Topographical investigations of human ovarian-carcinoma polymorphic epithelial mucin by scanning tunnelling microscopy

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Human polymorphic epithelial mucin is a high-molecular-mass glycoprotein that associates to provide protection to the epithelial-cell surface and may afford the malignant cell a selective advantage for growth. The scanning-tunnelling-microscopy micrographs obtained in the present study identify the purified human ovarian-carcinoma polymorphic epithelial mucin glycoproteins as rod-shaped molecules of mixed length. The dimensions of the individual molecules range from 25 to 45 nm in length and are 3–4 nm in width. The images further suggest that lateral association of the rods occurs.

INTRODUCTION

Human polymorphic epithelial mucins (PEM) are complex monomeric glycoproteins that are associated with human breast and ovarian carcinomas (Hilkens & Buijs, 1988; Taylor-Papadimitriou & Gendler, 1988). They have an apparent molecular mass that is in excess of 300 kDa, exhibiting a genetic polymorphism demonstrable at both the DNA and protein levels (Swallow *et al.*, 1986; Gendler *et al.*, 1988). This polymorphism is thought to arise from a variation in the number of tandem repeat units of a 20-amino-acid sequence that constitutes most of the protein content (Gendler *et al.*, 1988; Lancaster *et al.*, 1990; Ligtenberg *et al.*, 1990).

Secondary-structure predictions and hydropathicity calculations performed on the protein sequence have identified two domains in the tandem repeat. The first domain, extending over the first ten amino acids, has been predicted to be hydrophilic, containing a number of β -turns (Price *et al.*, 1990a). One of these predicted β -turns has been identified in n.m.r. studies on PEM-related peptide fragments (Tendler, 1990; Scanlon *et al.*, 1992). It is within this turn region that the determinants for the protein-specific antibodies are found (Price *et al.*, 1990a; Briggs *et al.*, 1991).

Electron-microscopy studies on PEM-related material have suggested that the molecules may be rod-shaped (Slayter & Codington, 1973), random coils (Rose *et al.*, 1984) or single non-linear extended strands (Bramwell *et al.*, 1986). In the present study we have purified and investigated the external topography of human ovarian-carcinoma PEM adsorbed to a conducting substrate using a scanning tunnelling microscope (STM). Unlike the electron microscope, the STM is able to probe the topography or surface electron density of biomolecules directly under ambient conditions (Binnig & Rohrer, 1985), with the data obtained providing structural information complementary to that from more established techniques such as n.m.r. spectroscopy and X-ray crystallography (Arscott *et al.*, 1989; Edstrom *et al.*, 1989; Davies *et al.*, 1990). The images presented here provide structural information on the intact PEM glycoprotein and its self-association.

MATERIALS AND METHODS

Purification of ovarian-carcinoma PEM

Ascitic fluid (430 ml) from an ovarian-carcinoma patient was filtered through a 120-mesh stainless-steel grid, centrifuged at 46000 *g* for 40 min and filtered through a Whatman no. 1 filter paper. The fluid was diluted 1 in 5 by addition of 2150 ml of 0.1 M-Tris/HCl, pH 7.6. An immunoadsorbent column was prepared by conjugating the murine IgG3 anti-epithelial mucin monoclonal antibody C595 (Price *et al.*, 1990b) to CNBr-activated Sepharose at 1 mg of antibody/ml of moist gel according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The diluted ascitic fluid was cycled through the immunoadsorbent column (bed volume 20 ml) at 50 ml/h for 65 h. The column was washed successively with 0.1 M-Tris/HCl, pH 7.6 (100 ml), 0.1 M-Tris/HCl containing 1 M-NaCl, pH 7.6 (50 ml) and 0.1 M-Tris/HCl, pH 7.6 (100 ml). Ovarian-carcinoma ascitic-fluid PEM was eluted from the column by the application of 0.1 M-diethylamine, pH 11.5. Fractions (2 ml) were collected into tubes each containing 0.5 ml of 1 M-Tris/HCl, pH 7.6, to neutralize the fractions.

Aliquots (10 μ l) of each fraction were dried in the wells of Terasaki microtest plates (A/S Nunc, Roskilde, Denmark). Adsorbed PEM was then detected by its capacity to bind the anti-mucin antibody C595 (added at 10 μ l/well from a 10 μ g/ml solution) using an indirect radioisotopic antiglobulin assay (Price *et al.*, 1990a). Those fractions retaining antigenic activity were pooled, dialysed against phosphate-buffered saline (8 mM- Na_2HPO_4 /15 mM- KH_2PO_4 /0.14 M-NaCl/2.7 mM-KCl, pH 7.3; PBS) and concentrated by dialysis against Aquacide II (Calbiochem, La Jolla, CA, U.S.A.). Previous studies have established that the major contaminant of preparations isolated in this way is human immunoglobulin (O'Sullivan *et al.*, 1990). This was separated from the PEM by reduction and alkylation of the preparation and application to a Sephacryl S-300 gel-filtration column (2.6 cm $\times$ 110 cm) (O'Sullivan *et al.*, 1990). Individual fractions showing antigenic activity were again identified by their capacity to react with the C595 antibody in a solid-phase

Abbreviations used: PEM, polymorphic epithelial mucins; STM, scanning tunnelling microscope; PBS, phosphate-buffered saline; ABTS, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt; HOPG, highly oriented pyrolytic graphite.

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radioisotopic-anti-globulin assay (Price *et al.*, 1990b). The final preparation of ovarian-carcinoma PEM, which eluted in the void volume of the gel-filtration column, was concentrated, dialysed against PBS and stored in aliquots at -20°C .

Normal urinary PEM was isolated using a comparable procedure, but, owing to the absence of contaminating human immunoglobulins, it was unnecessary to include a gel-filtration step (Price *et al.*, 1990b).

SDS/PAGE was performed as described by Laemmli (1970) using 7.5% acrylamide gels. After separation, proteins were either stained with 0.25% Coomassie Brilliant Blue or transferred to nitrocellulose membranes by Western blotting. The latter was performed by the method of Towbin *et al.* (1979) and the membranes were probed with the C595 antibody (20 $\mu\text{g}/\text{ml}$) as previously described (Price *et al.*, 1990b).

E.I.s.a. assays

Aliquots (50 μl) of a solution containing ovarian-carcinoma PEM (10 $\mu\text{g}/\text{ml}$ in PBS) was dispensed in the wells of Falcon 3912 Microtest III flexible plates (Becton Dickinson, Oxnard, CA, U.S.A.) and incubated overnight at 37°C . The plates were incubated with PBS containing 1% BSA for 1 h at room temperature and then washed four times with PBS containing 0.1% (v/v) Tween 20 (Sigma Chemical Co., Poole, Dorset, U.K.). Monoclonal antibodies (as hybridoma tissue-culture supernatants) were added in quadruplicate and incubated for 1 h at room temperature. The antibodies were removed and the plates washed by immersion in PBS containing 0.1% (v/v) Tween 20. Horseradish-peroxidase-linked rabbit anti-mouse immunoglobulin (Dako, High Wycombe, Bucks., U.K.) at a dilution of 1:400 (in PBS containing 1% BSA) was dispensed at 50 $\mu\text{l}/\text{well}$, incubated for 1 h at room temperature, and plates were again washed by immersion. The 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) substrate solution (0.05% ABTS in citrate phosphate buffer, pH 4.0, containing 0.01% H_2O_2) was added, and the absorbance in each well was measured at 405 nm.

STM analysis

A 5 μl portion of 0.1 μM ovarian-carcinoma PEM was deposited onto freshly cleaved highly oriented pyrolytic graphite (HOPG) (Union Carbide Corp., Cleveland, OH, U.S.A.), excess fluid was removed by blotting with filter paper after the sample had dried in air for 0.5 h. Images were recorded on a VG Microtech STM 2000 (Uckfield, Sussex, U.K.) system operating in air at ambient temperature and pressure. For the data presented, the instrument was operated in constant current (topographic) mode using a mechanically cut Pt/Ir tip, with a sample bias of 1V and a set point of 56pA. These settings select a tunnelling gap resistance of 18 G Ω and therefore a greater separation of tip and sample than is commonly used (Wilson *et al.*, 1991). In order to establish the performance of the instrument, routine images of the HOPG were obtained throughout the present study. The images presented have undergone no post-acquisition processing or data manipulation.

RESULTS AND DISCUSSION

The purified ovarian-carcinoma PEM from ascitic fluid was investigated by SDS/7.5%-PAGE and its subsequent transfer to nitrocellulose membranes by Western blotting. When immunostained with the C595 monoclonal antibody, there was a single region of stained material towards the top of the gel (Fig. 1). Close examination of 'under-stained' Western blots revealed

Fig. 1. SDS/Western-blot analysis of monoclonal antibody C595-defined antigen isolated by affinity fractionation of ascitic fluid from an ovarian-carcinoma patient

The nitrocellulose sheet was probed with C595 monoclonal antibody at 20 $\mu\text{g}/\text{ml}$ as described in the text. The position of the molecular-mass (M) markers are shown to the left of the gel.

that the high-molecular-mass band was in fact composed of two bands of similar electrophoretic mobility, an observation that is consistent with the characteristic polymorphism of these epithelial mucins. It was not possible to stain the PEM preparation with Coomassie Blue, and this has been attributed to the extensive glycosylation of these molecules (O'Sullivan *et al.*, 1990). No contaminants or fragments generated by the proteolytic degradation of the PEM were detected in the SDS/7.5%-PAGE gels (10 kDa limit of resolution). This result is in accord with previous studies which have demonstrated that the molecular size of isolated epithelial mucins, as assessed by the banding pattern of immunoblotting with antibodies, is preserved during purification (O'Sullivan *et al.*, 1990; M. R. Price, unpublished work) indicating the overall resistance to proteolysis of these extensively glycosylated glycoproteins.

A panel of murine anti-PEM monoclonal antibodies and control antibodies were examined for their reactivity with both ovarian-carcinoma PEM and normal urinary PEM. The latter was included in this study since it represents a reference preparation of PEM, the properties of which have been previously reported (Price *et al.*, 1990a,b) and against which the antibody C595 was raised. As shown in Table 1, the binding of the four control antibodies, C337, C365, C198 and 791T/36, was essentially equivalent to that obtained with the mouse myeloma P3NSO tissue-culture supernatant (which contains no mouse immunoglobulin). The four anti-PEM antibodies which define epitopes of three or four amino acid residues within the protein core of epithelial mucins (C595, NCRC-11, HMFG-1 and HMFG-2; Price *et al.*, 1990a), each reacted strongly with the two preparations of PEM. The antibody Ca1 reacted more strongly with the ovarian-carcinoma PEM as compared with its reaction with urinary PEM. This is best revealed by normalizing the signal for the binding of C595 antibody to each antigen to unity and then comparing all the other data. This being the case, the signal of 0.86 for the binding of Ca1 to ovarian-carcinoma PEM is considerably elevated in comparison with its reaction with normal urinary PEM (i.e. 0.20; Table 1). As the molecular-size phenotype of normal PEM molecules from the milk or urine of different individuals, as judged by banding patterns in the high-molecular-mass region of Western blots, is maintained in the tumour PEM (Griffiths *et al.*, 1987), this result is consistent with the observation that mucins of the malignant phenotype are frequently over-sialylated as a consequence of defective or aberrant glycosylation (Foster & Neville, 1984).

Topographical analysis of the purified ovarian-carcinoma PEM reveals that, on the HOPG surface, the glycoprotein aggregates into sheet-like structures. Fig. 2(a) shows a 350 nm $\times$

Table 1. Binding of monoclonal antibodies to epithelial mucins isolated from ovarian-carcinoma ascitic fluid and normal urine

Antibody	Isotype	Target antigen-[epitope]*	Antibody binding to mucin from:	
			Ovarian carcinoma	Urine
C595	IgG3	PEM-[RPAP]	1.104 ± 0.133 (1.00)†	1.576 ± 0.097 (1.00)
NCRC-11	IgM	PEM-[RPA]	1.524 ± 0.021 (1.38)	2.223 ± 0.085 (1.41)
HMFG-1	IgG1	PEM-[PDTR]	1.519 ± 0.030 (1.38)	1.299 ± 0.042 (0.82)
HMFG-2	IgG1	PEM-[DTR]	0.858 ± 0.085 (0.77)	1.092 ± 0.033 (0.69)
Ca 1	IgM	PEM-[X-sialic acid]	0.952 ± 0.070 (0.86)	0.313 ± 0.014 (0.20)
C337	IgG2a	CEA‡	0.025 ± 0.016 (0.02)	0.021 ± 0.004 (0.01)
C365	IgG1	CEA	0.065 ± 0.002 (0.06)	0.027 ± 0.034 (0.02)
C198	IgG1	NCA§	0.009 ± 0.013 (0.01)	0.045 ± 0.006 (0.03)
791T/36	IgG2b	Glycoprotein, gp72	0.045 ± 0.005 (0.04)	0.031 ± 0.001 (0.02)
P3NSO	—	—	0.039 ± 0.015 (0.04)	0.028 ± 0.013 (0.02)

* The epitopes for the first four antibodies are located within the mucin protein core (Price *et al.*, 1990b). The epitope for the antibody Ca1 is located within the carbohydrate side chains and involves sialic acid (Bramwell *et al.*, 1985).

† The signal for the binding of C595 antibody to each antigen was normalized to unity and all other signals have been calculated with respect to this value. All normalized data are presented in parentheses.

‡ CEA, carcinoembryonic antigen.

§ NCA, normal cross-reacting antigen.

350 nm image where one such aggregate is identified associated with three visible graphite steps. An interesting feature of this image is the 'artifactual' periodicity that is visible in the top right-hand portion of the scan. This STM-observed periodicity has been previously found on HOPG surfaces and is thought to be derived from sub-surface faults in the graphite layers (Clemmer & Beebe, 1991).

Higher-resolution 111.7 nm × 111.7 nm and 49.8 nm × 49.8 nm scans of the lower central region of the PEM aggregate are displayed in Figs. 2(b) and 2(c) respectively. The presented images show that the aggregates appear to be composed of associated rod-shaped molecules. The length of the observed individual molecules range from 25 to 45 nm. The widths of the individual PEM molecules were 3–4 nm. The packing of the PEM molecules along their long axis is such that maximum overlap of the individual PEM rods occurs.

In order to ensure that the topographical features observed were not an STM-observed HOPG artifact we attempted to move the PEM molecules on the graphite substrate. The parameters of the instrument were changed to a sample bias of 0.7 V with a set point of 500pA. This variation produces a gap resistance of 1.4 GΩ causing the piezo-ceramic crystal to attempt to move the tip closer to the HOPG surface (Lindsay *et al.*, 1990). The region displayed in Fig. 2(c) was re-scanned using these parameters, the instrument was then re-set to the original parameters and the same region re-imaged, as shown in Fig. 2(d). No complete molecules were observed on this portion of the substrate and the image quality was considerably reduced. These data confirm that the features observed were due to loosely associated molecules that appear to have been swept clear from the HOPG surface by the STM tip and not STM-observed HOPG artifacts (Salmeron *et al.*, 1990).

The bladder luminal membrane has previously been shown to contain PEM plaques of dodecameric subunits arranged in a

hexagonal lattice by the hydrophobic association of rod-like material along its long axis (Warren & Hicks, 1978). By this mechanism the PEM is thought to offer protection to the underlying epithelia of normal tissue to extremes of pH and may provide a selective advantage for tumour-cell growth (Bramwell *et al.*, 1983). The observation in the present study that the ovarian-carcinoma PEM aggregates along its long axis when deposited on the HOPG surface and not into a linear array of dodecameric subunits suggests that the hydrophobic substrate surface preferentially interacts with the maximum surface area of the purified PEM. This packing allows us to reveal the dimensions of the individual glycoprotein molecules.

Slayter & Codington (1973) have previously reported electron-microscopy studies on mucin-type glycoprotein purified from TA3-Ha cells after modified-trypsin treatment. Their investigations revealed that the PEM material is rod-shaped, with a width of 2.5 nm and a length of 20–700 nm. In a more recent electron-microscopy study, Bramwell *et al.* (1986) have reported PEM-related material to be a single non-linear extended strand with a mean length of 270 nm. Although it is difficult to compare these previous results with those presented here owing to the different PEM material and purification techniques utilized, our results appear to be in accord with those obtained by Slayter & Codington (1973). The difference in the observed length may reflect the fact that the authors cleaved the PEM from the cell with modified trypsin. In the present study the material originated from ovarian-carcinoma cells, although the process by which these molecules are released from the tumour in a soluble form is unknown (e.g. by sublethal autolysis of cell-surface-associated PEM, secretion of intracytoplasmic PEM or the release of the material from dead or dying cells). The appearance of the mucin in the study by Bramwell *et al.* (1986) may have been due to detergent-induced protein denaturation.

The protein core of PEM has been shown to be constructed of

Fig. 2. STM micrographs of the purified human ovarian-carcinoma PEM

Fig. 2(a) shows a 350 nm x 350 nm image of the PEM associated with three visible graphite steps. Higher resolution 111.7 nm x 111.7 nm and 49.8 nm x 49.8 nm micrographs of the lower central region of the PEM aggregate are displayed in Figs. 2(b) and 2(c) respectively. After bringing the tip closer to the HOPG substrate and re-scanning the region displayed in Fig. 2(c) as shown in Fig. 2(d) no complete molecules were observed, suggesting that the PEM molecules have been swept clear from the HOPG surface by the STM tip.

up to 60 tandem repeats of a 20-amino-acid sequence sandwiched between a C- and N-terminal fragment (Lancaster *et al.*, 1990; Ligtenberg *et al.*, 1990). Secondary-structure predictions and hydropathicity calculations (Price *et al.*, 1990a) have identified that the tandem repeat contains two distinct domains with the first ten residues encapsulating a hydrophilic domain. This region has been shown by n.m.r.-spectroscopy studies on PEM-related-peptide fragments to contain several elements of secondary structure, including a type I β -turn stabilized by a salt bridge (Tendler, 1990; Scanlon *et al.*, 1992). The n.m.r. studies, as well as independent computational investigations, suggest that the salt-bridge-locked type I β -turn forms a compact motif that is near the surface of the glycoprotein (Scanlon *et al.*, 1992), and indeed it is this turn region that is recognized by all of the anti-(PEM protein core) antibodies (Price *et al.*, 1990a). The finding that the intact glycoprotein is a linear rod of thickness 3–4 nm suggests that the molecule is constructed of a regularly packed

chain of salt-bridge-locked type I β -turn motifs separated by the hydrophobic second region of the PEM tandem repeat.

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