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Breast Gross Cystic Disease Protein 15 in Human Breast Cancer in Culture

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Abstract—*Media from explants of* 120 *human breast cancers cultured for* 24 h *were analysed for breast gross cystic disease protein* 15 (*GCDFP*-15). *The protein was detected in media from* 94 *tumours* (73%) *in concentrations varying from* 1.5 *to* 2100 ng/ml. *Levels were not related to menopausal status of the patient, disease stage, tumour oestrogen receptors or the derivation of tumour material. However, concentrations were significantly related to the degree of apocrine differentiation of the tumour and, in a subset of the cancers, capacity to release GCDFP*-15 *was positively correlated with incidence of progestogen and androgen receptors.*

There was also a negative quantitative correlation between production of GCDFP-15 *and glycoprotein hormone* α *subunit (but no relationship with CEA). In* 10 *tumours culture was continued for* 48 h. *Values for GCDFP*-15 *were always considerably lower in the* 24–48 h *media compared with those from* 0–24 h *and cytosols from post-culture explants contained no detectable GCDFP*-15. *In contrast, CEA levels were often comparable in* 0–24 *and* 24–48 h *media and explants after culture frequently contained substantial amounts of CEA. The high proportion of breast carcinomas producing GCDF*-15 *in relatively large amounts and its rapid release make it an interesting marker by which the* in vitro *activity of human breast cancers may be monitored.*

INTRODUCTION

GCDFP-15 is a major protein constituent of human breast cyst fluid [1]. The protein is also produced in large quantities by certain human breast cancers [1–3] but little is known about the characteristics of cancers which produce GCDFP-15. The aim of the present study was to determine factors which might influence GCDFP-15 levels, by examining cytosols and fluid from cultured explants of breast cancers.

MATERIALS AND METHODS

Patients

Tumour was obtained from 120 women with histologically proven breast cancer. This material was derived from the primary tumour in 105 cases and invaded axillary lymph node in 17 cases (both primary and lymph node were available in two patients). At the time of study 23 patients were premenopausal (experiencing regular menstrual periods), 85 were postmenopausal (at least 5 years since their last menstrual period), nine were perimenopausal and three patients had undergone a previous hysterectomy and their menopausal status was unclear. Routine clinical staging showed 22 patients to have distant metastatic disease; of the remaining 97 women with evidently early disease, 50 were shown histologically to have axillary lymph nodes involved with tumour at the time of primary surgery (lymph nodes being routinely obtained either by total axillary clearance or by lower axillary sampling).

Organ culture

Following excision, tumours were immediately placed on ice. Sufficient material was removed for histopathological diagnosis and oestrogen receptor measurements. The remainder was used in organ culture studies. Each tumour was cut into explants measuring 3 × 1 × 1 mm. Four explants were then mounted on lens paper on stainless steel grids placed in 30 × 10 mm Petri dishes. Waymouth's MB 752/1 medium containing *N*-glutamine and 20 mM hepes (2 ml) was added to each dish and incubated in 95% O_2/5% CO_2 at 37°C for 24 h. Cultured medium was removed for estimation of

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tumour markers and explants fixed in formal saline prior to histological examination. Depending on the quantity of material available, between two and six replicate cultures were set up for each tumour. Certain tumours were cultured for 48 h. After the initial 24 h culture period, medium was replaced with fresh fluid and cultured for a further 24 h. Both 0–24 and 24–48 h media were used for measurement of tumour markers. Post-culture explants were pulverized in liquid N_2 and extracted with 2 ml of culture medium. After centrifugation, the resultant cytosol was removed and used for the estimation of tumour markers. In these studies, cytosols were also obtained from replicate explants before culture.

Estimates of GCDFP-15

GCDFP-15 was measured by radioimmunoassay using a modification of the method previously described [4]. In brief, rabbit anti-GCDFP-15 antibody (diluted × 40,000, 100 μl), culture media (100 μl), ^{125}I-labelled GCDFP-15 (approx. 20,000 cpm/100 μl) were incubated with trasylol (50 unit/100 μl) in ammonium acetate buffer (0.1 M pH 7.0) overnight at room temperature. The antibody was then precipated with normal rabbit serum (× 1000, 100 μl) and donkey anti-rabbit serum (× 20, 100 μl) and left overnight at 4°C. Ammonium acetate buffer (1 ml) was added and mixed before centrifugation at 800 ***g*** for 10 min. The supernatant was decanted off and the tubes counted in a gamma well counter.

Other measurements

Carcinoembryonic antigen (CEA) and glycoprotein hormone α subunit were measured by radioimmunoassay, the former employing the method by Sturgeon *et al.* [5] and the latter that of MacFarlane *et al.*[6]. All steroid receptors were measured by saturation analysis, oestrogen receptors using the method of Hawkins *et al.* [7] and those for progestogen and androgen the method of Miller *et al.* [8].

Histological assessment of aprocrine features

These determinations were performed by one of us (AAS) without prior knowledge of the biochemical results. Characteristics upon which apocrine grading was based included the presence of (i) copious acidophilic granular cytoplasm, (ii) basophilic or vesicular nuclei with prominent nucleoli and (iii) bulbous contours at the glandular margins, when present.

RESULTS

Of the 120 breast carcinomas cultured, GCDFP-15 was detected in the media of replicate cultures in 94 (78%) and 22 tumours had undetectabe levels of GCDFP-15 (< 1.5 ng/ml) in the media of their replicate cultures. The remaining four tumours had undetectable levels in some replicate cultures but low amounts in others; their production was deemed equivocal and these tumours have not been considered in any further analysis. Media cultured in the absence of explants or explants which had been boiled in water for 10 min prior to culture did not contain detectable levels of GCDFP-15. The concentration of GCDFP-15 found in the culture media of the 94 breast cancers positive for the marker varied from 1.5 to 2100 ng/ml (median value 11 ng/ml). In view of the large range of values, factors which might influence levels were examined.

(a) Menopausal status: there was no significant association between menopausal status and levels of secreted GCDFP-15.

(b) Extent of disease: there was no significant difference in GCDFP-15 production in tumours obtained from patients who at the time of study either had clinically early breast cancer (with or without lymph node involvement) or advanced disseminated disease.

(c) Tumour derivation: no significant difference was apparent between the different sources of tumour material. It was possible to study both primary tumour and invaded lymph node in two patients: in one case levels of GCDFP-15 were higher in culture media from the primary whereas in the other values were higher in the lymph node.

(d) Tumour histology. Whilst no correlation was detected between tumour grade and level of GCDFP-15 released (data not shown), a highly significant relationship existed between the marker and degree of apocrine differentiation within the tumours. Thirty-six tumours showed no evidence of apocrine cells, 71 cancers possessed apocrine cells but these were not a predominant feature and 13 tumours were characterized by a marked degree of apocrine differentiation. There was a significant trend for the level of GCDFP-15 to be higher in fluids from tumours with apocrine characteristics, particularly when this was a predominant feature (Fig. 1). Thus most of the tumours failing to release GCDFP-15 were without apocrine cells and none of these tumours released more than 10 ng/ml. In contrast all tumours, in which apocrine differentiation was marked, were associated with culture media containing in excess of 30 ng/ml. The tumour releasing the greatest amounts of GCDFP-15 (2100 ng/ml) only displayed moderate apocrine features but this was a highly cellular tumour of unusual cell type—the same tumour also released the highest amount of CEA and α subunit.

(e) Tumour oestrogen receptors: 100 tumours were oestrogen receptor positive (values in excess of 5 fmol/mg cytosol protein), 19 were negative and one assay was invalid because the patient was

Table 1. *The relationships between GCDFP-15 production and (a) progestogen receptor and (b) androgen receptor status*

(a)	Progestogen receptor + ve	Progestogen receptor − ve	(b)	Androgen receptor + ve	Androgen receptor − ve
+ ve GCDFP-15	14	16	+ ve	11	19
− ve	2	18	− ve	2	18
	$\chi^2 = 7.41$	$P < 0.01$		$\chi^2 = 4.43$	< 0.05

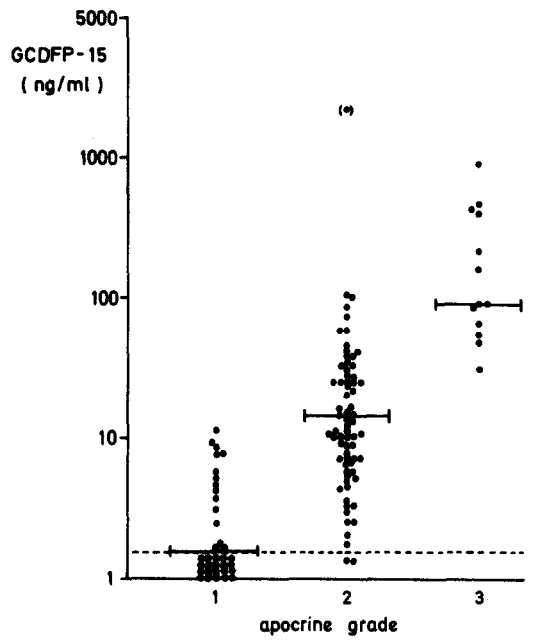

Fig. 1. *The association between levels of GCDFP-15 in cultured media and degree of apocrine differentiation in the tumour. Grade* 1 *tumours display no or minimal apocrine features. Grade* 2 *possess apocrine cells but this is not a marked feature and Grade* 3 *have apocrine characteristics as a predominant feature. The differences between each group is highly significant* ($P < 10^{-7}$) *by Wilcoxon Rank Test.*

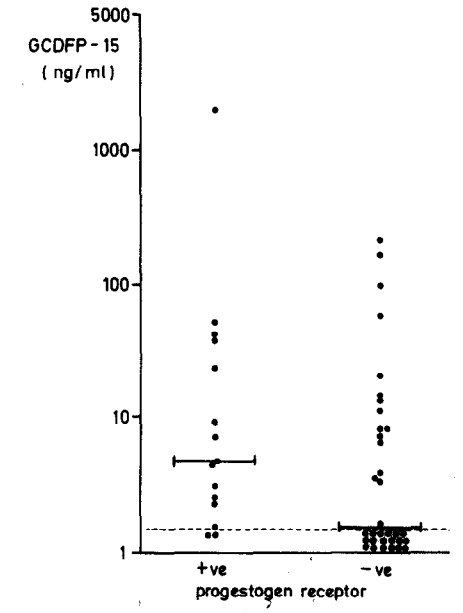

Fig. 2. *Comparison of GCDFP-15 levels in the cultured media from tumours with and without progestogen receptors. No statistically significant difference between the groups.*

receiving tamoxifen at the time of biopsy. There was no significant difference in amounts of GCDFP-15 produced by oestrogen receptor positive and negative tumours. Neither in oestrogen receptor positive tumours was there a quantitative relation between level of receptor and amount of marker in the media (data not shown).

(f) Other steroid receptors: progestogen and androgen receptors were measured in a subset of 50 tumours. Of these, 16 cancers (32%) were positive for progesterone receptors and 11 were positive for androgen receptors (22%), containing in excess of 5 fmol/mg cytosol protein in each case. There was a significant positive correlation between capacity to produce GCDFP-15 and the presence of progestogen receptor (Table 1) but quantitatively there was no significant difference between levels of GCDFP-15 found in media from progestogen receptor positive and negative tumours (Fig. 2). Androgen receptors were similarly positively associated with GCDFP-15 production (Table 1) and additionally levels of GCDFP-15 in androgen receptor positive tumours were significantly higher than in receptor negative tumours (Fig. 3).

(g) Other tumour markers: CEA was measured in the medium from 116 of the same breast carcinomas. In 95 tumours (82%) CEA was detected in all replicate cultures. Seventeen tumours had undetectable levels of CEA (< 3 ng/ml) and four tumours were classified as equivocal. No quantitative relationship was detected between production of the two markers (Table 2). Furthermore, levels of GCDFP-15 were not significantly different between tumours producing and not producing CEA. There was also no obvious quantitative correlation between levels of the markers in tumour releasing both products. Glycoprotein hormone α subunit was measured in the media from 98 of the tumours. In 26 tumours α subunit was detected in replicate cultures; 65 had undetectable levels (< 0.5 ng/ml) and seven were classified as equivocal. Although

Table 2. The relationships between GCDFP-15 production and (a) CEA and (b) glycoprotein hormone α subunit

(a)	CEA		(b)	Subunit	
	+ ve	− ve		+ ve	− ve
+ ve GCDFP-15	74	12	+ ve	19	55
− ve	19	3	− ve	8	8
	$\chi^2 = 0.00$	P = NS		$\chi^2 = 4.53$	$P<0.05$

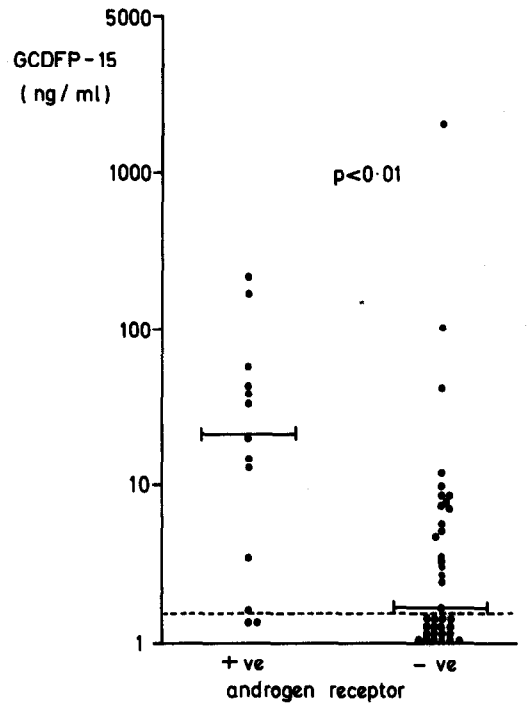

Fig. 3. Comparison of GCDFP-15 levels in the cultured media from tumours with and without androgen receptors. The difference between the groups is statistically different (P < 0.01 *by Wilcoxon Rank Test*).

there was a significant inverse qualitative association between GCDFP-15 and α subunit (Table 2) no quantitative relationship was apparent between the markers.

Ten tumours were cultured for 48 h with medium being replaced with fresh fluid after 24 h. The media for the two 24 h periods were analysed separately for GCDFP-15 and CEA. In comparison with levels in 0–24 h media, values of GCDFP-15 in the 24–48 h were always considerably lower, indeed apart from the two tumours producing the greatest amount at 24 h, levels were undetectable in the 24–48 h media (Table 3). In five systems, cytosols were prepared from post-culture explants and from replicate explants before culture. In each case GCDFP-15 was undetectable in cytosols from post-culture explaints. The marker was however detected in all but one of the preculture explants. The corresponding pattern of release for CEA is also shown in Table 2. Whilst levels in 24–48 h media were lower than those in 0–24 h media, the values were comparable. Additionally, in contrast to GCDFP-15, significant amounts of CEA were detected in the cytosols of post-culture explants. However, as with GCDFP-15, the combined values for CEA in the media and post-culture cytosols exceeded those for the marker in the pre-culture cytosol.

DISCUSSION

GCDFP-15 is a major protein found in human breast cyst fluids where it may be present in the milligram amounts [1]. It also appears to be produced by human breast cancers [1–2]. Levels in plasma may help in assessment of tumour burden [9] and sequential measurements following therapy may be useful in monitoring response to treatment [10].

We have shown in a large number of tumours that explants of breast cancer release GCDFP-15 during culture. In the present series about 80% of breast carcinomas liberated measurable amounts of the marker into the culture medium, an incidence very similar to that detected by immunohistochemical studies [11]. Whilst the remaining 20% of tumours apparently did not produce GCDFP-15, this was not due to the absence of malignant cells within these explants, tumour presence being confirmed histologically after culture. Furthermore, most GCDFP-15 negative tumours produced other markers such as CEA.

The high proportion of breast carcinomas producing GCDFP-15 in large quantities makes its synthesis of interest and factors which might influence production in cultured explants have been examined. Levels of GCDFP-15 were not influenced by the patient's menopausal status, stage of disease, tumour oestrogen receptor activity or whether the biopsied material was from primary tumour or lymph node. It thus seems unlikely that tumour production of the protein increases during the developmental stages of the disease or is a reflection of tumour aggressiveness. The lack of correlation with oestrogen receptors and lymph node invasion means that GCDFP-15 is also unlikely to be of prognostic

Table 3. *Effects of culture on marker levels (ng/ml)*

GCDFP-15	1	2	3	4	5	6	7	8	9	10
Pre-culture explants	—	—	—	—	9.2	10.8	2.7	<1.5	8.0	9.8
Day 1 media	104	170	59	39.6	15.3	11.9	8.9	<1.5	12.2	12.5
Day 2 media	7	46	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5
Post-culture explants	—	—	—	—	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5
CEA										
Pre-culture explants	—	—	—	—	10.4	38.0	5	17	51	17
Day 1 media	21.3	156	13	<3	7.3	25.1	<3	15	59	16
Day 2 media	11.3	119	5	<3	5.7	21.0	<3	11	32	10
Post-culture explants	—	—	—	—	6.2	31.0	8	17	48	18

significance although clinical follow-up of the patients in this study will be necessary before this may be definitively resolved. Interestingly, tumour production of GCDFP-15 was positively associated with progestogen receptors. Although a positive relationship has been reported by others [11], it is important to note that this previous observation related to quantitative levels of GCDFP-15 whereas in the present study the association is qualitative and a quantitative difference in GCDFP-15 between progestogen receptor positive and receptor negative tumours was not detected. Because of the association with progesterone receptor and variation in expression in normal breast during the menstrual cycle [2] it has been suggested that GCDFP-15 levels may be regulated by oestrogen [11, 12]. The data in the present study would not support this contention. No relationship of GCDFP-15 has been found with the oestrogen receptor and it must be emphasized that the qualitative relationship with the progestogen receptor result from tumours without GCDFP-15 production being more likely to be progestogen receptor negative; carcinomas expressing GCDFP-15 were equally likely to be positive or negative for the progestogen receptor and levels of the marker released during culture are independent of progestogen receptor status.

As has been reported by others, the major factor which determines the expression of GCDFP-15 by breast cancers appear to be degree of apocrine differentiation within the tumour [11, 13]. Thus, apart from a single carcinoma, tumours releasing large amounts of GCDFP-15 into culture media all possessed apocrine characteristics as a marked histological feature; the single exception was an extremely cellular tumour of unusual cell type which also released large quantities of other marker proteins. If it is assumed that GCDFP-15 is marker of apocrine activity, the observations that 80% of breast carcinomas produce GCDFP-15 would suggest that a higher proportion of tumours possess cells with apocrine features than has been recognized previously [14–18] by standard histological criteria.

Androgens appear to regulate apocrine secretion [19] and to stimulate GCDFP-15 production [20]. The latter findings would be supported by results from the present study in which a positive correlation was found between GCDFP-15 and androgen receptors. Levels of GCDFP-15 in androgen receptor positive cancers were significantly higher than those in receptor negative tumours. However the presence of androgen receptor protein did not appear to be a prerequisite for GCDFP-15 expression since one half of receptor negative tumours also released the marker during culture. Some tumours which display apocrine features contain high concentrations of endogenous androgens [21] which could occupy vacant receptor sites, and thus lead to these tumours being falsely classified as androgen receptor negative. However, it is unlikely that this phenomenon occurred in all cases.

Other factors regulating the release of GCDFP-15 still have to be identified but they are likely to be different from those controlling other markers. Not only was there no qualitative relationship between GCDFP-15 and CEA but, in tumours producing both markers, there was no quantitative relationship between levels in the culture media. CEA and GCDFP-15 also displayed different patterns of release into the culture media. Most GCDFP-15 was released within 24 h and the marker was absent or present in only small amounts in 24–48 h media and post-culture explants. In contrast, CEA was released more gradually and there were significant amounts in both 24–48 h media and post-culture explants. The large difference in molecular size beween the two proteins may be one factor accounting for these variations, GCDFP-15 being a much smaller molecule than CEA. The rapid release of GCDFP-15 into culture medium may be useful in determining factors influencing its synthesis. It also means that changes in production are more easily detected than with other secretory products which have slower turn-over rates.

In summary, GCDFP-15 is a protein which is rapidly released from the majority of breast cancers during short-term culture. Its production is particularly associated with tumours displaying apocrine features histologically. The significant relationship

with androgen receptors also suggest that GCDFP-15 may be useful as a marker of androgenic sensitivity.

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