Localisation of Ca and HMFG2 antigens in breast tissue by immunoperoxidase, immunofluorescence, and immunoelectron microscopy

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SUMMARY The reactivities of Ca1 and HMFG2 monoclonal antibodies were compared on paraffin wax embedded breast tissues using indirect immunoperoxidase. The expression of Ca antigen, like HMFG2, is not exclusive to malignancy: Ca was present in 41/53 (77%) and HMFG2 in 42/53 (79.2%) non-malignant conditions and both were present in 33/35 (94%) carcinomas. Similar results were obtained when cryostat sections were used. Both antigens showed striking similarities in their topographical distributions, although quantitative differences were seen. Their cellular and sub-cellular localisations were investigated by double labelling immunofluorescence and immunogold electron microscopy, which showed that the expression of Ca and HMFG2 antigens was closely associated on cell membranes but that the epitopes were distinct.

Monoclonal Ca1 antibody, which defines an antigen Ca,¹ has been reported to react selectively with malignant lesions taken from a variety of tissues.² Subsequent studies have shown a significant degree of reactivity with non-malignant tissue.³⁻¹¹ Several monoclonal antibodies directed against epithelial membrane antigens react with breast tissue.¹²⁻¹⁴ The staining pattern reported for one of these, the human milk fat globule 2 (HMFG2) antibody,¹² appeared similar to our observations with Ca1 in a preliminary study of breast tissue.³ This study was undertaken to compare the occurrence, distribution, and staining patterns of Ca and HMFG2 antigens on formalin fixed, paraffin wax embedded and frozen tissues derived from malignant and benign lesions of the breast and to determine their cellular association by double label immunofluorescence and immunogold electron microscopy.

Material and methods

TISSUES

Formalin fixed, paraffin wax embedded breast tissues obtained from 4 normal, 49 benign, and 35

malignant lesions were used in the retrospective study. Fresh tissues from 20 benign and 24 malignant breast lesions obtained at operation and snap frozen in liquid nitrogen were used for the prospective study.

METHODS

Immunoperoxidase

Formalin fixed, paraffin wax embedded sections (6 μ m) were dewaxed and rehydrated to water before examination by a modification of the indirect immunoperoxidase procedure (Table 1).² Monoclonal antibodies Ca1 (IgM, culture fluid, Wellcome Diagnostics Ltd, reconstituted with 1.0 ml of distilled water) and HMFG2 (IgG1, culture fluid, Seward Laboratories) were diluted 1/10 and 1/5 respectively in phosphate buffered saline (PBS) and 10% bovine serum albumin (BSA) (Miles Laboratories) for use. As controls, monoclonal antibodies R4/23 (IgM, culture fluid, reactive against human dendritic reticulum cells)¹⁵ and WMB 36 (IgM, culture fluid, reactive with a meningococcal antigen)¹⁶ were used undiluted.

Immunofluorescence

Cryostat sections (6 μ m) were air dried, wrapped in

Table 1 Immunoperoxidase procedure

Dewaxed, rehydrated, formalin fixed, paraffin wax embedded sections
10/1 (vol/vol) methanol: H ₂ O ₂ (30%), 30 min, room temperature (blocks endogenous peroxidase activity)
Incubate with monoclonal antibody overnight, 4°C
Incubate with conjugate, 30 min, room temperature
Incubate with 500 µg/ml 3,3' diaminobenzidine hydrochloride (Sigma) containing 0.015% H ₂ O ₂ in phosphate buffered saline (PBS), pH 7.5, 5 min, room temperature
Counterstain with Mayer's haemalum, dehydrate, clear, and mount in DPX
Sections were thoroughly washed in PBS (3 × 5 min) between each step

cellophane, and stored at -20°C. Before use they were brought to room temperature, unwrapped, fixed in methanol (96%) for 30 s, and air dried. After a wash in PBS (5 min), the sections were incubated with the appropriate monoclonal antibodies (overnight, 4°C), washed with PBS (3 × 5 min), and incubated with fluorescein isothiocyanate (FITC) labelled rabbit antimouse Ig (1/20 in PBS, 30 min at room temperature, prepared in this laboratory). After a final wash in PBS (3 × 5 min), the tissues were mounted in PBS buffered glycerol containing 0.1% p-phenylenediamine¹⁷ before examination by fluorescence microscopy.

Double labelling immunofluorescence

Cryostat sections, treated as described above, were incubated with a mixture of Ca1 and HMFG2 monoclonal antibodies (1/1 vol/vol) overnight at 4°C. After a wash in PBS (3 × 5 min), sections were first incubated with tetramethylrhodamine (TRITC) labelled rabbit antimouse IgG (1/5 in PBS, 30 min at room temperature, Cappel Laboratories), washed in PBS (3 × 5 min) and then incubated with FITC labelled sheep antimouse IgM (1/20; in PBS, 30 min at room temperature, prepared in this laboratory). The TRITC conjugate also reacted with mouse IgM and was rendered IgG specific by preabsorption with mouse IgM. After a final wash in PBS (3 × 5 min) the tissues were mounted as described above and examined by a fluorescence microscope equipped with incident light illumination: excitation filter BP 450-490 barrier filter BP 520-560 for FITC; and excitation filter BP 546/12 barrier filter LP 590 for TRITC.

Double labelling immunogold

Freshly obtained breast tissue from a case of infiltrating duct carcinoma, known to express both Ca and HMFG2 antigens, was fixed with 1% formaldehyde in 0.1 M cacodylate buffer (1 h at room temperature), dehydrated in a graded series of ethanol, and embedded in LR white resin (London

Resin Company, Basingstoke). As a primary screen, areas of the tissue reactive with Ca1 and HMFG2 antibodies were first identified by light microscopy. All reagents were diluted with 0.5 M Tris buffer, pH 7.4, containing 1% (vol/vol) Tween 20, 1% (wt/vol) ovalbumin, and 0.1% gelatin.¹⁸ Sequential semithin (2 µm) sections were incubated with Ca1 (1/10) or HMFG2 (1/10) overnight at 4°C, washed in buffer (2 min), incubated with rabbit antimouse Ig (1/10, 30 min at room temperature), washed again in buffer (2 min), and finally incubated for 1 h at room temperature with an undiluted suspension of 40 nm gold spheres coated with goat antirabbit IgG (Janssen Pharmaceutica). The sections were washed with water (2 min), counterstained with 1% toluidine blue, and examined by light microscopy.

Ultrathin sections (90-100 nm thick) of areas reactive with both Ca1 and HMFG2 were prepared for subsequent double label immunoelectron microscopy. One face of the section was incubated with Ca1 monoclonal antibody (1/10 overnight, 4°C) followed by rabbit antimouse Ig (1/10, 30 min at room temperature), and lastly for 1 h with the 20 nm goat antirabbit IgG gold probe (1/20, Janssen Pharmaceutica). The grids were floated on drops of each reagent and sections were washed with buffer (5 × 1.5 min) between all incubations. Care was taken to avoid wetting the reverse side of the section. After a final wash in water the sections were air dried and coated with a protective film of carbon and, finally, with a supporting layer of celloidin. The opposite face of the section was then similarly treated with HMFG2 monoclonal antibody (1/10), rabbit antimouse Ig, and finally the 5 nm antirabbit gold probe (1/50, Janssen Pharmaceutica). The sections were contrasted with osmium tetroxide vapour before examination by electron microscopy. The labelling was then repeated using the 5 nm gold for Ca1 detection and the 20 nm gold for HMFG2 detection to avoid the possibility of labelling artefacts.¹⁹

Results

Localisation of Ca and HMFG2 antigens in formalin fixed, paraffin embedded and frozen breast tissue

The distributions of Ca and HMFG2 antigens in normal and benign breast tissue are shown in Tables 2 and 3. Of the 53 tissues studied, 41 (77%) were stained by Ca1 and 42 (79.2%) by HMFG2. Wherever the two antigens were coexpressed the tissue distribution was similar, although the staining intensity was variable. Ca and HMFG2 antigens were not detected in the mammary epithelium of two normal breasts. The lactating breast expressed HMFG2 strongly on the luminal surface of the acini and in the luminal secretion. Ca antigen was present

Table 2 *Distribution of Ca and HMFG2 antigens in normal and benign paraffin embedded breast tissue*

Condition	No	Ca1				HMFG2			
		Ducts		Acini		Ducts		Acini	
		LS	Cytoplasm	LS	Cytoplasm	LS	Cytoplasm	LS	Cytoplasm
Normal	2	—	—	—	—	—	—	—	—
Lactating	2	—	—	F(1)+	—	—	—	F(1)++	—
Papilloma	2	F(2)	Luminal surface of papillae or ducts				Similar to Ca1		
Fibroadenoma	17	F(9)+	F(1)++	—	—	F(10)++	F(1)+	—	—
		D(1)++							
Total no tissues =	23	No positive with Ca1 = 13 (56.5%)				No positive with HMFG2 = 14 (60.9%)			

LS = luminal surface of the epithelium.

F (focal) = positive scattered ducts or acini <10% of the total number.

D (diffuse) = positive ducts or acini >50% of the total number.

+—+++ = intensity of staining.

(%) = % tissues stained.

Table 3 *Distribution of Ca and HMFG2 antigens in sections of paraffin embedded fibrocystic disease of the breast*

Condition	No	Ca1			HMFG2		
		Epithelium		Myoepithelial cells	Epithelium		Myoepithelial cells
		LS	Cytoplasm	Cytoplasm	LS	Cytoplasm	Cytoplasm
Apocrine metaplasia	12	D(12)++—+++	F(4)++	—	D(12)++—+++	F(4)+	—
Adenosis	18	F(17)++—+++	F(2)++	F(3)++	F(16)++—+	F(2)+	F(3)++
Epitheliosis	12	F(11)++—+++	—	—	F(11)++—+++	—	—
		D(1)			D(1)		
Ducts	30	F(28)++—++	—	—	F(28)++—++	—	—
Cysts	30	F(28)++—++	—	—	F(28)++—++	—	—
Total no tissues =	30	No positive with Ca1 = 28 (93%)			No positive with HMFG2 = 28 (93%)		

LS = luminal epithelial surface.

F (focal) = scattered positive ductules.

D (diffuse) = almost all areas.

+—+++ = intensity of staining.

in smaller amounts and with similar distribution to HMFG2. Inter- and intralobular ducts were negative for both antigens.

We noted a similar distribution of both antigens in 10/17 fibroadenomas. The expression was invariably focal and random, but in one case almost all ducts showed positive epithelium. In nine tissues the antigens were localised on the luminal surface (Fig. 1a) and in one strong intracytoplasmic staining was detected (Fig. 1b). In papillomas focal surface positivity of the epithelium was noted.

The expression of both antigens in 28/30 cases of fibrocystic disease was either focal or diffuse (Table 3). All cases of apocrine metaplasia showed strong staining of the entire luminal surface and secretion and in 4/12 strong focal intracytoplasmic granular staining was present (Fig. 2). The ductules comprising foci of adenosis were also positive for Ca and HMFG2 antigens, but the number of positive ductules varied from case to case. In two cases strong intracytoplasmic staining was noted. Both antigens were expressed, mainly on the surface of the epithelium, in areas of epitheliosis.

An interesting finding was the identification of

both antigens in the cytoplasm of myoepithelial cells in three cases of fibrocystic disease. In these areas cytoplasmic staining of the adjacent epithelium was also noted; hence it is difficult to distinguish between expression or absorption.

In no case was staining seen with control monoclonal antibodies of unrelated specificity and Ca1 staining was not abolished by pretreating the sections with trypsin or preincubating with protein rich buffers.²

Table 4 shows the distributions of Ca and HMFG2 antigens in breast carcinomas. The same 33 of 35 (94.3%) tissues were stained by both Ca1 and HMFG2. Most ductal infiltrating carcinomas (18/24), regardless of histological grade or staging, showed surface and cytoplasmic staining of the malignant cells in more than half of the total tumour mass, although the density varied from site to site (Fig. 3). There was striking topographical similarity in the distribution pattern of both antigens with slight variations in intensity. In two tubular carcinomas both antigens were expressed on the luminal surface of ducts and tubules. There was a distinct quantitative difference in the expression of Ca and

Fig. 1 Fibroadenoma (paraffin section) stained with Ca1 and HRP antimouse Ig showing (a) luminal duct epithelial border ($\times 80$) and (b) intracytoplasmic staining ($\times 300$).

HMFG2 antigens in infiltrating lobular carcinoma. HMFG2 antigen was strongly expressed in most malignant cells in 4/5 cases; even the intracytoplasmic lumina were surrounded by a positive rim. By contrast, Ca antigen was expressed in occasional scattered cells in the four positive cases.

In order to determine whether tissue processing influenced the results, the reactivity and staining patterns of Ca and HMFG2 antigens were studied on cryostat sections from freshly collected material using immunofluorescence (Table 5). Immunofluorescence was selected because immunoperoxidase gave weak diffuse staining on cryostat sections irrespective of the level of monoclonal antibody used. There was no detectable difference in the incidence of reactivity with both antibodies when compared with the formalin fixed material.

Simultaneous detection of Ca and HMFG2 antigens by the double labelled immunofluorescence and immunogold procedures

Ca and HMFG2 antigens had close topographical distributions in all tissues examined. In order to determine the relative cellular localisation with greater precision, simultaneous staining with Ca1 and HMFG2 antibody was performed on single cryostat sections using immunofluorescence and on single ultrathin sections by the immunogold procedure.

When separate sections were treated with either Ca1 (IgM) or HMFG2 (IgG1) antibody followed sequentially by TRITC antimouse IgG and FITC antimouse IgM, fluorescent staining was observed only with the homologous conjugate. This confirms the specificity of the conjugates and the suitability of the microscope filter combinations used. When a

Fig. 2 Apocrine metaplasia duct epithelium (paraffin section) incubated with (a) Ca1, (b) HMFG2, and (c) control monoclonal antibody and HRP antimouse immunoglobulin showing staining of the luminal border and in the cytoplasm. $\times 300$.

Table 4 Distribution of Ca and HMFG2 antigens in paraffin embedded sections of breast carcinoma

Condition	No	Ca1		HMFG2	
		Epithelium		Epithelium	
		Surface	Cytoplasm	Surface	Cytoplasm
Ductal	24	F(5)++++ D(18)++++	F(10)++	F(4)++ D(19)++++	F(10)+++
Tubular	2	F(1)+++ D(1)+++	—	— D(2)+++	—
Lobular	5	F(4) occasional scattered cells (+++)	—	F(2)++ D(2)+++ F(3)++	F(2)++ D(2)+++ F(2)++
Medullary	3	F(3)+	F(2)+	—	—
Mucoid	1	—	—	—	—
Total no tissues =	35	No positive with Ca1 = 33 (94.3%)		No positive with HMFG2 = 33 (94.3%)	

F = luminal surface of scattered ducts (<5% of total number).

D = luminal surface or cytoplasm of malignant cells and ducts >50% of the total number.

mixture of the antibodies was applied to a single section, both antigens were detected in all eight malignant and five benign tissues studied. In the majority, both labels were detected in the same groups of cells, confirming the similar topographical distribution of Ca and HMFG2 antigens on a single

preparation (Fig. 4). Preincubation of the section with an excess of Ca1 or of HMFG2 antibody did not inhibit binding of the other antibody, which confirms that the double labelling seen was not due to sub-optimal levels of antibody in the mixture.

The relative sub-cellular localisation of Ca and HMFG2 antigens in selected ultrathin sections of an infiltrating duct carcinoma was examined by double labelling immunogold electron microscopy. Experiments, in which Ca1 antibody was labelled with the 20 nm gold probe and HMFG2 with the 5 nm gold probe, showed that Ca and HMFG2 were closely associated on the membranes of the same cells (Fig. 5a), although HMFG2 was occasionally present in the absence of Ca (Fig. 5b). The same result was obtained when the probes were reversed, thereby excluding the possibility of artefacts due to probe size.

Discussion

This study confirms earlier observations that immunohistochemical staining with Ca1 on tissue sections fails to discriminate between malignant and benign lesions of the breast.^{3,4,11} The use of fresh material failed to improve this differentiation, which

Fig. 3 Lobular carcinoma (paraffin section) of the breast stained with Ca1 and HRP antimouse immunoglobulin showing cytoplasmic staining and heterogeneity of antigen expression. $\times 280$.

Table 5 Occurrence of Ca and HMFG2 antigens in cryostat sections of breast tissue detected by indirect immunofluorescence

Condition	No	Reactivity with	
		Ca1	HMFG2
Benign lesions			
Fibrocystic disease	9	7	8
Fibroadenoma	11	5	7
Total	20	12	15
% tissues stained		60	75
Malignant lesions			
Infiltrating duct carcinoma	24	24	24
% tissues stained		100	100

Fig. 4 Fibrocystic disease (cryostat section) double labelled with (a) FITC antmouse IgM (Ca1) and (b) TRITC antmouse IgG (HMFG2). Note the occurrence of staining in similar groups of cells with both antibodies. $\times 160$.

is in agreement with results reported on fine needle breast aspirates.⁶ The similarity between results obtained with formalin fixed and fresh frozen tissues excludes the possibility that poor specificity of Ca1 for malignant cells is due to artefacts resulting from component polymerisation during fixation.

Although breast carcinomas invariably expressed intracytoplasmic staining with both antibodies, intracytoplasmic reactivity was noted in the ductal epithelium of fibroadenomas or fibrocystic disease, albeit infrequently on paraffin embedded tissue. It has been suggested that intracytoplasmic staining of benign epithelium may be associated with lesions which progress to malignancy,²⁰ but only retrospective follow up studies will provide conclusive data.

Our comparative immunohistochemical studies with Ca1 and HMFG2 antibodies on sequential sections showed a striking similarity in the topographical distribution of both antigens, although quantitative differences were noted. This similar localisation suggests that the expression of epitopes recognised by Ca1 and HMFG2 antibodies is intimately associated. Synchronous expression of Ca and HMFG2

antigens is not, however, always present.

Physicochemical and immunohistochemical studies indicate several similarities between Ca and HMFG2 antigens.^{21 22} Both are tumour associated membrane glycoprotein antigens, which are also expressed in normal, hyperplastic, or benign epithelium.^{3 11 12} Ca1 and HMFG2 antibodies recognise determinants on high molecular weight components derived from mammary carcinoma cell lines. The antibody binding is blocked by wheat germ lectin, which reacts with β -(1,4)-N-acetylglucosamine and sialic acid residues. The determinants recognised by HMFG2 antibody exhibit a greater diversity of molecular size (80–400K) than those defined by Ca1 (350–390K), but this difference may be a reflection of the analytical methods used.^{21 22} In view of the physicochemical similarities and the almost identical distribution described above, we examined the relation of the two antigens at light microscopy and ultrastructural level.

We have shown that both antigens are similarly distributed, although they may not be simultaneously expressed. Double labelling immunofluores-

cence showed that both antigens were present on the same group of cells on the luminal surface as well as in the cytoplasm of mammary epithelium. The

finding that both antigens can be detected simultaneously on the same section and that Ca1 and HMFG2 binding was not inhibited by preincubation

Fig. 5 *Infiltrating duct carcinoma double stained by the immunogold procedure using (a) 20 nm gold probe for Ca and 5 nm for HMFG2 (b) 5 nm gold probe for Ca and 20 nm for HMFG2. Note the cellular coexistence of Ca and HMFG2 in (a) and HMFG2 in the absence of Ca in (b).*

with an excess of the heterologous antibody indicates that the antigenic epitopes, although closely situated, are not identical and that they are topographically sufficiently distant to prevent inhibition due to configurational effects.

These conclusions were confirmed and extended to the cellular level by the simultaneous staining with immunogold preparations of defined diameter and examination by electron microscopy. These double staining results for the first time provide conclusive evidence that Ca and HMFG2 antigens are closely, but not exclusively, associated at the cellular level, topographically separate, and retain distinct epitope specificity. Their expression may be related to the functional activity of individual cells and this phenomenon needs further investigation.

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