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Acknowledgements—We are much obliged to Jaim Prilusky and Yuval Ginosar for technical assistance and to the Israel Ministry of Health, the John D. and Catherine T. MacArthur Foundation, and the Sherman Foundation for support. The work was also supported by the Jakubskind Cymmerman Research Prize to Z.A.

Eur J Cancer, Vol. 28A, No. 6/7, pp. 1090–1098, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Immunohistochemical and Biochemical Characterisation of the Expression of a Human Embryonal Carcinoma Cell Proteoglycan Antigen in Human Germ Cell Tumours and other Tissues

Malcolm D. Mason and Martin F. Pera

In the embryonal carcinoma (EC) cell line GCT 27, monoclonal antibody GCTM-2 recognises an epitope on a 200 kD pericellular matrix keratan sulphate proteoglycan. Immunohistochemical analyses demonstrated staining of tissue sections from 21 out of 22 human non-seminomatous germ cell tumours, and from 22 out of 28 sections of seminomas. In normal human fetal tissues gut epithelium and muscle stained strongly, and certain other epithelia stained moderately. In adult tissues, the distribution of the epitope was similar, but staining intensity was weaker. Neoplastic tissues showed reactivity with embryonal rhabdomyosarcoma and colorectal carcinoma, but no other non-germ cell tumours. Immunofluorescence microscopy showed that GCTM-2 also stained cell lines from human colorectal carcinoma, embryonal rhabdomyosarcoma and choriocarcinoma. In contrast to EC cells the epitope in these other cell types required permeabilisation of the cells to be visualised, and the protein bands in immunoblots lacked extensive modification with keratan sulphate and were smaller. Thus, GCTM-2 reacts with an epitope which has a previously unrecognised tissue distribution; its expression as a pericellular matrix proteoglycan is predominantly a characteristic of human EC cells.
Eur J Cancer, Vol. 28A, No. 6/7, pp. 1090–1098, 1992.

INTRODUCTION

THE SERUM markers α-fetoprotein and human chorionic gonadotropin are useful in monitoring patients with testicular non-seminomatous germ cell tumours (NSGCT) [1]. These secreted polypeptides are products of differentiated yolk sac cells and trophoblastic lineage respectively, which appear in a proportion of testicular teratomas [2]. Despite the utility of these markers, it would be desirable for cell biological studies and for certain clinical applications to define markers expressed on embryonal carcinoma stem cells themselves, rather than on their differentiated derivatives. A preliminary study has suggested that the GCTM-2 antigen merits investigation as one such clinical marker [3].

A number of cell surface antigens defined by monoclonal antibodies have been described in association with embryonal carcinoma. Many of these monoclonals react with carbohydrate antigens and are of the IgM class [4]. Monoclonal antibody GCTM-2 was shown to recognise a pericellular matrix EC proteoglycan which was susceptible to degradation by keratanase but not other glycohydrolases or lyases [5]. More recent studies on the purified GCTM-2 antigen confirmed that it is a keratan sulphate proteoglycan. The further evidence supporting this conclusion included aminoacid and sugar analyses of the antigen; the reactivity of the antigen with Alcian blue dye; high affinity of the antigen for anion exchange resins; metabolic labelling of

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Revised 15 Jan. 1992; accepted 17 Jan. 1992.

the antigen with inorganic sulphate and release of this labelled sulphate following keratanase treatment and the appearance of aggregates of purified antigen in electron microscopy (S. Cooper *et al.* Dept of Zoology, Oxford University. *Biol J* in press). The epitope recognised by the GCTM-2 antibody probably lies on the proteoglycan core protein, since the antigen–antibody interaction is enhanced by keratanase treatment, but is not altered by *N*-glycosidase digestion, or mild alkaline hydrolysis, which would be expected to release *N*-linked or *O*-linked carbohydrate, respectively; chondroitinase ABC and heparitan sulphate lyase also had no effect on the antigen–antibody interaction [5, and Pera, unpublished observations].

Proteoglycans are a diverse group of molecules with a range of functions, and are most often found in the extracellular matrix [6]. They are composed of one or more glycosaminoglycan chains covalently bound to a core protein, frequently with *N*-linked or *O*-linked oligosaccharides [7]. Given recent evidence that proteoglycans are important in the regulation of growth and differentiation [8], it is noteworthy that the GCTM-2 antigen disappears from the cell surface during spontaneous differentiation *in vitro* of the embryonal carcinoma cell line GCT 27X-1, a clonal derivative of GCT 27 [9].

In these studies we sought to further characterise the tissue distribution and biochemical characteristics of the antigen recognised by GCTM-2.

MATERIALS AND METHODS

Cell culture

GCT 27 cells were maintained in culture under the conditions described previously [10]. On reaching confluence the cells were harvested with 0.05% trypsin and 0.02% EDTA and replated at a 1:10 split ratio. The HX 18 cell line was established from a human colorectal carcinoma [11] and was grown under the same conditions as described for GCT 27, except that confluent monolayers were subcultured at a split ratio of 1:4. The cell line HX 170 was established from xenografts in nude mice of a human embryonal rhabdomyosarcoma and was grown as described [12]. The human choriocarcinoma cell line BeWo [13] was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury). BeWo cells were grown in Ham's F12 medium supplemented with 20% fetal calf serum, glutamine at a concentration of 2 mmol/l, and hydrocortisone at 1 mg/ml. The cells were subcultured when subconfluent at a split ratio of 1:6. Mouse hybridoma GCTM-2 [5] was grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum (Myo-clone, Gibco). Cells were harvested twice weekly, and replated at a split ratio of 1:10. The cell supernatant was harvested, supplemented with 1 mmol/l phenylmethyl sulphonyl fluoride (PMSF) and used in this form.

Tissue samples

Specimens taken at orchidectomy for removal of a testicular tumour were fixed in 100% ethanol, and embedded in paraffin blocks. Additionally, blocks from formalin-fixed testicular tumours kept in histopathology laboratory archives were obtained from 15 specimens, and sections were cut from these blocks for immunohistochemical study. Germ cell tumours were classified histologically according to the WHO system [14]. Midtrimester fetal tissue was obtained from the tissue bank at the Royal Marsden Hospital (approval was obtained from the Royal Marsden Ethics Committee for the use of this material in this study). The specimens were dissected, fixed in 100% ethanol, and embedded in paraffin blocks before sectioning. Normal adult tissue was obtained at surgery, when its resection formed part of the procedure. It was fixed in 100% ethanol, and embedded as described above. Additionally, some normal adult tissue was obtained from formalin-fixed autopsy specimens. Non-germ cell tumours were obtained from formalin-fixed material held in the archives of the histopathology department of the Royal Marsden Hospital.

Immunohistochemistry

5 μm sections were cut from the blocks onto glass slides, dewaxed in three changes of xylene, and then rehydrated by passing them through 100, 70 and 40% (vol:vol) ethanol in water, and then through a final rinse in distilled water. Rehydrated sections from formalin-fixed material were treated with 0.1% Bacto Trypsin (Difco Laboratories) at 37°C for 1 h prior to staining, as preliminary studies had shown this to enhance staining to the levels seen with ethanol-fixed material. Control sections were treated with normal mouse serum diluted 1:400 in phosphate-buffered saline (PBS) plus 1% bovine serum albumin (BSA) as a source of control mouse immunoglobulin of the same class as GCTM-2 (IgM). GCTM-2 was applied to the test section as neat hybridoma supernatant. The slides were incubated at room temperature for 1 h (ethanol-fixed material) or for 1.5 h (formalin-fixed material). The slides were rinsed in PBS/BSA between this and each subsequent step. Next, rabbit anti-mouse immunoglobulin conjugated to biotin (Amersham) was applied to the sections at a 1:100 dilution in PBS/BSA, and incubated for 30 min at room temperature. Streptavadin conjugated to horseradish peroxidase (Amersham) was applied at a concentration of 1:300 vol/vol in PBS/BSA and incubated for 30 min at room temperature. Finally, 3,3-diaminobenzidine (Sigma), 0.5 mg/ml in PBS, plus hydrogen peroxide to a final concentration of 0.02% vol/vol, was added as substrate, and incubated for 3 min. The sections were counterstained with haematoxylin, dehydrated in two changes of 100% ethanol followed by 100% xylene, and mounted in D.P.X. (BDH). Those control sections in which extensive staining was observed (usually associated with massive necrosis) were deemed to be unassessable and were excluded from the analysis. Staining with GCTM-2 was only scored as 'positive' where no such staining was observed in corresponding control sections.

Indirect immunofluorescence

Cultured cells were grown on sterile multiwell slides (Flow) under the conditions described above. When grown to a suitable density, the cells were fixed in a 1:1 (vol:vol) solution of methanol/acetone over solid CO_2, and were stored at −20°C. Slides were stained and mounted as described [5]. For immunofluorescent staining of live cells, 10^6 cells were harvested, and resuspended in fresh medium. GCTM-2 antibody was added to the cell suspension to a dilution of 1:3. The cells were incubated at 4°C for 30 min, rinsed in PBS after centrifugation at 500 *g*, and resuspended in ice cold PBS. After a second wash, they were resuspended in diluted anti-mouse immunoglobulin/fluorescein isothiocyanate conjugate as described [5], and incubated at 4°C for 30 min. They were then mounted and viewed as described above.

Immunoblotting

Monolayer cultures of GCT 27, HX 18, HX 170 and BeWo were treated with a small volume of 50 mM Tris–HCl buffer, pH 7.4, with or without keratanase (keratan sulphate 1,4-β-D-galactanohydrolase from *Pseudomonas* sp. IFO-13309, ICN

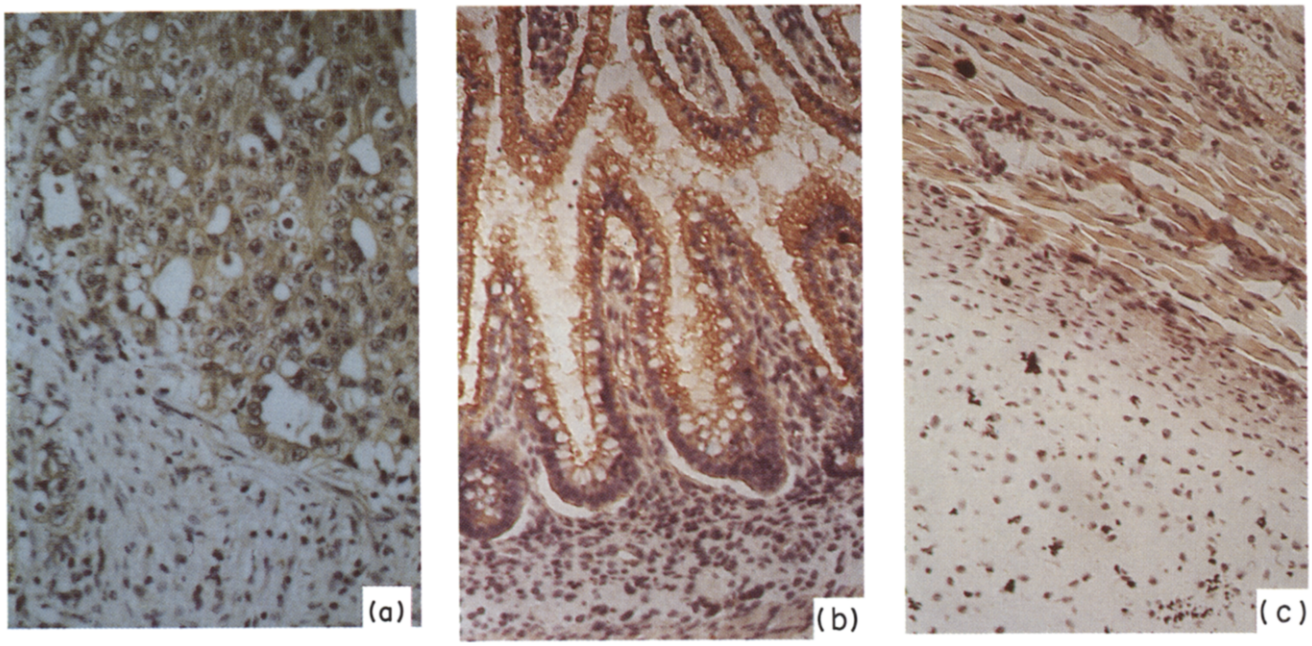

Fig. 1. (a) Human testicular teratocarinoma stained with GCTM-2. Embryonal carcinoma is stained but not surrounding mesenchymal tissue. (b) Second-trimester human fetal small intestine showing intense staining of mucosal epithelium by GCTM-2. (c) Second-trimester human fetal larynx stained with GCTM-2. Skeletal muscle fibres are stained but not adjacent cartilage.

Biomedicals) to a final concentration of 2.5 units/ml, and incubated for 1 h at 37°C. The preparation was then harvested into a small volume of sodium-dodecyl sulphate (SDS) containing sample buffer plus PMSF, sonicated, boiled for 5 min, and then run on 10% polyacrylamide gels [15]. Prestained marker proteins applied to the gel were α-macroglobulin, β-galactosidase, fructose-6-phosphate kinase, pyruvate kinase, fumarase, lactate dehydrogenase and triosephosphate isomerase (Sigma). Following separation, the proteins were transferred to a nitrocellulose membrane using flat graphite electrodes (Pharmacia), and the blot was processed and stained with GCTM-2, biotinylated anti-mouse immunoglobulin, and streptavadin peroxidase [5].

RESULTS

Immunohistochemistry on germ cell tumours

12 out of 13 NSGCT stained with GCTM-2; the single specimen which did not stain consisted of mature tissues from a specimen of differentiated teratoma. Furthermore, staining was also seen in 17 out of 23 seminomas, and in all three sections from tumours consisting of combined seminoma/NSGCT. Following treatment of formalin-fixed sections with PBS/trypsin, each of nine NSGCT, five seminomas, and one intratubular germ cell tumour (in which the subtype could not be determined) stained with GCTM-2. Figure 1a shows a section of teratocarcinoma stained with GCTM-2 in which there is staining of areas of EC but not of the surrounding primitive mesenchyme. Within the mesenchyme, staining was observed in a few isolated cells of uncertain nature. The staining pattern of EC cells was cytoplasmic as well as surface; this is in keeping with the known natural history of some extracellular matrix proteoglycans, which are assembled from core proteins in the cytoplasm before being packaged and exported to the cell surface [6, 7]. Staining was also observed in areas of carcinoma *in situ* where these were present.

Immunohistochemistry on mid-trimester fetal tissue

Table 1 summarises the results of immunohistochemical staining of ethanol-fixed specimens of second-trimester fetal tissue. The strongest staining was seen in gut epithelium, and in all types of muscle (skeletal, smooth and cardiac). Several other epithelia also stained more weakly with GCTM-2. Within the epithelium of the small bowel, it was noted that the strongest staining was generally seen at the apex of the villi, while many of the crypts stained relatively less strongly. No staining was observed in mesenchyme, cartilage, brain, lymph node, dermis, renal glomeruli or testis. Figure 1b shows a section of fetal small intestine with strong staining of the mucosal epithelium. Figure 1c shows a section of fetal larynx in which staining of skeletal muscle is observed but not of the adjacent cartilage.

Immunohistochemistry on adult normal tissues

Table 2 lists reactions of adult normal tissues with GCTM-2. Wherever possible these were obtained during surgery and fixed immediately in 100% ethanol. Some tissues (brain, breast, liver, testis, pancreas and lymph node) were obtained from formalin-fixed material obtained at surgery or post-mortem. The pattern of reactivity resembled that of fetal tissues, but the level of staining was generally much less intense.

Immunohistochemistry on non-germ cell tumours

The results of immunohistochemistry on sections from formalin-fixed non-germ cell tumours is shown in Table 3. The only tumours in which staining was seen were colonic adenocarcinoma, and embryonal rhabdomyosarcoma. No staining was seen in a variety of other tumours, including several which might be considered in the diagnosis of a malignancy of uncertain origin in a child or young adult.

Immunofluorescence studies on cultured cells

Fixed cell preparations from GCT 27, HX 18, HX 170 and BeWo all stained with GCTM-2 by indirect immunofluorescence (Fig. 2). In contrast, when suspensions of live cells were stained, though 100% of GCT 27 cells examined were reactive, only 30% of HX 18 cells, and none of the HX 170 or BeWo cells reacted with GCTM-2 (Fig. 3). This suggests that the epitope is on the

Table 1. Immunohistochemistry on second trimester fetal tissue

	No. positive/ no. tested
Strongly positive	
Gut epithelium	3/3
Muscle	10/10
Moderate	
Trophoblast	4/4
Liver	4/4
Kidney (epithelium of distal tubules and collecting ducts)	3/3
Weak	
Lung epithelium	2/2
Bladder epithelium	2/2
Oesophageal epithelium	2/2
Tracheal epithelium	2/2
Thyroid	2/2
Negative	
Cornea	0/2
Brain	0/2
Cartilage	0/4
Skin	0/3
Mesenchyme	0/2
Testis	0/3
Spleen	0/2
Pancreas	0/2
Thymus	0/2
Lymph node	0/2
Renal glomeruli	0/3
Adrenal	0/2

Table 2. Immunohistochemistry on adult normal tissues

	No. positive/ no. tested
Strongly positive	None
Weakly positive	
Colonic mucosa	2/2
Skeletal muscle	3/3
Smooth muscle	3/3
Renal tubules	1/2
Liver	2/2
Pancreas	2/2
Breast epithelium	2/3
Adrenal Cortex	2/2
Negative	
Skin	0/3
Tongue epithelium	0/2
Bladder epithelium	0/2
Mesenchyme	0/2
Testis	0/2
Brain	0/2
Lymph node	0/2
Renal glomeruli	0/2

Table 3. Immunohistochemistry on non-germ cell tumours

	No. positive no. tested
Strongly positive	
None	
Moderately positive	
Colon carcinoma	1/4
Embryonal rhabdomyosarcoma	2/3
Negative	
Adenocarcinoma breast	0/3
kidney	0/2
submandibular gland	0/1
Transitional cell carcinoma—bladder	0/3
Squamous carcinoma—head and neck	0/2
Ewing's sarcoma	0/2
Neuroblastoma	0/2
Hodgkins disease	0/2
Non-Hodgkin lymphoma	0/2
Wilms' tumour	0/2
Malignant melanoma	0/2

surface of all GCT 27 cells, 30% of HX 18 cells, and is inaccessible in live HX 170 and BeWo cells.

Immunoblotting of whole cell lysates

Immunoblotting on whole cell lysates from GCT 27 yielded a characteristic band at 200 kD, which was degraded on pretreatment of the cell lysate with keratanase (Fig. 4a), down to a size limit of 55 kD, as previously reported [5]. In this series of experiments immunoblotting also revealed staining higher than the 200 kD position, a feature that has been noted with immunoblots on purified GCTM-2 proteoglycan preparations, and which appears to be due to the tendency for the proteoglycan to self-aggregate (S. Cooper, *et al.*, Dept Zoology, Oxford University. *Biol J* in press). Immunoblotting on whole cell lysates from HX 18, HX 170, and BeWo yielded single bands at approximately 55–70 kD, and in contrast to GCT 27, pretreatment of the cell lysates with keratanase did not modify the position of the band or the intensity of the staining (Fig. 4b,c,d). However, preparations from HX 18 also yielded faint immunoreactivity at approximately 200 kD, which was more apparent in the keratanase-treated lysate (Fig. 4c).

DISCUSSION

Screening of a panel of cell lines with monoclonal antibody GCTM-2 showed that the epitope was expressed in embryonal carcinoma, visceral yolk sac carcinoma, and a poorly differentiated colorectal carcinoma [5]. Previously published biochemical and electron microscopic studies have indicated that the GCTM-2 antigen lies on a pericellular matrix keratan sulphate proteoglycan in human EC cells [5], and more recent work has confirmed the proteoglycan nature of the antigen and its keratan sulphate glycosaminoglycan content (S. Cooper *et al.*, Dept Zoology, Oxford University. *Biol J* in press).

In this study we have shown that in histological sections of testicular tumours GCTM-2 strongly stained embryonal carcinoma, and, in the differentiated areas of one teratocarcinoma, primitive muscle and columnar epithelium, in keeping with the distribution of staining seen in fetal tissues. Additionally,

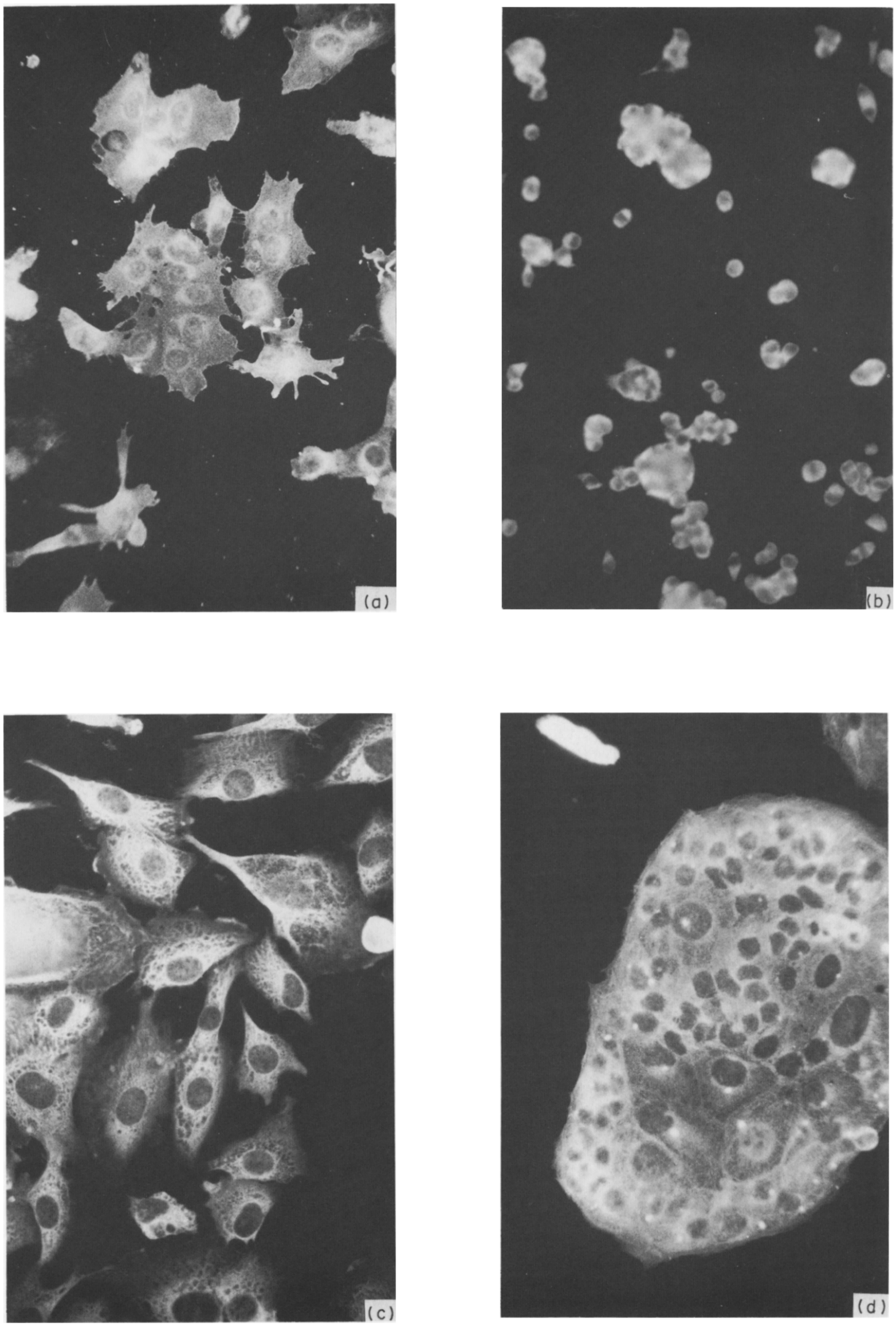

Fig. 2. Immunofluorescence on fixed cells with monoclonal antibody GCTM-2: (a) GCT 27; (b) HX 18; (c) HX 170; (d) BeWo.

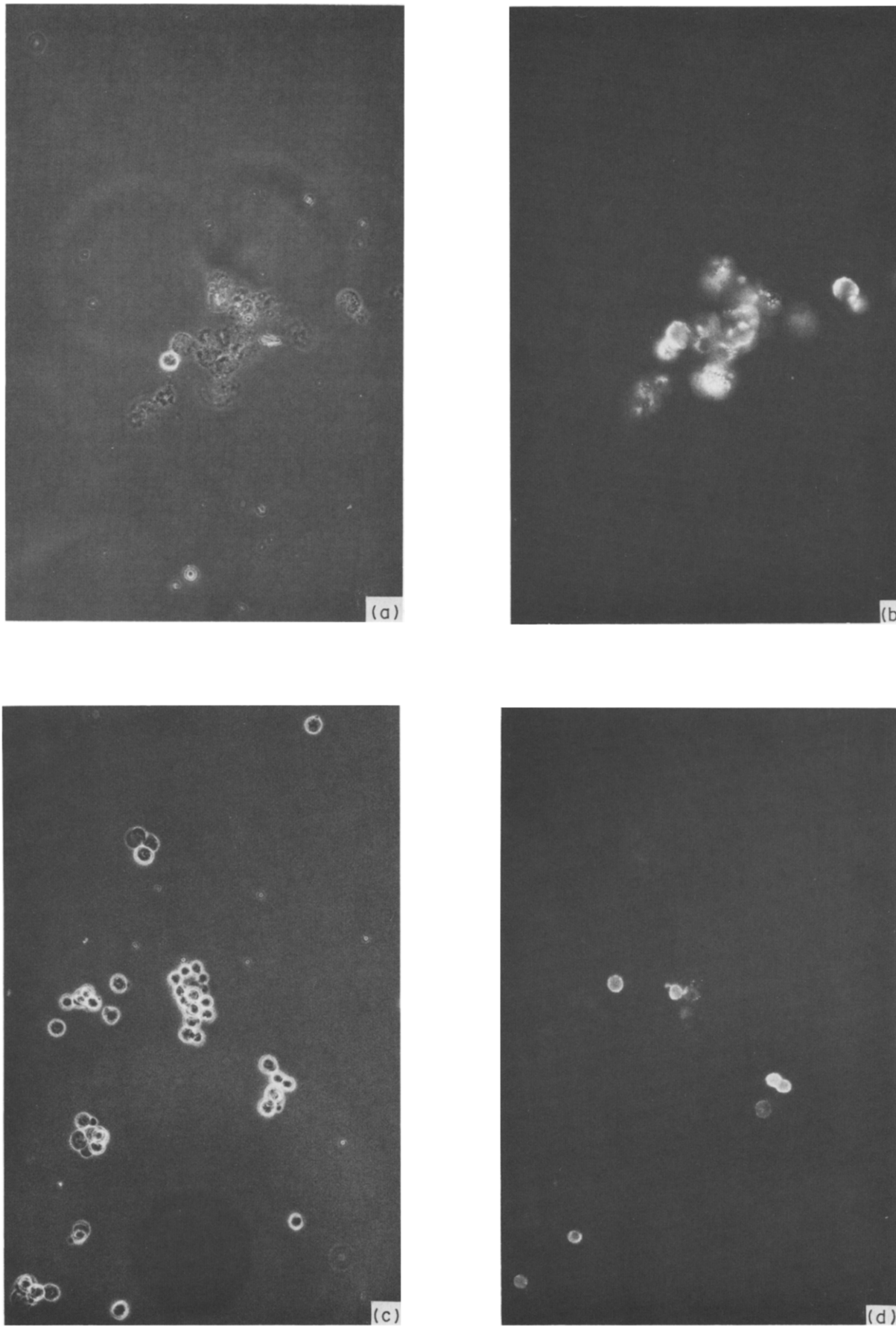

Fig. 3. Continued overleaf.

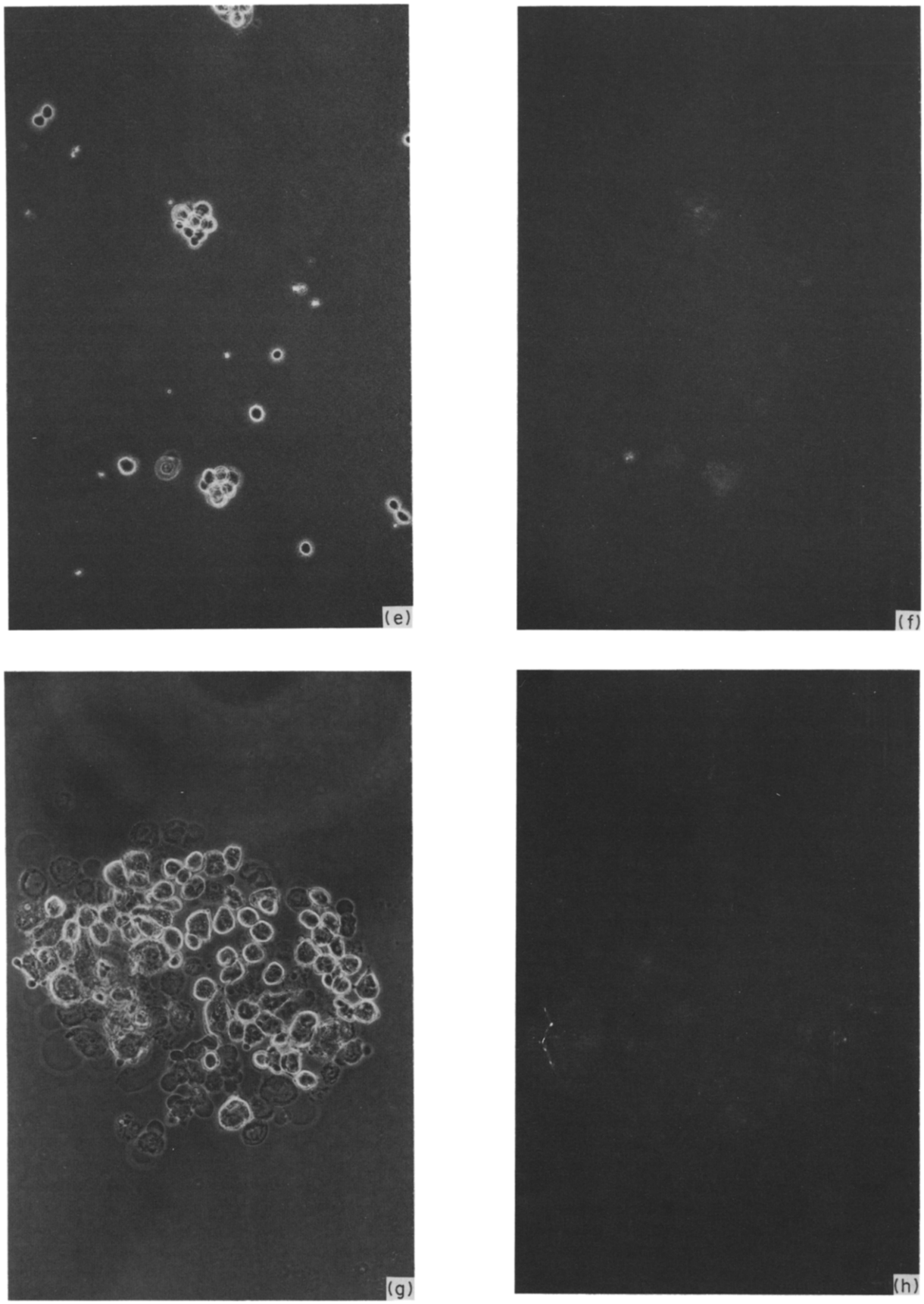

Fig. 3. Immunofluorescence on live cells with GCTM-2: (a) GCT 27 under phase-contrast; (b) same field as (a) under fluorescence; (c) HX 18 cells under phase-contrast; (d) HX 18 cells under fluorescence showing staining of subpopulation of cells in same field as (c); (e) HX 170 cells under phase-contrast; (f) HX 170 cells under fluorescence showing no staining; (g) BeWo cells under phase-contrast; (h) BeWo cells under fluorescence showing no staining of same field as (g).

GCTM-2 strongly stained 78% of seminomas tested. This heterogeneity in GCTM-2 expression in seminoma is in keeping with recent observations on intermediate filament expression which indicate that seminoma is not a homogeneous entity [16]. Furthermore, heterogeneity in staining for GCTM-2 has been observed within an individual seminoma, as well as between seminoma specimens (M.P.). So far, it has not been possible to separate staining from non-staining seminomas on clinical grounds.

GCTM-2 did not stain a number of non-germ cell tumours that were tested, with the exception of embryonal rhabdomyosarcoma and colorectal carcinoma where moderate staining was observed. Consistent with this was the observation that cultured cell lines from these tumour types expressed the GCTM-2 epitope, as did the choriocarcinoma cell line BeWo. However, the biochemical characteristics and cellular localisation of the antigen in these cell lines are different to that in EC. Enzymatic digestion of carbohydrates prior to immunoblotting of whole cell lysates of GCT 27 revealed that the antigen was sensitive to keratanase digestion. However, immunoblotting of whole cell lysates from HX 18, HX 170 and BeWo visualised protein bands considerably smaller than the 200 kD band in EC, whose electrophoretic mobility was not altered by treatment of the lysate with keratanase. This result suggested that in these cells the antigen was smaller, and lacked the extensive modification with keratan sulphate that was seen in EC. However, the faint immunoreactivity at approximately 200 kD in HX 18 preparations and the enhancement of this staining after keratanase treatment (Fig. 4) suggest the possibility that a proportion of these cells may express the epitope on an antigen similar to the EC proteoglycan. Immunofluorescent staining of live cells with GCTM-2 provided confirmation of the surface localisation of the antigen in EC. By contrast, although all fixed HX 18 cells reacted with GCTM-2, only 30% of live cells did, and no live HX 170 or BeWo cells were reactive, suggesting that in these cell types the antigen is inaccessible to the antibody on the surface, and therefore only internal. It remains to be determined whether the external epitope expressed on 30% of HX 18 cells is carried in the same form as the EC antigen or on the smaller internal antigen.

At one time, it was thought that the strongly hydrophilic proteoglycans had a purely structural or supportive function in tissues. However, most extracellular matrix proteins, and many growth factors can bind to glycosaminoglycans [8], and one core protein has been shown to contain epidermal growth factor like sequences [6]. The importance of proteoglycans in regulating cell growth and differentiation, particularly by binding to growth factors, has only recently come to light [8, 17–20]. The differentiation-dependent expression of the GCTM-2 antigen on human EC cells suggests its possible role in the presentation of cell attachment molecules and growth factors required for stem cell renewal.

The distribution of the GCTM-2 antigen in normal tissues does not conform to any previously described pattern, including that of known keratan sulphate proteoglycans [21]. It was thought for some time that keratan sulphate proteoglycans were confined to cornea and cartilage, but recent evidence suggests that they are more widespread than this [21]. Biochemical analysis of the EC proteoglycan core protein indicates that it is rich in serine, glycine, glutamic acid, and proline, and could be consistent with a number of hexapeptide repeats (S. Cooper *et al.*, Dept Zoology, Oxford University. *Biol J* in press), as found in the large aggregating bovine cartilage proteoglycan [22].

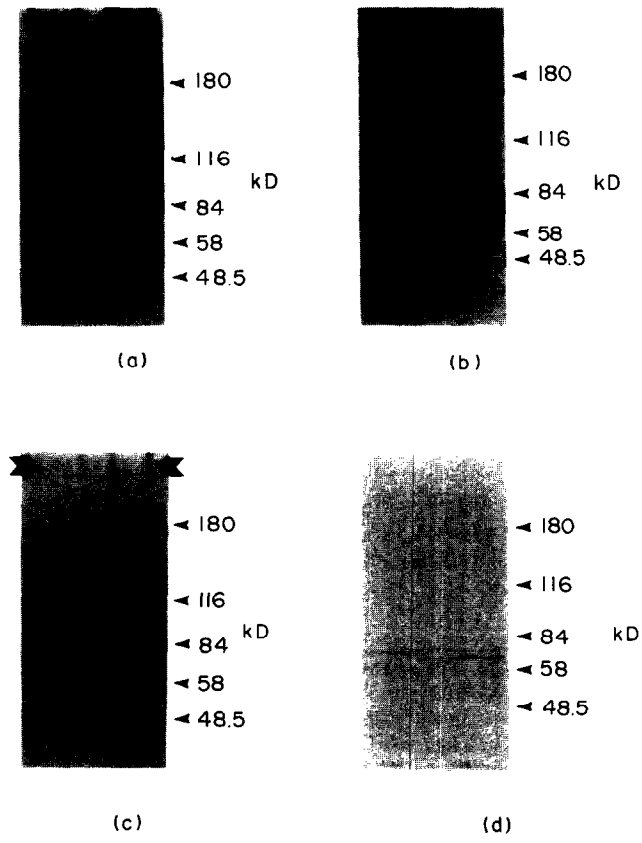

Fig. 4. Immunoblotting with GCTM-2 on whole cell lysates. *Left hand channels*—untreated; *right hand channels*—keratanase treated: (a) GCT 27; (b) HX 170; (c) HX 18; (d) BeWo. Faint immunoreactivity is seen at and above 200 kD to edge of the tracks in (c) (arrows).

However, the aminoacid composition of the core protein of the corneal keratan sulphate proteoglycan distinguishes it from this EC proteoglycan [23]. Furthermore, expression of the EC proteoglycan was not seen in normal fetal cornea and cartilage, and antibodies raised against the keratan sulphate proteoglycans of cornea and cartilage did not show reactivity with human EC cells. (M.P.). The keratan sulphate containing proteoglycan fibromodiulin is distinguished from our EC proteoglycan by its expression in mesenchymal tissues [24]. It is interesting that the expression of keratan sulphate proteoglycans has also been observed following the induction of differentiation of murine F9 EC cells in tissue culture [25].

The GCTM-2 proteoglycan is one of a number of surface antigens found on EC cells, from which it can be distinguished, including SSEA-3, SSEA-4, ABH blood group determinants, TRA-1-60, TRA-1-81, and 8-7D [26–29]. Monoclonal antibody FC 10.2 reacts with an EC membrane glycoprotein of molecular weight 200 kD [30]. The FC 10.2 antigen in EC has been shown to be a carbohydrate chain related to the type 1 blood group sequence [31]. The monoclonal antibody 5T4 was raised against a glycoprotein preparation from human syncytiotrophoblast plasma membrane, and reacts with a 72 kD glycoprotein on syncytiotrophoblast in human NSGCT, but only weakly with EC or yolk sac carcinoma [32]. Finally, a new surface antigen has been identified in EC using the monoclonal antibody 5F9, but it has not been biochemically characterised [33]. That it too is distinct from the GCTM-2 antigen is suggested by the absence of 5F9 staining in seminomas, and by the surface staining in

BeWo cells, though both of these observations require further confirmation.

The differing cellular localisation and biochemical structure of the antigen in EC and in other cells cannot be explained at present. It is possible that the antibody is cross-reacting with an epitope on unrelated proteins in the latter. However, the size of the antigen in these cells is close to that of the core protein in the EC proteoglycan. Furthermore, preliminary results using a second generation monoclonal antibody raised against the purified core protein also indicate reactivity with HX 170 cells on immunofluorescence (M.P.). A more likely possibility is that the GCTM-2 antigen is heavily modified with keratan sulphate and then secreted in EC, but remains internal in other expressing cells, in which the functions of the antigen may be different. In summary, the present results indicate that the GCTM-2 antigen is associated with an extracellular matrix proteoglycan in EC cells and possibly in a proportion of HX 18 cells, but not in other cell types tested. Further studies, using additional probes to the purified proteoglycan, will clarify the nature of the antigen in other tissues.

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Acknowledgements—We thank Professor B. Gusterson for his valuable suggestions and Professors A. Horwich and G. Steel for their advice and encouragement. This work was supported by the Cancer Research Campaign.