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Release of Malignancy-related Fucopeptides from Ascites Tumour Cells in Association with Membrane Vesicles

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Abstract—*Membrane vesicles were found to be released from Landschütz ascites cells* in vivo *and* in vitro. *Vesicle fractions were highly enriched in the large fucopeptides which are a known feature of malignant cell membranes. Incubation with trypsin* in vitro *is commonly used to release these fucopeptides from tumour cells. The present results show that the enzyme acts by increasing the release of lipid vesicles enriched in these components, rather than by liberating soluble tryptic products. Thus, the parent molecules in the cell would appear to be integral membrane proteins which are insensitive to direct cleavage by trypsin.*

INTRODUCTION

THE PRESENCE in malignant cell membrane glycoproteins of increased proportions of larger, more complex glycoproteins is a phenotypic alteration closely correlated with transformation and tumourigenicity [1, 2]. The alteration stems from increased oligosaccharide branching, usually accompanied by extra sialylation. It is generally discerned by brief exposure of fucose-labelled cells to trypsin, followed by pronase degradation of released fucoproteins and fucopeptides to smaller components, which are examined by molecular size gel filtration. In an earlier study of Landschütz mouse ascites cells [3] we used this method to examine ascitic fluid from tumour mice and also supernatants from cells incubated *in vitro*. Considerable release of the large malignancy related fucopeptides was shown to take place *in vivo* into ascitic fluid. Under *in vitro* conditions, however, release appeared to be minimal unless trypsin was present. These results referred to incubation supernatants and ascitic fluid samples which had been centrifuged at 500 ***g*** and then at 11,950 ***g*** to remove cells and debris, respectively. Thus, the analyses would have included not only soluble glycopeptides but also any such components which might have been released in vesicle form.

There is much evidence that membrane vesicles are actively shed from ascites type tumours, both *in vivo* and *in vitro*. An antigen-rich vesicle fraction was described by Raz *et al.* [4, 5] for ascitic fluid of YAC tumour. Van Blitterswijk *et al.* [6, 7] found a vesicle fraction exfoliated from GRSL ascites cells to be enriched in tumour-associated antigens and also to have a high cholesterol to phospholipid ratio, indicating derivation from relatively rigid plasma membrane domains. Their additional observation that these vesicles were rich in sialic acid suggested to us that this might be reflecting selective enrichment in the large, highly sialylated glycopeptides associated with malignant cell membranes.

To test this possibility supernatants from *in vitro* incubation of Landschütz cells and ascitic fluid from mice bearing that tumour were prepared as previously [3] and then subjected to ultracentrifugation. Pellets consisting largely of membraneous vesicles were obtained and the contribution of these pellets to the molecular size profiles for the total released fucopeptides was examined. It will be shown that such vesicle pellets are highly enriched in the larger fucopeptides.

MATERIALS AND METHODS

Labelling and harvesting of cells

Landschütz ascites tumour cells, maintained in inbred Schofield albino mice by weekly passage, were metabolically labelled by intraperitoneal injection of L-[1-^{14}C]fucose (10 μCi), L-[1-^{3}H]fucose

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(40 μCi) or [1,2(*n*)-^{3}H]cholesterol (40 μCi) in 0.5 ml Dulbecco A phosphate-buffered saline, pH 7.3 (PBS) into mice bearing 7-day tumours. Two days later the tumour was collected into PBS and the suspension centrifuged for 5 min at 500 ***g***. The supernatant ascitic fluid/PBS was retained if needed and the cells washed twice in PBS with centrifugation at 500 ***g***. They were resuspended in PBS for counting and assessment of viability (trypan blue exclusion) by phase-contrast microscopy. Preparations showing less than 95% viability were discarded.

In vitro *incubation*

Cell counts were adjusted to 3–5 × 10^7/ml and equal numbers (10^9 or more cells) incubated in a shaking water bath at 37°C in either PBS alone or PBS containing 0.1 mg/ml trypsin (Sigma, twice crystallized bovine pancreas, type 111). As previously [3], incubation times were 20 min when trypsin was present or 1 h in PBS alone. Release of surface glycoproteins by trypsin is complete in approx. 10 min [1] and prolonged exposure may lead to cell damage. With PBS alone however, a 1 h incubation period was needed for release of sufficient material for analysis. Viability after incubation was always greater than 90%. After sedimentation of the cells at 500 ***g*** for 10 min the supernatants were freed from debris by centrifugation at 1950 ***g*** for 10 min. They were then either processed directly for fucopeptide analysis or ultracentrifuged for removal of crude vesicle pellets and then processed for analysis. Such samples will be designated 'whole supernatants' and 'vesicle-free supernatants', respectively.

Ascitic fluid

Ascitic fluid/PBS mixtures, obtained during collection of tumour cells from mice, were centrifuged at 1950 ***g*** for 10 min and the supernatants analysed directly or after ultracentrifugation.

Ultracentrifugation

This was at 105,000 ***g*** for 90 min at 4°C in a Sorvall OTD 65B, using a T 865 rotor. The resulting crude vesicle pellets were suspended in small volumes of PBS for analysis.

Electron microscopy

Crude vesicle pellets were rinsed in PBS and incubated for 1 h in PBS containing 2.3% glutaraldehyde. After three rinses in PBS the blocks were post-fixed in 1% Os_2O_4 (buffered with 0.1 M phosphate, pH 7.4) for 1 h. They were dehydrated and embedded in Araldite using standard procedures, sectioned and stained with 2% uranyl acetate for 1 h. The samples were examined by transmission electron microscopy (Philips, Model 210).

Processing of samples for fucopeptide analysis

Supernatants and ascitic fluid samples were dialysed, concentrated by freeze-drying, digested with pronase, re-dialysed (after protein precipitation) and freeze-dried, as described previously [3]. Crude vesicle pellets from ultracentrifugation were subjectd to pronase digestion immediately after sedimentation and treated as above. All differentially labelled samples to be compared by co-chromatography were combined before addition of pronase.

Gel chromatography

The freeze-dried samples were suspended in 1 ml volumes of appropriate elution buffer for chromatography. Molecular size gel filtration was on columns of Sephadex G-50 fine (Pharmacia) or a 2:1 mixture of Biogel P-100 (Biorad) and Sephadex G-50. Blue Dextran 2000 (Pharmacia) and phenol red were used as molecular weight markers. Ion exchange chromatography of ascitic fluid fucopeptides was carried out using columns of DEAE/Sephacel (Pharmacia). Details of column sizes, elution buffers and fraction volumes are designated in the captions to the figures.

Counting of radioactivity

Aliquots of fractions from the column were mixed with 10 ml toluene/Triton X-100 (2:1) containing 0.4% PPO and 0.05% POPOP. Radioactivity was counted in an LKB Rackbeta (Model 1217) and results expressed as percentage of total radioactivity eluting from the columns.

RESULTS

Release of fucose and cholesterol from labelled cells in pelletable form

Tumour was labelled *in vivo* with [^{14}C]fucose and [^{3}H]cholesterol and the cells collected 2 days later. Equal numbers were incubated in PBS alone and in PBS containing trypsin and supernatants prepared and ultracentrifuged as described in Materials and Methods. Small opaque ultrapellets were obtained under both incubation conditions. Counting of radioactivity in whole supernatants (1950 ***g***) and in ultrapellets therefrom (Table 1) showed that the pellets contained 10–11% of the total fucose and 83–87% of the total cholesterol in the whole supernatants. Table 1 also shows that the overall release of both labels was increased by approx. 3-fold in the presence of trypsin, this being reflected in the pellets as well as in the soluble fractions.

Ascitic fluid, collected at the same time as the labelled cells, also yielded ultrapellets containing radioactive cholesterol and fucose.

Electron microscopy showed all pellets to be largely composed of heterogeneous membrane vesicles and particles. The main vesicle populations

Table 1. Radioactive counts (dpm × 10^{-2}) *in whole supernatants and associated ultrapellet fractions from incubation of cells labelled with* [^{14}C]*fucose and* [^{3}H]*cholesterol*

Experiment	Incubation conditions: PBS alone Whole	PBS alone Pellet	PBS + trypsin Whole	PBS + trypsin Pellet	Increase due to trypsin: Whole	Increase due to trypsin: Pellet
	[^{14}C]*Fucose*					
(a)	137.8	16.8	356.9	22.8		
(b)	437.5	77.8	1162.0	175.0		
(c)	124.0	8.9	487.8	36.9	3.32-fold	3.36-fold
(d)	121.9	9.3	498.8	52.7	± 0.80 [4]*	± 1.93 [4]*
	Pellet fucose % of total = 11.2 ± 4.9 (4)*		Pellet fucose % of total = 9.90 ± 3.9 (4)*			
	[^{3}H]*Cholesterol*					
(a)	68.2	58.6	161.3	110.0		
(b)	117.6	103.5	299.3	283.5		
(c)	n.d.	24.6	n.d.	95.3	2.45-fold [2]	2.89-fold
(d)	n.d.	36.1	n.d.	109.7	(2.36;2.54)	± 0.83 [4]*
(e)	n.d.	n.d.	107.4	91.3		
	Pellet cholesterol % of total = 87.0 (2) (85.9;88.0)		Pellet cholesterol % of total = 82.7 ± 13.4 (3)*			

*Mean values ± S.D.; no. of experiments in parentheses; n.d. = not determined.

ranged in size from 50 to 1000 nm. In all three, a smaller population, poorly resolved at 80,000 magnification, was also present. These electron micrographs closely resembled those obtained by various other authors for extracellular membrane vesicles from ascites type tumours [5, 6] and from cultured rat glioma cells [8].

Fucopeptide characterization of components released in pelletable and soluble form

Cell suspensions prepared from tumour which had been differentially labelled with [^{14}C]- or [^{3}H]fucose were incubated in PBS alone or PBS containing trypsin. The supernatants were processed, either directly or after removal of ultrapelletable material, and differentially labelled samples co-chromatographed.

Typical fucopeptide profiles from Sephadex G-50 chromatography of whole supernatants are shown in Fig. 1. The leftward shift in the trypsinate profile relative to that for PBS indicates the presence of a higher proportion of large fucopeptides in the trypsin-released material. These trypsinate and PBS profiles are reminiscent of those obtained by various authors on comparing trypsin-released glycopeptides from normal and malignant cells. They also closely resemble our own results [9] for normal and CLL lymphocytes exposed to trypsin. In Landschütz ascites, as in most other tumour cell types, the earlier eluting areas of the trypsinate profile represent increased sialylation of oligosaccharide residues [3]. It should be noted here that Fig. 1 refers to fractionation on Sephadex G-50 but that

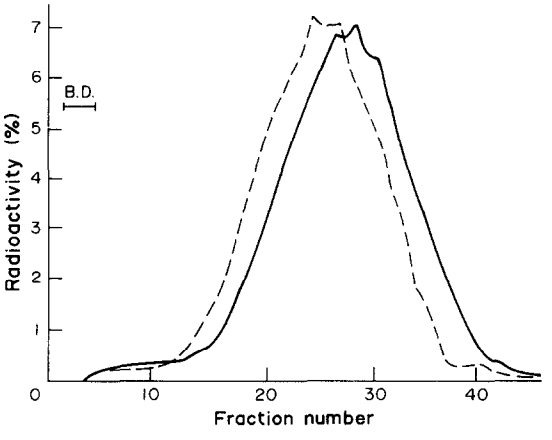

Fig. 1. Molecular size gel filtration of fucopeptides released into PBS alone (^{3}H, ———) *or containing trypsin* (^{14}C, -----). *Pronase-digested supernatants were co-chromatographed on a Sephadex G-50 column* (1.0 × 90 cm^2) *and eluted with* 0.1 M *Tris-acetate buffer, pH* 9.0, *containing* 0.1% *SDS*, 0.01% *EDTA and* 0.1% *mercaptoethanol. Fractions* (0.8 ml) *were collected and aliquots counted for radioactivity. Blue Dextran* (*BD*) *indicates void volume.*

subsequent profiles were obtained using a mixture of Biogel P-10 and Sephadex G-50 (2:1) for better resolution.

The contribution of the vesicle fractions to the fucopeptide characteristics of the whole supernatants was examined by the procedures described below. The figures shown are typical examples for several separate experiments.

(1) The crude vesicle pellet was removed from a trypsinate of [^{3}H]fucose-labelled cells and the remaining fucopeptides co-chromatographed with those of a whole trypsinate from cells labelled with

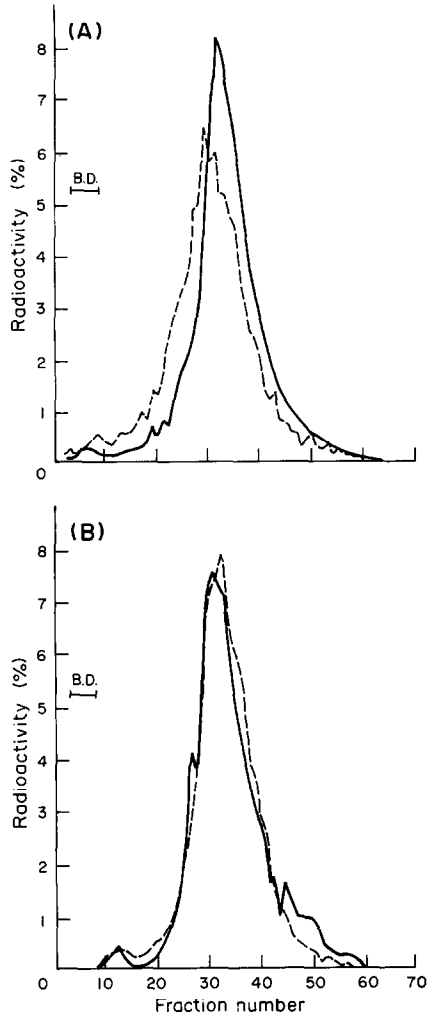

Fig. 2. Molecular size gel filtration of fucopeptides of vesicle-free supernatants on Biogel P-10/Sephadex G-50 (2:1). Columns 1.0 × 90 cm² and were eluted with 0.1 M Tris-acetate buffer, pH 9.0, containing 0.1% SDS, 0.01% EDTA and 0.1% mercaptoethanol. Fractions (0.5 ml) were collected and aliquots counted for radioactivity. A. Vesicle-free trypsinate (^{3}H, ———) co-chromatographed with whole trypsinate (^{14}C -----). B. Vesicle-free trypsinate (^{3}H, ———) co-chromatographed with vesicle-free PBS supernatant (^{14}C, -----).

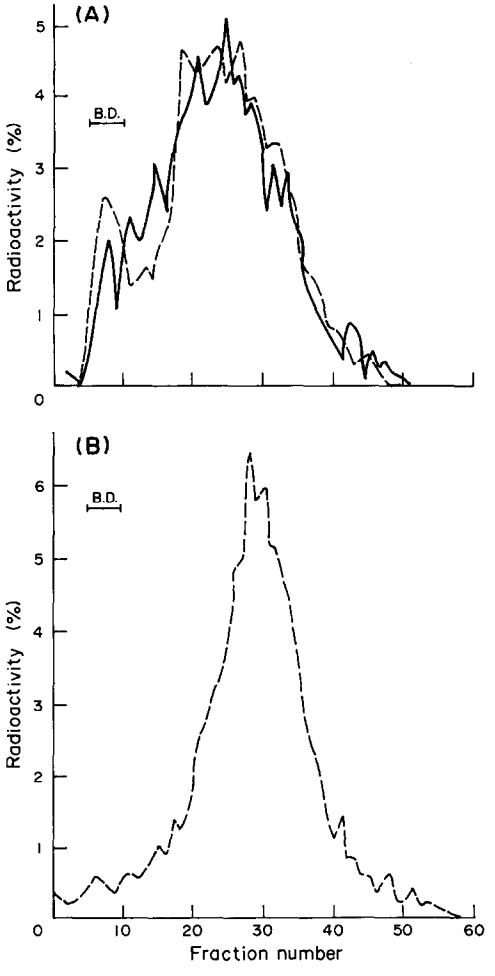

Fig. 3. Molecular size gel filtration of vesicle fucopeptides on Biogel P-10/Sephadex G-50 (2:1). Column size, elution conditions and fraction volumes as in Fig. 2. A. Co-chromatography of vesicle fucopeptides from trypsinates (^{3}H, -----) and PBS supernatants (^{14}C, ———). B. Fucopeptide profile of a whole trypsinate under the same conditions plotted for comparison.

[^{14}C]fucose. Figure 2A shows that removal of the vesicle pellet abolished the earlier-eluting character of the original trypsinate, resulting in a shift towards lower molecular size.

(2) Fucopeptides of vesicle-free trypsinate from [^{3}H]labelled cells were co-chromatographed with those of vesicle-free PBS supernatant from cells labelled with [^{14}C]fucose. The profiles were virtually identical (Fig. 2B) indicating again the important influence of the vesicle fractions on the molecular size difference observed with whole supernatants. It is also seen that while some release of larger components occurs in soluble form (fractions 12–25, Fig. 2B) under both incubation conditions, the molecular nature of these entities and of the main peak material in general is virtually unaffected by the presence of trypsin.

(3) The crude vesicle pellets from trypsinates (^{3}H) and PBS supernatants (^{14}C) were directly compared by co-chromatography (Fig. 3A). The leftward displacement of the two peaks, when compared with whole trypsinate, as illustrated below in Fig 3B, indicates that both vesicle samples are equally enriched in large fucopeptides and are of generally similar character, although some slight differences exist.

The degree of enrichment of the vesicles in high molecular weight components can be assessed from the percentage of total eluting fucose which is located in the area of interest (fractions 12–25) of the profiles. In this respect, mean values (± S.D.) for trypsin and PBS vesicles were 52.8 ± 5.6% [3] and 52.7 [2], respectively, as compared with 19.4 ± 1.0% [3] for whole trypsinates. This indicates a 2.8-fold enrichment of the vesicle fractions over the whole trypsinates.

As already shown in Table 1, vesicle pellets from

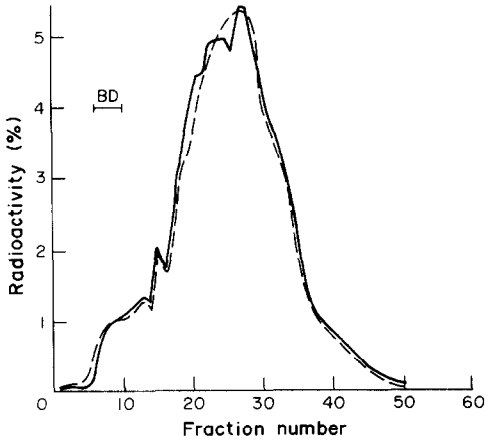

Fig. 4. Molecular size gel filtration of ascitic fluid vesicle fucopeptides on Biogel P-10/Sephadex G-50 (2:1). Column size, elution conditions and fraction volume as in Fig. 2. Fucopeptides of delipidated vesicles (^{3}H, -----) were co-chromatographed with those of non-delipidated vesicles (^{14}C, ———).

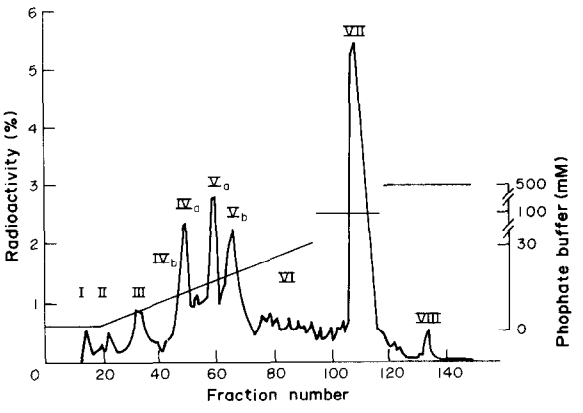

Fig. 5. Ion-exchange chromatography of fucopeptides from ascitic fluid vesicles. Samples were applied to a column (1.6 × 25 cm^2) of DEAE/Sephacel equilibrated with 2 mM phosphate buffer (pH 6.8) and eluted with 40 ml of the same buffer followed by 100 ml each of 2 mM and 30 mM buffer, 40 ml of 100 ml buffer and, finally, 60 ml of 500 ml buffer. Fractions (2.0 ml) were collected and aliquots counted for radioactivity.

trypsinates contained approximately three times more cholesterol and fucose than the PBS controls. It is now seen that vesicles from either source also show a 2.8-fold enrichment in membrane glycoproteins carrying the larger fucopeptides. Thus, the difference in PBS and trypsinate elution profiles originally observed for whole supernatants (Fig. 1) is due to increased release of vesicles in the presence of trypsin.

Crude vesicle pellets from ascitic fluid were also examined. To test the possibility that the fucopeptide samples might be contaminated with labelled fucolipid, the vesicle fraction from ^{3}H-labelled ascitic fluid was co-chromatographed with a similar (^{14}C-labelled) sample from which the bulk of glycolipid had been removed by triple extraction with chloroform/methanol (2:1 v/v). As seen in Fig. 4, this procedure did not noticeably alter the fucopeptide profile. It is also evident that a large proportion of the total radioactivity elutes between fractions 12 and 25, indicating the enrichment of these *in vivo*-released vesicles with high molecular weight fucopeptides. In contrast to the *in vitro* situation, however (*cf.* Fig. 2A), removal of the vesicle fraction did not alter the fucopeptide profile of whole ascitic fluid (data not shown). Thus, in the case of ascitic fluid both the soluble and the vesicle fractions are rich in larger fucopeptides.

The fucopeptides of ascitic fluid vesicles were further characterized by ion exchange chromatography. Figure 5 shows that they are resolved on a molecular charge basis as eight peaks. Peaks I–VII evidently represent the same species as previously observed [3] in whole ascitic fluid and whole trypsin supernatants. The vesicle fraction from ascitic fluid is, however, notably enriched in peak VII material. This comprised 5.4% of the total eluted radioactivity in Fig. 5, which is almost double the corresponding value of 2.8% observed for whole ascitic fluid or trypsinates in the earlier study. Figure 5 also shows the presence in ascitic fluid vesicle fractions of an even more highly charged fucopeptide species, peak VIII, eluting with 500 mM buffer. Thus, the vesicle fraction from ascitic fluid is enriched in components of high charge as well as high molecular size.

DISCUSSION

In an earlier paper [3] we showed that Landschütz ascites tumour cells, in common with many other tumour types, released large, highly sialylated fucopeptides on treatment with trypsin. The molecular size profiles for the total fucopeptides of trypsinates were found to differ from those of control supernatants in showing a shift towards earlier elution. These fucopeptides were also shown to be spontaneously released *in vivo* into the ascitic fluid of host mice. The main points arising from the further studies reported here are as follows:

(i) On ultracentrifugation of the supernatants from incubation of the cells in PBS alone or in PBS containing trypsin pellets were obtained which consisted largely of membraneous vesicles. Similar pellets were also obtained from ascitic fluid.

(ii) The pellets were highly enriched in the large fucopeptides associated with malignancy.

(iii) The action of trypsin in promoting release of the large fucopeptides from cells appears to be through increased release of ultrapelletable membrane material.

Our finding that membrane vesicles are spontaneously released from Landschütz ascites cells *in vitro* and *in vivo* is an agreement with the observations of Van Blitterswijk *et al.* [6, 7] and Raz *et al.*

[4, 5] on GRSL and YAC leukaemia ascites cells, respectively. Electron microscopy showed our vesicle fractions to be similar to theirs. The vesicles examined by Van Blitterswijk *et al.* [6] were enriched in sialic acid and our present results suggest that this reflects selective shedding of the complex, highly sialylated fucopeptides of malignant cell membranes in vesicle form.

There is much evidence that shedding of membrane vesicles *in vivo* may be important for tumour growth and behaviour. Exocytosed vesicles have been shown to carry factors influencing metastatic capacity [10] and to contain procoagulant activity [11]. They can also have inhibitory effects on cellular immunity [5, 12] and an explanation for this may lie in their enrichment in complex oligosaccharide moieties, as indicated in the present report. For example, triantennary glycans are known to have suppressive effects on immune function [13, 14] and a triantennary, fully sialylated component is a feature of the high molecular weight glycopeptides associated with malignancy, at least in the case of virus-transformed BHK cells [15, 16].

While there have been many studies characterizing the malignancy-related glycopeptides, little is known about their location on cell membranes or their functional significance. The alteration appears to be a general one, not confined to any one class of glycoprotein [17] and it may have a role in the impaired communication shown by cancer cells [18]. Our finding that these glycopeptides are mainly associated with the vesicle, rather than the soluble fractions of *in vitro* supernatants, supports earlier evidence [17] that they are derived from integral membrane proteins. The results using trypsin also indicate that the parent glycoproteins are relatively insensitive to direct cleavage by this enzyme. It is possible that complexity of oligosaccharide branching and increased sialylation protect these components against direct effects of exposure of the cells to trypsin [19].

The release of the malignancy-related glycopeptides from tumour cells *in vitro* is generally regarded as requiring trypsin. The present results show, however, that some spontaneous release into PBS does occur, in association with a small vesicle fraction, and that the effect of trypsin is to increase the amount of this fraction. Trypsin and other proteases are known to produce changes in the lipid bilayer [20, 21] and it is likely that the surface blebbing observed in trypsin-treated cells [22] is a precursor of vesicle formation. More recently, Kirkpatrick *et al.* [23] observed marked release of phospholipids when trypsin was used to harvest cultured monolayer cells. Their suggestion that this might represent release of membrane vesicles is supported by our present results. Ascitic fluid vesicle pellets showed fucopeptide enrichment in the same areas of high molecular weight and charge as those released *in vitro*. In ascitic fluid, however, as opposed to *in vitro* supernatants, high molecular weight fucopeptides known to have originated from the tumour cells [3] were also present to a marked extent in soluble form. The presence *in vivo* of proteases of wide specificity might be expected to bring about degradation of vesicle components by analogy with the pronase digestion used in preparing the released fucopeptides for analysis. Other factors *in vivo*, such as antigenic modulation [7] and selective hepatic clearance of non-sialylated galactose-terminating glycopeptides [24] could also effect the molecular size and charge distribution of ascitic fluid fucopeptides.

Finally, the demonstration that the vesicle fractions are specifically enriched in these fucopeptides offers a convenient way of obtaining membrane fractions containing the parent, malignancy-associated glycoproteins for further study.

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