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Detection of tumor-associated membrane proteins in prostate and bladder carcinomas by means of protein blotting

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Summary. Analysis of membrane proteins by Western blotting has revealed both overexpression of proteins of molecular weight 10–200 kD (in particular, of proteins of MW less than 43 kD) and increased glycosylation in a xenografted human small cell undifferentiated prostatic carcinoma, and in two xenografted human bladder tumor cell lines compared with preparations from normal human tissue. Of potential functional significance were: a) a 43 kD protein in the bladder line, UCRU-BL-13, which demonstrated increased synthesis and a marked increase in the degree of glycosylation, and b), a 28 kD ConA-binding protein in prostatic tissue which was absent in normal tissue, present in intermediate quantity in a benign hyperplasia and greatly overexpressed in small cell carcinoma. This study demonstrates the utility of the protein blotting/autoradiography technique for the investigation of tumor membrane proteins.

Key Words: Membrane proteins – Western blotting – Bladder cancer – Prostate cancer – Lectin binding

Introduction

The identification and characterisation of membrane-localised tumor specific markers has been the subject of many investigations. In particular, lectins with different carbohydrate binding specificities are useful tools for obtaining information concerning the cell-surface phenotype of metastatic tumor cells [5, 6, 11]. Recently, we observed strong surface glycosylation of two human bladder cancer cell lines compared with normal bladder tissue [19]. In the current study, we have utilized the technique of "protein blotting" or "Western blotting" [22, 23] to analyse tumor-derived membrane proteins. In this technique, membrane proteins separated by sodium dodecyl sulphate polyacrylamide electrophoresis are electrotransferred to a nitrocellulose sheet. This sheet can be cut into several strips which can then be probed simultaneously for lectins, antibody reactions, etc. In this study, we probed the immunoblots with a panel of lectins. The technique has demonstrated clear differences between the membrane protein phenotypes of urological cancer cell lines and their normal counterparts.

Materials and methods

Preparation of single cell suspensions

Single cell suspensions of the following solid tissues were prepared as described [13] by incubating finely minced tissue in a mixture of collagenase (Sigma Chemical Co., St. Louis, Mo., USA) and DNAse (Sigma):

(a) a primary small cell undifferentiated carcinoma of the prostate (SCUCP) which had been xenografted in nude mice. The establishment and characterisation of this xenograft line, UCRU-PR-2, was described elsewhere [13, 16, 24]. The fourth serial passage of this tissue in nude mice was used, designated PR2/4.

(b) a biopsy specimen of benign hyperplasia of the prostate.

(c) normal prostate tissue and bladder transitional epithelium obtained from normal liver transplant donors.

In addition, continuous cell lines derived from human bladder transitional cell carcinoma previously established in our laboratory were harvested using 1 mM ethylene diamine tetraacetic acid (FLOW Laboratories, North Ryde) as previously described [19]. These lines were UCRU-BL-17/2 and UCRU-BL-13/0.

Isolation of crude and plasma membrane pellets

Cells were disrupted using 2% Tween-40 (Sigma Chemical Co., St. Louis, Mo., USA) [4] in the presence of the analytical grade protease inhibitors, phenylmethylsulfonylfluoride (PMSF, 1 mM, evaporated from acetone solution) and benzamidine-hydrochloride (1 mM). Cell suspensions were rotated for 24 h at 4°C and consisted of a total of

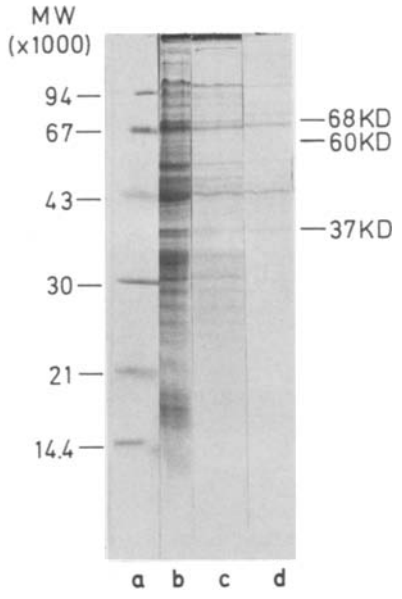

Fig. 1. Separation by SDS-PAGE of different tissue membrane preparations. Lane a: Low molecular weight markers – phosphorylase B (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (21 kD) and lysozyme (14.4 kD). Lane b: Crude membrane preparation of a xenograft of human small cell undifferentiated carcinoma of the prostate. UCRU-PR-2 (passage 4; PR2/4). Lane c: Plasma membrane preparation of PR2/4. Lane d: Crude membrane preparation of normal prostatic tissue

1.7×10^8 cells in the case of prostate tissue, or 2.7×10^7 cells from the bladder cell lines. Crude plasma membranes were prepared and purified as described by Mountford et al. [14] and Iwanik et al. [12], respectively. The membrane bands from the sucrose density gradients were removed by aspiration and diluted 1:4 with 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.2. All marker enzyme activities were assayed on membranes which had been frozen overnight at −70°C.

Marker enzymes

The following marker enzymes were assayed using previously established methods: γ-glutamyl transpeptidase [17] as plasma membrane marker; acid phosphatase [18] as the marker for lysosomes; lactate dehydrogenase [26] as a cytoplasmic marker and cytochrome c oxidase [8] as the mitochondrial marker. Protein content was measured using the Biuret method [27] with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis

The materials used for electrophoresis: acrylamide (twice crystallized), N,N'-methylene bisacrylamide (twice crystallized), ammonium persulphate, TEMED and sodium dodecyl sulphate (SDS) were from BDH (UK). Glycine, Tris (hydroxymethyl) aminomethane (Trizma base, reagent grade), and 2 mercapto-ethanol (2-ME) were obtained from Sigma Chemical Co., St. Louis, Mo. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described [7, 21] except that gels were longer (14 cm) and 9–27% gradient gels (0.75 mm thickness) rather than homogeneous gels were employed. An SE400 Sturdier Slab gel electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, CA, USA) was used with a Pharmacia ECPS 3000/150 Constant Power Supply (Uppsala, Sweden). The membrane pellets prepared from xenograft tissue or cultured cells, treated with 5% 2-ME, were subjected to electrophoresis at 200v for 15 h. Low molecular weight markers were from Bio-Rad. Gel loadings of the different membrane preparations were standardized according to cell number (not protein) and protein loadings per well were: normal bladder, 2.6 μg; UCRU-BL-17/2, 11.7 μg; UCRU-BL-13/0, 56.7 μg; normal prostate, 5 μg; benign hyperplasia 5 μg; UCRU-PR-2/4, 72 μg.

Electrophoretic transfer of proteins to nitrocellulose

Proteins were electrotransferred to nitrocellulose membrane (NC, 0.1 μm, Schleicher and Schuell, Dassel, FRG) as previously described [7, 21] using a Trans-Blot system (Bio-Rad, Richmond, CA, USA).

Probing of NC membrane strips and autoradiography

After electrophoretic transfer to NC, the NC was cut into 5 cm wide sheets in such a way that each sheet contained the transferred proteins of the different prostate and bladder cell preparations. Each sheet was incubated with one of a panel of biotinylated lectins (E-Y Laboratories, San Mateo, CA, USA) at a final solution concentration of 5 μg/ml diluted in 0.1% Tween-20 (Sigma) in phosphate buffered saline (PBS) for 2 h followed by incubation with ^{125}I-streptavidin (Lot 38,31 μCi/μg, Amersham, UK) at 1×10^6 cpm/sheet for 45 min. The lectins used were *Ulex europaeus agglutinin* (UEA), *Concanavalin A* (ConA), *soybean agglutinin* (SBA), and *Dichlorus bifloris* (DBA), which have affinity for α-L-frucose, mannose, methyl 2-acetamido-2-deoxy-2-D-galactoside and methyl 2-acetamido-2-deoxy-2-D-galactose respectively. The NC strips were washed (30 min) with 0.05% Tween-20 in PBS, air-dried at toom temperature, loaded into a Kodak X-omatic cassette together with photographic film (Fuji, Rx-safety), and stored at −70°C for 24–120 h before development.

Protein staining

Polyacrylamide gels were stained with Coomassie Brilliant Blue G-250 (CBB-G250, Bio-Rad, Richmond, CA, USA) using the enhancing method [15]. NC transfers were stained with Amido Black (Bio-Rad).

Results

SDS-PAGE of membrane proteins from prostate and bladder tissues

CBB stained protein patterns showed marked qualitative and quantitative differences between PR2/4 crude and plasma membrane and normal prostate crude membrane samples (Fig. 1). The PR2/4 crude

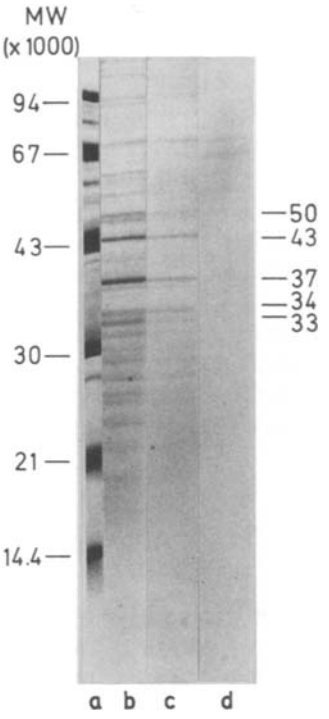

Fig. 2. Separation by SDS-PAGE of different bladder cell membrane preparations. Lane a: Low molecular weight markers (as Fig. 1). Lanes b–d: crude membrane preparations of UCRU-BL-13/0 (b), UCRU-BL-17/2 (c) and of normal bladder tissue (d), respectively

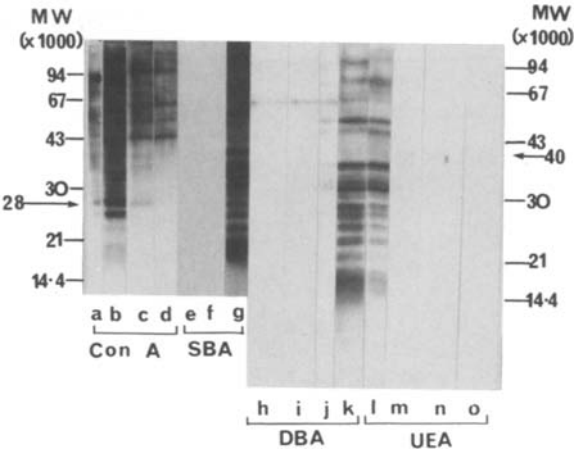

Fig. 3. Identification by autoradiography of the lectin-binding components in various prostatic tissues transferred to NC after SDS-PAGE. Lanes a–d: probed with ConA. Lanes e–g: probed with SBA. Lanes h–k: probed with DBA. Lanes l–o: probed with UEA. Lanes a, j, m: plasma membrane preparations of PR2/4. Lanes b, g, k, l: crude membrane preparations of PR2/4. Lanes c, f, i, n: crude membrane preparations of a benign hyperplasia of the prostate. Lanes d, e, h, o: crude membrane preparations of normal prostatic tissue

membrane sample contained 44 protein bands in the MW range 10–200 kD with 11 bands below 30 kD (lane b) compared with only 23 bands (3 bands below 30 kD) (lane d) in preparations of normal prostatic tissue. There was a significant degree of homology between the protein profiles from crude PR2/4 and normal prostate membrane, e.g., common protein bands at 95 kD, 67 kD, 43 kD and 37 kD. However, the quantities of these proteins were 3–5 fold greater in the tumor sample. Several bands which were present in crude membrane preparations from PR2/4 e.g., components of MWs 68 kD, 60 kD, 37 kD and several others with MW <35 kD, were absent in preparations from normal prostate tissue (see arrows, Fig. 1).

In the plasma membrane fraction derived from PR2/4, there were 31 protein bands with the most intense staining occurring the MW region 30–100 kD (lane c). The most obvious differences between the crude and plasma membrane preparations occurred in the MW region less than 30 kD, where there was much more marked staining in the crude membrane than in the purified plasma membrane sample.

SDS-PAGE analysis of crude membrane preparations of the two bladder tumour cell lines, BL17/2 and BL13/0, showed 24 and 37 bands, respectively, following enhanced CBB-staining (Fig. 2, lanes c and b respectively). The protein content of membrane preparations from the BL13/0 cell line was much higher than that of the BL17/2 on a per cell basis, as reflected in the intensity of the bands obtained. E.g., 50 kD, 43 kD, 37 kD, 34 kD and 33 k-d components (see arrows in Fig. 2).

Probing of proteins transferred to NC after SDS-PAGE for lectin-binding activity

Lectin binding had previously been assessed flow cytometrically using fluorescein labelled lectins, and indicated strong binding of the lectins, UEA, ConA, PNA, and SBA by single cell suspensions derived from the xenografted tumor, PR2/4 (results not shown). Similarly, this techniques has revealed very strong glycosylation of the surface of two bladder tumor cell lines, BL13/0 and BL17/2 [19]. For this reason, protein blots obtained from membrane preparations derived from different tumors or from normal tissues were probed with lectin probes.

Lectin binding by electrotransferred membrane proteins from prostate tissue are shown in Fig. 3. Each of 4 lectins bound with greater intensity to crude membrane proteins of PR2/4 (lanes b, g, k, l) than those of normal prostate (lanes d, e, h, o) or of a prostate sample showing benign hyperplasia (lanes c, f, i, n). The relative differences in lectin binding intensity showed the same trend as the CBB-staining differences observed in the total protein patterns shown in Fig. 1.

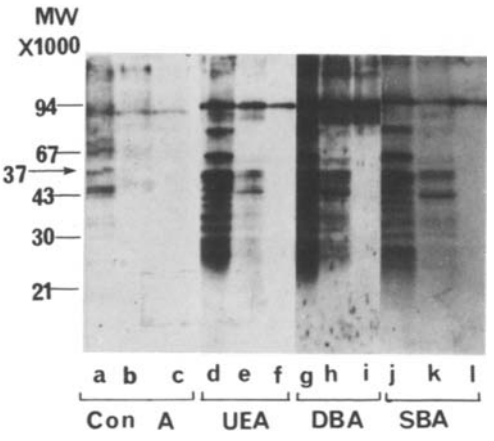

Fig. 4. Identification by autoradiography of the lectin-binding components in membrane preparations of two human bladder tumor cell lines transferred to NC after SDS-PAGE. Lanes a–c: probed with ConA. Lanes d–f: probed with UEA. Lanes g–i: probed with DBA. Lanes j–l: probed with SBA. Lanes a, d, g, j: crude membrane preparations of BL13/0. Lanes b, e, h, k: crude membrane preparations of BL17/2. Lanes c, f, i, l: crude membrane preparations of normal bladder tissue

An exception was seen in the case of SBA binding where membranes from normal prostate (lane e) failed to show any binding whereas preparations from tumor tissue showed very intense binding (lane g).

The lectin binding patterns of purified PR-2/4 plasma membrane are shown in lanes a, j, m. Poorer staining of UEA and DBA by plasma membrane, compared with crude membrane preparations indicated binding of these lectins by internal membrane components.

A ConA-binding component of MW 28 kD displayed differential expression in all 3 prostate tissue types: an absence in normal tissue (lane d), intermediate expression in a benign hyperplasia (lane c) and a large amount in PR2/4 (lane b). In addition, this component was plasma membrane-localized as indicated by its appearance in lane a. There was a striking homology in the binding patterns of SBA, DBA and UEA (and to a lesser extent, ConA) by the small cell carcinoma of the prostate, particularly in the region of MW less than 40 kD lanes g, k, l, b, respectively).

Figure 4 demonstrates the binding of lectins to protein blots derived from crude membrane fractions of normal bladder tissue and of two bladder tumor cell lines, BL17/2 and BL13/0. For certain proteins, the respective intensities of lectin binding by the proteins in both bladder cell lines closely resembled the corresponding total protein patterns (Fig. 2); e.g., a 37 kD component displayed much greater lectin binding in BL13/0 than in BL17/2 with a similar difference in the CBB-staining of this component between the two cell lines (see arrows, Fig. 4). However, in most cases, the increase in glycosylation in BL13/0 compared with that in BL17/2 was greater than the relative increase in protein staining. E.g., a 43 kD component was more highly expressed in BL13/0 than in BL17/2, and was glycosylated (as shown by lectin binding) in BL13/0, but not in BL17/2 (apart from slight, almost negligible binding by ConA). From Fig. 2, it is apparent that a significant quantity of this protein was expressed in BL17/2.

As noted with the prostatic tisue, there was a close resemblance between the binding patterns of UEA, DBA and SBA (and to a lesser extent, ConA) to membrane components of both bladder tumor cell lines.

Discussion

In this study, the use of protein blotting [22, 23] has been successfully applied to distinguish differences between preparations of membrane-derived proteins from normal and tumorigenic prostate and bladder cells. This technique has considerable advantages over the more widely used gel electrophoresis (either agarose or polyacrylamide) as the sole analytical method for identification [1–3, 10]. In protein blotting, detection only requires small quantities of reagents relative to those needed when probing electrophoretic gels, loss of resolution due to band diffusion is not a problem and NC sheets with transferred proteins can be stored and assayed or reassayed even after several months. The most important advantage of blotting is the accessibility of the immobilized proteins to reagents allowing the performance of analyses that are difficult or impossible in gels.

By using protein blotting, with enhanced CBB-staining, together with sensitive lectin probes, we have observed marked qualitative differences in membrane protein composition between normal tissue, a benign hyperplasia and malignant SCC of the prostate, particularly in the MW region less than 43 kD. In particular, a 28 kD ConA-binding protein was overexpressed in the SCUCP, expressed to a lesser extent in benign hyperplasia of the prostate, and absent in normal prostatic tissue. This component was plasma membrane localized and hence may be a suitable candidate for production of a tumor-specific cell surface antibody probe. It did not correspond to prostate specific antigen (33 kD, 25) nor to prostatic acid phosphatase (approximately 100 kD). We have not yet examined prostatic adenocarcinoma by Western blotting because of lack of availability of material.

Baylin et al. [3] identified 12 cell-surface proteins ranging from 30–70 kD that distinguished lung small

cell carcinomas (SCC) from non-SCC cells with three of these (MWs 60 kD, 57 kD and 30 kD) possibly being unique SCC gene products. In our study we have also found 3 plasma membrane components of corresponding MW that were present in prostate SCUCP and not in normal prostatic tissue. It remains to be determined whether the two sets of proteins are related. In contrast to the study by Baylin and coworkers we found additional overexpression of a series of lower MW proteins in prostatic SCC in the MW range 10–30 kD.

In this study, a bladder tumor cell line, Bl-13/0, exhibited a greater number and quantity of membrane proteins compared with another cell line, BL-17/2, particularly in the MW region <43 kD. The two lines were both derived from transitional cell carcinomas of the bladder, but the BL-17/2 line expresses features of both squamous and adenocarcinoma [20], whilst the BL-13/0 line is predominantly a mixture of cells with features of squamous or transitional cell carcinoma (unpublished observations). A similar overexpression of low molecular weight components was observed in the prostatic small cell undifferentiated carcinoma compared with normal or benign tissue.

Marked homology between the individual lectin binding patterns (particularly SBA, DBA and UEA) for both prostatic SCUCP and the two bladder tumors signified that in each of these cancers, these LMW proteins possessed multiple glycosylation along their chains with the carbohydrate moieties, N-acetyl-D-galactosamine, methyl 2-acetamido-2-deoxy-D- galactose and α-L-fucose. These findings confirmed previous studies which showed marked glycosylation of the cell surface of bladder tumor cells as compared with normal bladder tissue as detected by flow cytometric assessment of lectin binding [19].

The membrane protein phenotypes of both bladder and prostatic cancer cells were distinctively different from those of the corresponding normal tissue, and there were also differences between human bladder cancer cell lines derived from two patients. Two types of change were seen: a marked increase in the relative amounts of the protein present in membrane preparations from both bladder and prostate cancer compared with normal tissue (Figs. 1 and 2) as well as increased glycosylation observed may have arisen from gross abnormalities at the gene level with such changes leading to a switching on of the synthesis of a series of low molecular weight proteins (<43 kD) as well as from changes in carbohydrate processing.

A 43 kD protein showed markedly increased expression in BL13/0 compared with BL17/2 (Fig. 2). Not only was there increased synthesis of this protein, but in addition, marked glycosylation (with D-N acetylgalactosamine, methyl 2-acetamide-2-deoxy-D-galactose, mannose and α-L-fucose residues) of this protein was observed in BL13/0 compared with that in BL17/2 (Fig. 4). The functional role of this is being pursued. This did not correspond to carcinoembryonic antigen (200 kD, [9]).

In summary, the use of protein blotting in this study has provided a rapid identification and preliminary characterisation of the membrane protein phenotypes of several urological cancers. In addition, the usefulness and potential of the described technique in the investigation of specific tumor markers has been demonstrated in a manner not previously reported. The information obtained from this study has provided direction for current studies on possible molecular mechanisms responsible for induction of neoplasia. The identification of specific membrane protein markers could lead to the production of monoclonal antibody probes resulting in a refinement of current histological classifications of urological cancers, and will be the subject of future work in our laboratory.

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