ISOLATION OF PLASMA-MEMBRANE COMPONENTS FROM CULTURED HUMAN PANCREATIC CANCER CELLS BY IMMUNO-AFFINITY CHROMATOGRAPHY OF ANTI-β₂M SEPHAROSE 6MB

S. PÅHLMAN†, I. LJUNGSTEDT-PÅHLMAN†, A. SANDERSON*, P. J. WARD*, A. GRANT AND J. HERMON-TAYLOR

From the Department of Surgery, St George’s Hospital Medical School and *Blond Laboratories, Queen Victoria Hospital, East Grinstead, Sussex

Received 28 June 1979 Accepted 23 July 1979

Summary.—Human pancreatic exocrine adenocarcinoma cells established in tissue culture expressed both HLA and β₂-microglobulin (β₂M). Plasma-membrane components of this pancreatic cancer cell line were purified from plasma membrane fractions enriched by sucrose density-gradient centrifugation, using immunoaffinity chromatography on immobilized anti-human β₂M antibody. Both rabbit and mouse monoclonal anti-β₂M IgG were used, with a 20–25-fold overall purification of 5'-nucleotidase. The method was applicable to 5×10⁷ cells and permitted the solubilization of membranes retained on the column, with the selective desorption of components not associated with β₂M before the subsequent elution at pH 3 of β₂M-associated macromolecules. The acid eluate contained one major and two minor bands in the 40–45,000 mol.-wt range with two additional enriched components of 18,000 and 22,000 dalton. A major carbohydrate-containing component of high mol. wt was also found to be associated with the pancreatic cancer-cell plasma membrane.

Oncofetal macromolecular products of common solid human tumours, such as carcinoembryonic antigen, α-fetoprotein and pancreatic oncofetal antigen (Banwo et al., 1974; Gelder et al., 1978), are unlikely to be of value in the development of specific sensitive early diagnostic methods, since they are not iso-immunogenic. More information is required about the plasma-membrane components of human tumour cells which may excite recognition by the host immune system. The isolation of such components, which must precede their biochemical and immunological characterization, is associated with two central technical problems. The first is the difficulty of extracting tumour-cell plasma-membrane fractions from often sclerotic primary tumour tissue heavily infiltrated with normal host cells. The establishment of a human pancreatic cancer cell line in this laboratory (Grant et al., 1979), which preserves its morphological characteristics as xenografts in immune-deficient animals, should provide an apparently homogeneous source of pancreatic cancer cell membrane. The problem then becomes the availability of sufficient starting material and the need for separation methods of greater selectivity. The present investigation is concerned with the development of an immuno-affinity procedure for the isolation of plasma membranes and their components from cultured human pancreatic cancer cells, using immobilized antibody to human β₂-microglobulin (β₂M). The method, which permits selective adsorp-

† Present address: Institute of Biochemistry, Biomedical Centre, University of Uppsala, S-751 23 Uppsala, Sweden.
tion and desorption, is likely to be applicable to other human cell populations expressing this antigen.

MATERIALS AND METHODS

Radiolabelling of cultured cells

Human pancreatic exocrine adenocarcinoma cells derived from a primary lesion were cultured and harvested as previously described (Grant et al., 1979). Endogenous labelling of proteins and rough endoplasmic reticulum (RER) was carried out using $^{14}$C-amino acids or $^3$H-uridine respectively. About 10 $\mu$Ci/10$^7$ cells of $^{14}$C-amino acids (U.$^{14}$C protein hydrolysate 50 mCi/m atom carbon, Radiochemical Centre, Amersham) was incubated overnight with almost confluent pancreatic cancer cells in 10% FCS supplemented medium (Hams F12, Flow Laboratories, U.K.). 10 $\mu$Ci/10$^7$ cells of $^3$H-uridine (25 Ci/mmol, Radiochemical Centre, Amersham) was incubated for 24 h with almost confluent cells under similar culture conditions. In other studies exogenous labelling of surface proteins and carbohydrates was carried out on cells in suspension using 125I lactoperoxidase-glucose oxidase (Hyne, 1973) or galactose-oxidase--NaB$^3$H$_4$ (Gahmberg & Andersson, 1977) respectively. Before labelling by the latter method, the cells were incubated at 37°C with 15 u/ml neuraminidase for 1 h. Both the galactose oxidase (KABI, Sweden) and neuraminidase (Boehringer, U.K.) were reported to be free of proteases (Gahmberg & Andersson, 1977). After iodination or titration of the cell surface, viability was always greater than 90% as determined by trypan-blue exclusion.

Membranes preparation

Membranes were prepared from $\sim 5 \times 10^7$ cells (about 25 mg protein). The cells were detached from the culture flasks using 0.02% EDTA (Grant et al., 1979) washed with PBS and suspended in 1 ml of 1mM NaHCO$_3$ + 0.2mM MgCl$_2$ and immediately centrifuged at 500 g at 4°C for 5 min. The supernatant contained cytoplasmic proteins but only minute amounts of plasma membrane, since the cells became leaky but did not disintegrate under these conditions. The supernatant was discarded and the pellet resuspended in 3 ml of 1mM NaHCO$_3$ + 0.2mM MgCl$_2$ at 4°C. The cells were then immediately homogenized by 5-7 strokes with a tightly fitting Dounce homogenizer (F.T. Scientific Inst. Ltd, Tewkesbury, Glos) and transferred to 7 ml of sucrose solution to give a final concentration of 0-25m sucrose + 1.5mM EDTA in a total volume of 10 ml. Nuclei and residual whole cells were removed by centrifugation at 500 g at 4°C for 5 min. The supernatant was used as a crude membrane preparation for the further fractionation of plasma membrane.

Preparative procedure

Sucrose-gradient centrifugation.—Discontinuous gradients were prepared by layering 3.5 ml of 20, 35 and 45% sucrose w/w in 1mM NaHCO$_3$ + 1.5mM EDTA into 15ml tubes. Gradients were loaded with 3 ml of the crude membrane preparation (~1 mg protein/ml) and centrifuged at 100,000 g for at least 5 h (MSE 6 x 15 S.W. rotor). The gradients were collected from the bottom of the tube into 1-1.5ml fractions. The sucrose density of the fractions was measured with a refractometer.

Immunoaffinity chromatography.—Human $\beta_2$M was isolated from the urine of patients with Wilson's disease as described by Berggård & Bearn (1968) and purified to apparent homogeneity. Rabbits were immunized with 0.1 mg $\beta_2$M emulsified with Freund's adjuvant and serum obtained at intervals after boost inoculations. Sera were pooled and established as having $\beta_2$M specificity by double diffusion in gels against pure antigen; control wells with other human serum proteins were consistently negative. Specific anti-human $\beta_2$M IgG was prepared from pooled serum by DEAE chromatography and established to be free from other serum proteins by immuno-electrophoresis.

Mouse anti-human $\beta_2$M was obtained as follows: a hybridoma cell line which secreted anti-$\beta_2$M IgG was established (Kohler & Milstein, 1976) by fusion of NS1 myeloma cells with spleen cells from mice sensitized with human peripheral-blood lymphocytes. After growth in HAT medium and cloning, a line was identified which produced monoclonal anti-$\beta_2$M IgG antibody. Large-scale production of this monoclonal IgG was achieved in (BALB/c $\times$ DBA/2) F$_1$ hybrid mice by isolation of serum when large tumour growth was noted. Purification of $\beta_2$M-specific antibody was achieved by adsorption and acid elution from a column of $\beta_2$M-
substituted Sepharose 4B (2 mg β2M/g wet gel). Eluted material was identified as IgG by immunoelectrophoresis and established as monoclonal by isoelectric focusing. Anti-β2M titres were established in a radioimmunoassay as previously described (Sanderson, 1977). Anti-β2M antibody was coupled to Sepharose 6MB (a gift from the research department of Pharmacia Fine Chemicals AB, Sweden) after activation in this laboratory using cyanogen bromide (Axen et al., 1967). In order to favour binding of the antibody to the exterior of the gel beads, the Sepharose was highly activated (0.2 g CNBr/g wet gel; Grant et al., 1978).

Initially, IgG fractions of rabbit anti-human β2M serum were coupled to CNBr-activated gel at a concentration of 2 mg antibody/g wet gel. This antibody was replaced in later studies with purified monoclonal mouse anti-human β2M substituted at 0.05 mg/g wet gel. Sample volume and column size in the chromatographic procedures varied with the different experiments, details of which are given in the legends to Fig. 2 and 3. The crude membrane sample was diluted 1:1 with the operational buffer (10 mM Tris-HCl, pH 8.2, +0.1 M NaCl +1.5 mM EDTA) and added to the anti-β2M Sepharose 6MB in the column. The slurry was gently mixed with a glass rod at intervals over a 1 h period, after which the gel was allowed to settle for another hour. Unbound material in the supernatant was removed and the column washed overnight with operational buffer (3 ml/h). Protein-bound radioactivity in the flow-through material was counted in washed TCA precipitates. Membranes retained by the gel were solubilized with detergents, Nonidet-P40 (NP-40, BDH, U.K.), Mulgoen or Deoxycholate, 2.5%, NP-40 in the operational buffer was mixed with the gel bed to a final concentration of 0.5% NP-40 and the slurry again stirred at intervals for 1 h, before elution with further operational buffer containing 0.5% NP-40. Adsorbed β2M and components linked to β2M were then eluted with 50 mM glycine-HCl, pH 3.0, +0.5% NP-40. Control experiments with non-substituted Sepharose 6MB were also performed. Fractions were concentrated on a Minicon B15 concentrator (Amicon Corp., U.S.A.). Binding of whole cells to the anti-β2M column was also investigated by an adsorption procedure similar to that described for the membrane fractions.

Analytical methods
Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE) was performed in a discontinuous buffer system essentially as described by Neville (1971). Slab gels with a 4% stacking gel and a 11% separation gel were used. Samples containing 10–100 µg protein in 80 mM boric acid, 80 mM Tris buffer (pH 8.64) were reduced with 0.5% β-mercaptoethanol in the presence of 0.02% EDTA, 0.3% SDS and 5% suerose, and boiled for 1 min. Electrophoresis was carried out for 4 h at a maximum of 4 W. The gels were then fixed in 20% sulphosalicylic acid, stained with 0.05% Coomassie BB R-250 in methanol-acetic-acid–water (50:7:43) and destained in methanol–acetic-acid–water (25:7:68). 125I-labelled proteins in dried gels were visualized autoradiographically: 3H- and 14C-labelled components were fluorographed according to the method of Laskey & Mills (1975). The Pre-sensitized X-ray films, X-Omate H (Kodak), were exposed for 1–4 weeks. Protein was estimated by the Lowry method in the presence of SDS. Bovine serum albumin was used as standard. 5' nucleotidase activity of the membrane preparations was determined according to Avrukh & Wallach (1971) using [14C]AMP (Amersham) as tracer radioactivity. For this assay and for all other radioactive samples in water, a scintillation cocktail consisting of 1 l toluene, 0.5 l Triton X-100, 5 g PPO and 50 mg POPOP was used.

Immunofluorescent staining of cells for HLA and β2M
Suspensions of viable cultured human pancreatic cancer cells (10⁶ cells/100 µl PBS) were incubated with monoclonal mouse anti-human HLA W6/32 (a gift from Dr A Williams; Barnstable et al., 1978) and anti-β2M antibodies at room temperature for 1 h. The cells were washed twice with PBS, resuspended in 100 µl and labelled with 10–20 µl FITC- or rhodamine-conjugated anti-mouse or anti-rabbit IgG (Nordic Immunological Laboratories, Maidenhead) for 30 min at room temperature. In control experiments monoclonal mouse anti-rat AgB or normal rabbit serum respectively were used. Cells were mounted in PBS glycerol (1:1 v/v) and examined with a Zeiss ultraphot IIIb with epi-fluorescent illumination.
RESULTS

Membrane preparation and sucrose-gradient centrifugation

The cultured human pancreatic cancer cells were noticeably more resistant to disruption by hypotonic medium and Dounce homogenization than human lymphocytes or fibroblasts (Graham, 1975). 0.2 mM Mg\(^{2+}\) was required during homogenization to prevent nuclear rupture, but when the cell homogenate was transferred to isotonic sucrose, EDTA became necessary to prevent membrane aggregation associated with the presence of Mg\(^{2+}\) (Avruch & Wallach, 1971). Under these circumstances the yield was in the range 65–75% of available plasma membrane as assessed by 5' nucleotidase activity; omission of Mg\(^{2+}\) and EDTA reduced the membrane yield to only 20–25%.

The results of sucrose-gradient centrifugation are shown in Fig. 1. With \(^3\)H-labelled surface glycoproteins, a major peak of radioactivity which banded on top of the 35% sucrose coincided with a protein and 5' nucleotidase peak (Fig. 1A). The radioactivity on top of the sucrose gradient (Fractions 1–4, Fig. 1A) is unbound \(^3\)H which penetrated the intact cells during the labelling procedure. This could be removed by dialysis after homogenization, since subsequent SDS-PAGE of these fractions and fluorography showed no radioactivity associated with macromolecules.

Fig. 1B shows the results obtained with \(^{14}\)C amino acid-labelled cells. The peak of radioactivity in Fraction 1–4 corresponds to the similar protein peak in Fig. 1A, and represents cytoplasmic proteins and some free labelled amino acids. The 5' nucleotidase activity does not precisely coincide with the distribution of radioactivity, suggesting the presence of other components in the plasma-membrane fractions. The results shown in Fig. 1C with labelled RNA suggest that the major 5' nucleotidase peak is contaminated with RER. In all 3 experiments a second plasma-

![Fig. 1.](image-url)
Membrane peak was detected, banding on top of the 45% sucrose zone. The amount of material varied from one experiment to another, but visual observation suggested that this fraction consisted of aggregated membranes. The overall enrichment of the plasma-membrane fractions represented only a 4–5-fold purification and, with the limited amount of starting material available, it was clear that a preparative procedure of greater selectivity was necessary.

**Anti-β2M-Sepharose 6MB column chromatography**

At least 95% of the cultured pancreatic cancer cells stained for both HLA and β2M by indirect immunofluorescence. Both mouse monoclonal and rabbit anti-human β2M were available in sufficient quantity to prepare respective immunoadsorbents. In the experiments using rabbit IgG, gels were substituted with ~2 mg antibody per g wet agarose. Higher substitution would not, according to Weston & Scorer (1978), increase the capacity of the gel, and might increase nonspecific adsorption (Titanji & Pählman, 1978). Since the membranes were unlikely to penetrate the 6% agarose beads, coupling conditions were chosen which favoured substitution on the exterior of the beads (Grant et al., 1978). The monoclonal antibody preparation had an approximately 40 times higher titre than the rabbit anti-β2M antibody. Gels with monoclonal antibodies were substituted with 50 μg antibody per g gel.

The elution profile of the crude membrane preparation with labelled surface glycoproteins on monoclonal anti-β2M Sepharose 6MB column is shown in Fig. 2. Elution of bound material with 0.5% NP-40 brought off a peak of radioactivity which contained 5′nucleotidase activity. Subsequent elution at pH 3 desorbed a trace of protein, which after concentration was visualized by SDS-PAGE (Fig. 4). NP-40 was found to be the most suitable

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**Fig. 2.** — Purification of plasma membrane components on anti-β2M Sepharose 6MB. 5 ml of crude membranes (~1 mg protein/ml) with labelled surface glycoproteins (galactose oxidase-NaB₃H₄) were applied to a 10 ml gel bed (6 x 1.5 cm) substituted with mouse monoclonal antibody. Retained material was eluted with 0.5% NP-40 in the operational buffer followed by 50 mM glycine-HCl (pH 3) + 0.5%, NP-40. Flow rate was 3 ml/h; 3 ml fractions were analysed for radioactivity (▲—▲) and 5′nucleotidase activity (□—□).
of the 3 detergents tested, and did not affect 5' nucleotidase activity. Mulgofen was as efficient as NP-40 in desorbing the plasma membrane proteins, but inactivated 5' nucleotidase over a 24h period. Deoxycholate was less efficient than NP-40 and activated 5' nucleotidase about 1.5-fold.

A 10ml bed of anti-β₂M-substituted gel retained 25-40 μg of protein. In the experiment shown in Fig. 2, about 60% of the applied 5' nucleotidase activity did not bind to the gel and when this flow-through material was rechromatographed on a fresh anti-β₂M column only a small fraction was retained. The same phenomenon was seen with intact tumour cells, since about 40% of cells applied consistently failed to bind to the anti-β₂M column, despite positive staining of the unbound population for surface β₂M by immunofluorescence. The proportion of both membranes and cells which failed to bind increased if adsorption was carried out as a batch procedure. About 8% of ¹²⁵I-labelled membranes were retained when applied to a column of unsubstituted sepharose 6MB, and 4% when mixed with unsubstituted gel in a batch procedure. This suggests that only a small proportion of the adsorbed material in the column experiments was due to the physical entrapment of aggregated or large membrane fragments in the packed gel.

The selectivity of the immuno-affinity chromatography with respect to RER is shown in Fig. 3. In two separate experiments, under identical conditions, crude membrane preparations with either ¹²⁵I-labelled plasma membranes or ³H-uridine-labelled RER were chromatographed on anti-β₂M Sepharose 6MB. Desorption with the nonpolar detergent Mulgofen eluted a peak of ¹²⁵I-labelled plasma membranes, but only a trace of ³H-labelled RER. The proportion of RER (9%) that eluted with detergent was similar to the proportion retained nonspecifically to unsubstituted gel, suggesting physical entrapment as previously described.

Fig. 4A shows the protein stain of an SDS-PAGE of crude membranes and fractionated plasma membrane components. Fig. 4B shows the fluorograph of the same gel, the glycoproteins of which were labelled by the galactose-oxidase-sodium-borotritide method. Fig. 4C is a fluorograph of the ¹⁴C-amino-acid-labelled plasma-membrane-enriched fraction. A progressive selection of components from the crude membrane preparation is seen as fractionation proceeds. The acid-eluted material from the anti-β₂M column contains one major and two minor components in the 40-45,000 mol. wt range; two other enriched components of ~mol. wt 22,000 and 18,000 are seen in this fraction (Fig. 4A, lane 4). The most striking feature of the corresponding fluorograph is the major radioactive component trapped on top of the 4% stacking gel and present in all fractions including
MEMBRANE COMPONENTS FROM CANCER CELLS

Fig. 4.—SDS-PAGE followed by fluorography of fractionated plasma-membrane components. (A) Slab gel stained for protein—Lane 1: Crude membrane preparation from cells labelled with galactose-oxidase-NaB₃H₄. Lane 2: The plasma-membrane-enriched fraction from the sucrose gradient in Fig. 1A. Lane 3: NP-40-eluted material from α-β₂M column shown in Fig. 2. Lane 4: pH3-eluted material from α-β₂M column shown in Fig. 2. Protein standards: human serum albumin (204,000, 136,000 and 68,000), human IgG (50,000 and 25,000), ovalbumin (43,000) and cytochrome c (13,800). (B) Corresponding fluorograph. (C) Fluorography of the ¹⁴C amino acid-labelled plasma-membrane-enriched fraction (Fraction 6) from the sucrose gradient shown in Fig. 1B (comparable with Lane 2 of (A)).

the NP-40 eluate from the affinity column, but absent from the acid-eluted fraction. This high-mol.-wt surface component which does not appear in the protein stain and contains little incorporated ¹⁴C-amino acid (Fig. 4A, 1–3 and Fig. 4C, respectively) must be highly glycosylated. Radio-labelling of other membrane glycoproteins with galactose-oxidase–NaB₃H₄ was poor (Fig. 4B).

DISCUSSION

The human pancreatic exocrine cancer cell line previously described (Grant et al., 1979) clearly expresses both the HLA antigens and β₂M in conditions of in vitro culture and, despite the abnormalities in chromosome content, has functional Chromosomes 6 and 15. The expression of these components is preserved during serial passage. In Daudi, by contrast, a human lymphoblastoid cell line Chromosome 15 is missing and 6 is cryptic (Goodfellow et al., 1975). However, Daudi grows well in suspension culture, whereas the pancreatic cancer cells, like cell lines derived from other solid tumours, require a matrix for growth. This limits the number of cells that can be made available for study, and means that preparative procedures for the isolation of their plasma membranes must combine maximum resolution with high yield, conditions which
are difficult to attain with centrifugation techniques alone.

The expression of $\beta_2$M at the cell surface, and the availability of sufficient specific antibody as a ligand, permitted the development of an affinity chromatography system for the selective absorption plasma membranes. This had the additional advantage of offering selective desorption from the column of membrane-bound macromolecules not associated with $\beta_2$M before the elution of $\beta_2$M-containing histocompatibility antigens and other components which might be associated with the malignant state (Kvist et al., 1978; Tada et al., 1978; Beutler et al., 1978). Similar methods have been applied to the isolation from solid tissue of papain-solubilized $\beta_2$M-associated components in human malignant melanoma, breast carcinoma, colonic carcinoma and hepatoma (Thomson et al., 1978; Rauch et al., 1978).

Despite the observation that at least 95% of the cultured tumour cells were positive by immunofluorescent staining for $\beta_2$M, only 50% of the plasma membranes were apparently able to bind to the gel. It seems unlikely that this could be due to the formation of inside-out plasma-membrane vesicles, itself an unusual event, since a similar fraction of whole cells remained unbound. It is more likely to be due to heterogeneity at the cell surface in the density of $\beta_2$M expression or differences in its accessibility to free as opposed to CNBr-immobilized Ig. Similar unbound cell populations have been described in the lectin-affinity chromatography of rabbit thymocyte membranes (Brunner et al., 1977) and in the affinity separation of lymphocytes on immobilized insulin (Ljungsted-Pålman et al., 1977).

The specificity of the immunoadsorbent appeared to be high. The trace of RER retained could either be due to physical entrapment as a result of aggregation, or to ionic and hydrophobic interactions with immobilized Ig (Titanji & Pålman, 1978). The small amount of material available and the interference of detergent in the assays made it difficult to quantify the purification achieved, but it was of the order of 10-fold for the affinity step alone with an overall purification of 20–25-fold.

It has been suggested that tumour-specific macromolecules may be associated with $\beta_2$M and HLA antigens (Kvist et al., 1978; Tada et al., 1978; Beutler et al., 1978; Thomson et al., 1978; Rauch et al., 1978). The components eluted from the immuno-absorbant at pH 3 resemble similar material isolated by Thomson et al. (1978) from the solid tissue of other human tumours. The biological significance of the components derived from the pancreatic cancer cell line in the present study awaits the application of suitably specific cytological assays.

During the initial steps in the preparation of membranes, the cultured human pancreatic cancer cells were found to be much more resistant to disruption in a hypotonic environment than peripheral-blood lymphocytes or cultured human fibroblasts (Graham, 1975). Under hypotonic conditions the cells became leaky of cytoplasmic components, presumably due to defects in the plasma membrane, but the peripheral envelope itself did not disintegrate. We also found that the pancreatic cancer cell surface labelled poorly with galactose-oxidase–NaB$^3$H$_4$ and $^{125}$I-lactoperoxidase, the only exception being the large glycosylated component seen on top of the 4% stacking gel in the SDS-PAGE. It is probable that this component contributes to a substantial glycoalcalyx which might be responsible for some structural stabilization of the plasma membrane. It might also serve to protect the cell surface from enzymic modification and immunological attack.

We thank Dr Alan Williams for the W6/32 monoclonal antibody, and Dr J Graham (Department of Biochemistry) and Dr C. Wylie (Department of Structural Biology) at St George's Hospital Medical School for their help. Dr J. Walshe of Addenbrooke's Hospital, Cambridge, kindly provided the $\beta_2$-microglobulin-containing urine. This investigation was supported by a grant from the Cancer Research Campaign. Drs S. and I. Pålman were funded by travelling fellowships from "OE och Edla Johanssons vetenskapliga stiftelse", "Byråchefen Sten-
holms stipendieförsäljning”, “Gälo stiften”, “Aptekarsocietetens forskarstipendium” och “IF stifelse för farmaceutisk forskning”.

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