fatty acid content.

Amino acid analysis of the new compound was carried out, after hydrolysis with 6 N HCl at 105°C overnight [20], by HPLC (Waters, Manual no.CU85999, Milford, MA).

^{1}H NMR spectra were recorded on a Bruker AM 400 spectrometer, at 400 MHz. 20 mM EDTA in $2H_2O$ was added before measurements. The water signal was partially suppressed by gated irradiation of its proton signal. Chemical shifts are referred to external DSS.

3. RESULTS AND DISCUSSION

The supernatant of HCT-8R cells shows narrow lipid resonances in the ^{1}H NMR spectrum (fig.1: a, partially relaxed spectrum to minimize the lactate signal; b, fully relaxed spectrum). These signals are analogous to those attributed to acyl chains of neutral lipids of a proteolipid complex isolated from the sera of tumor-bearing patients [5] and to those originating from the plasma membrane of

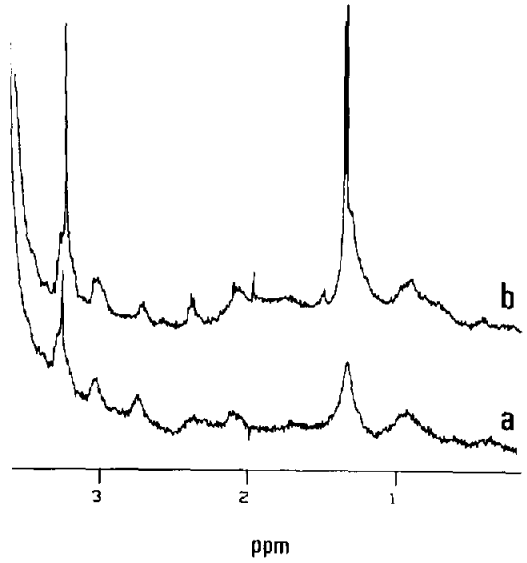

Fig.1. (a) ^{1}H NMR spectra of vesicles released by intact HCT-8R cells at room temperature by using an inversion recovery sequence to minimize the lactate signal; (b) fully relaxed spectrum.

tumor cells [6,21]. Therefore, we attribute the signals in fig.1a to lipids present in vesicles released by cancer cells. A contribution of phospholipids, besides neutral lipids, to the ^{1}H NMR spectrum is suggested by ^{31}P NMR: a phosphodiester signal around −0.5 ppm from H_3PO_4 is always present in the spectrum of this sample and is produced, to an extent of at least 30%, by phospholipid molecules [7].

The lipid composition of vesicles released by intact cells (VC) is reported in table 1. In agreement with data on the complex isolated by Mountford et al. [5] from sera of tumor patients, large amounts of triglycerides (TG) have been found. The phospholipid (PL) content of the vesicles is only slightly affected by cytoplasm deprivation of cells (VM) (table 1). Similar cholesterol (Ch) to lipid ratios were observed for VC and VM samples (table 1). The release process appeared to reach completion at 40 min as the new saline solution replaced after this time (VDC) did not contain lipids (table 1). However, these exhausted cells were able to restore partially the shedding process if allowed to recover metabolism and pH neutrality by maintaining them in serum-free culture medium continuously bubbled with 5% CO_2 in air (VRC,

Table 1

Lipid content of vesicles released at room temperature by HCT-8R cells (VC)

	VC[a]	VC4°	VM	VDC	VRC
PL (μg)	19.3 ± 0.5	n.d.	16.7 ± 0.5	n.d.	8.3 ± 0.5
Ch (μg)[b]	7.6 ± 1.0	n.d.	7.1 ± 1.0	n.d.	7.3 ± 1.0
TG (μg)	10.0 ± 1.0	n.d.			
Unknown lipid (μg)	1.6 ± 0.5	n.d.	1.8 ± 0.5	n.d.	1.4 ± 0.5

[a] When the cell sample was vortex-mixed during the shedding time, the amount of released material was approximately triplicate, maintaining the same relative ratios among the different molecules

[b] Ch is partially esterified

Data on vesicles released by cytoplasm-deprived cells (VM), exhausted cells (VDC), partially recovered cells (VRC) and a cell sample maintained at 4°C during the release (VC4°) are given for purposes of comparison. All data are the averages of at least three independent measurements. n.d., not detectable

table 1). When the cell suspension is held at 4°C, the release process is inhibited and no vesicles can be found in the supernatant (table 1), in agreement with previous findings on vesicles recovered from culture media [22].

Higher alkaline phosphatase activity observed in vesicle samples compared to plasma membrane (table 2) indicates that vesicles are selected domains of plasma membranes. Contamination by endoplasmic reticulum (ER) can be excluded on the basis of the lack of the ER marker NADH-cytochrome reductase in both plasma membrane and released vesicles. Similar results were reported by Lerner et al. [13] on vesicles recovered from culture medium of different tumor cells.

The plasma membrane composition (fig.2) is

Table 2

Enzymatic activities of plasma membranes (PM), vesicles released by HCT-8R cells at room temperature (VC), and vesicles released by cytoplasm-deprived cells (VM)

	PM	VC[a]	VM[a]
NADH-cytochrome reductase	0	0	0
Alkaline phosphatase	1.0	7.7	8.6

[a] From [7]

Activities are related to mg proteins

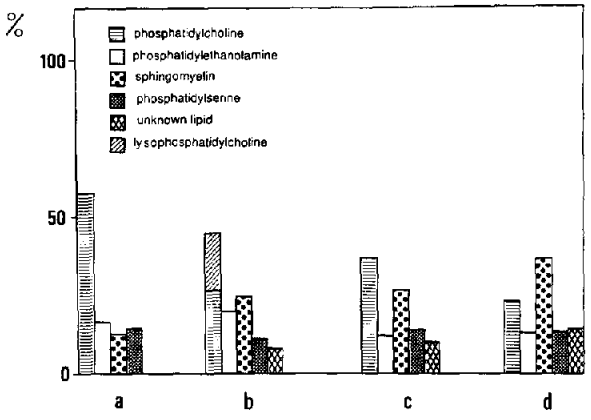

Fig.2. Phospholipid composition of (a) isolated plasma membranes of HCT-8R cells; (b) VC; (c) VM; (d) VRC. Phosphatidylserine is PS in b–d.

characterized by a high sphingomyelin (SM) content, in agreement with published data on the lipid composition of plasma membranes from eukaryotic cells [23]. The PL composition of vesicles released by intact cells (fig.2b) reveals the presence of lysophosphatidylcholine (LPC), most likely due to partial degradation of phosphatidylcholine (PC) by some phospholipase active during the release process. A higher concentration of SM is also present in these vesicles as compared to the corresponding plasma membranes. Similar results are obtained in VM (fig.2c) and VRC (fig.2d) samples. The high SM content found in the vesicles provides additional experimental evidence that they are shed by the plasma membrane as selected lipid domains with an unusual composition, very similar, in terms of lipid analysis, to that of vesicles from different tumor cells [12].

A PL fraction, with the same one-dimensional TLC mobility as that of PS or phosphatidylinositol standards, is present in all vesicle samples. This fraction has been indicated as PS in the following, but neither the specific spray reagents nor two-dimensional TLC confirmed the identity with the above-mentioned standards. Its solubility in organic solvents is very similar to that of PS. Another lipid, not containing phosphorus, detected using I_2 vapour, with the one-dimensional mobility of GM1 ganglioside, has been indicated as an unknown lipid (fig.3a). This compound does not contain carbohydrate and sialic acid residues,

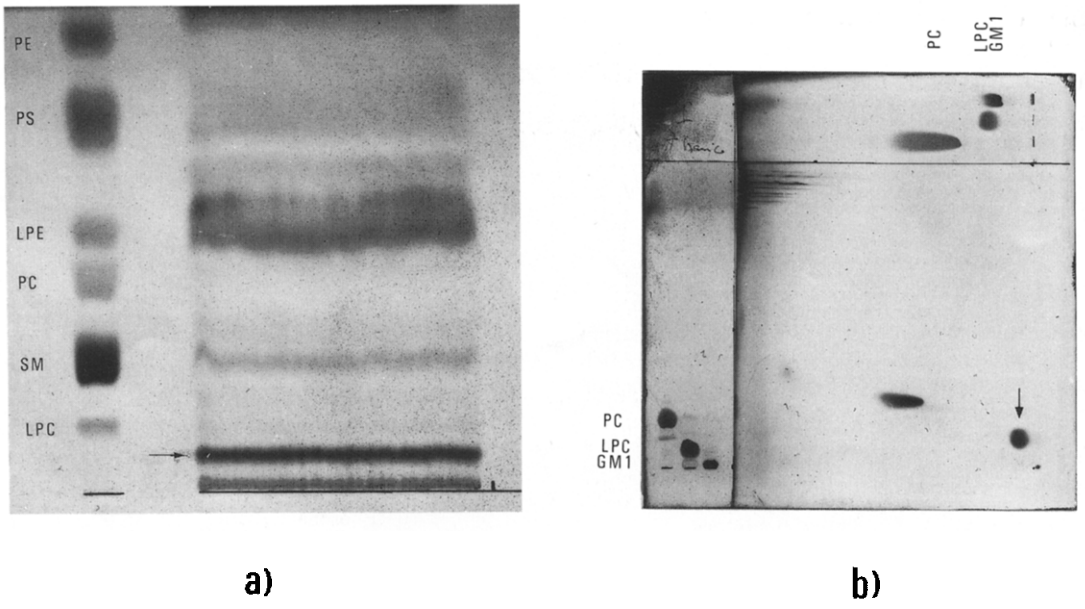

Fig.3. TLC of lipids extracted from vesicles released by HCT-8R cells (VC), detected by I_2 vapour. (a) One-dimensional TLC of all lipids; the arrow indicates the unknown lipid (due to the heterogeneous composition of the fatty acid chains, PC and PE migrate at higher rates than the standards). (b) Two-dimensional TLC of the unknown lipid; its spot is indicated by the arrow (the other spot is a phospholipid, sometimes scraped together with the unknown compound).

as shown by negative staining with the specific reagents and migrates as a single spot in two-dimensional TLC (fig.3b). The presence of a short oligopeptide chain as the head group of this peculiar molecule has been demonstrated by amino acid analysis following acid hydrolysis. Table 3 lists the amino acid composition of the oligopeptide moiety. Large quantities of the basic amino acids Lys and His were found but acidic residues are also well represented.

Preliminary experiments on $\overline{PS}$ also demonstrated the presence of amino acid residues, mainly Lys and His, in its head group. Analysis of the structure of this compound, recovered on the TLC plate, indicates that this is actually a family of closely related molecules, the study of which is currently in progress, requiring further work.

Table 4 shows the relative fatty acid composition of the phospholipids and the unknown compound.

Table 3

Amino acid composition (expressed as mol%) of the oligopeptide moiety of the unknown lipid found in the vesicles

Amino acid	mol%
Asn	4.1
Thr	2.1
Ser	5.7
Glu	9.0
Gly	3.0
Ala	4.0
Leu	10.0
Phe	6.0
His	31.0
Lys	22.0
Arg	3.4

Table 4

Fatty acid composition of vesicles released by HCT-8R cells

	a	b	c
PC	64.6	31.6	5.1
LPC	65.5	30.2	4.3
PE	70.0	26.0	4.2
SM	72.0	26.3	2.2
$\overline{PS}$	61.0	36.4	2.5
Unknown lipid	59.8	36.4	3.8

Data are averages of at least three independent measurements. (a) Saturated, (b) monosaturated, (c) polyunsaturated

SM and PE display the highest amount of saturated chains, while the strict similarities in percentages of double bonds observed for PS and the unknown lipid suggest a close relationship between their metabolic patterns.

4. CONCLUSIONS

The vesicles recovered from the supernatant of HCT-8R cell samples are selected micromaps of the plasma membrane, as demonstrated by both enzyme and lipid analysis (table 2 and fig.2). We have, therefore, demonstrated that very similar structures can be isolated from the supernatants of high-density cell samples and from cell culture media, as performed usually [12–14].

Some structural similarities exist for the vesicles described here and the proteolipid complex isolated from sera, in particular the high TG and cholesterol ester content found in the released vesicles (table 1) and serum proteolipid complex [5]. Bearing in mind that RNA is also bound to the vesicles released by HCT-8R cells [7,8], we suggest that an as yet unexplained link exists between the presence of an RNA-proteolipid complex in sera of tumor-bearing patients [5] and the very general phenomenon of vesicle shedding from tumor cells.

Another relevant result is the finding that uncommon compounds are recovered in large amounts from the vesicle sample. These compounds do not seem to be related to the known classes of acylated proteins (recent review [24]). Due to the similarities in the degree of saturation of the fatty acid chains of PS and of the new lipid, we are investigating the connections between the two molecules. This point deserves further investigation in view of a possible function of these compounds as membrane anchors, as has been suggested for many membrane phosphatidylinositol-anchored proteins [25].

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