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Radioimmunoassayable Insulin-like Growth Factor-I in Human Breast Cyst Fluid

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Abstract—*Insulin-like growth factor-I (IGF-I) was measured in breast cyst fluid and serum collected at the same time. In addition, epidermal growth factor* (EGF) *was measured in breast cyst fluid. The radioimmunoassay inhibition curve of cystic IGF-I was parallel to that of authentic IFG-I. Cystic immunoreactive IGF-I had an elution pattern similar to IGF-I from Sep-Pak C_{18} silica columns and from gel-filtration chromatography using Sephacryl* 200. *The material from Sephacryl* 200 *gave a radioimmunoassay curve which was parallel to IGF-I.*

The median (range) amount of IGF-I in cyst fluid was 9.3 ng/ml (0.8–33.3 ng/ml) *and was* 5–10% *of that found in serum, although there was no significant correlation between the two levels. The median (range) level of cystic EGF was* 416 ng/ml (14–1640 ng/ml). *There was a weak negative correlation between cystic EGF and cystic IGF-I* ($P < 0.01$).

Some women presented with multiple cysts and for these cysts there was a high degree of concordance of levels for both IGF-I or EGF.

Unlike serum there was little or no protein present in cyst fluid which bound IGF-I.

INTRODUCTION

WOMEN with gross cystic disease have a 2–3-fold increased risk of breast cancer [1]. It is likely that this is associated with atypical hyperplastic epithelial changes [2, 3]. Cystic fluid is probably derived from abnormal epithelial cells and therefore provides a unique opportunity to study the electrolyte and hormonal background of cystic change. Many studies have been made on the composition of cyst fluid in an attempt to obtain an insight into the mechanism of this increased risk [see 4].

Lippman *et al.* have recently suggested that growth factors are of importance in the autocrine or paracrine control of breast carcinogenesis [5]. Compared with serum, there are highly elevated levels of epidermal growth factor (EGF) in cyst fluid [6] but the factors cited by Lippman *et al.* [5] comprised transforming growth factors α and β (TGF-α and TGF-β) and insulin-like growth factor-I (IGF-I).

No information was available on IGF-I and EGF determined simultaneously on the same cyst fluid and therefore these have been measured. In addition, IGF-I was measured in synchronously collected serum samples.

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MATERIALS AND METHODS

Cyst fluid

Cyst fluid was obtained by aspiration from 80 women attending the Clinical Oncology Unit, Guy's Hospital. None of these patients had underlying breast cancer. Blood was also collected at the same time and serum prepared. Specimens of cyst fluid and serum were stored at −20°C until analysis.

This was part of a large study examining hormones and growth factors and as a result the available amounts of cyst fluid were insufficient in some cases to assay both IGF-I and EGF in the same sample. Thus of the 80 women 68 and 64 had IGF-I measured in cyst fluid and blood, respectively, and 67 had EGF measured in cyst fluid.

Insulin-like growth factor-I assay

The levels of IGF-I were measured by radioimmunoassay using an antiserum (UBK 487) kindly supplied by Drs. Underwood and Van Wyk and iodinated IGF-I (catalogue number IM 172; 2000 Ci/mmol; Amersham International plc). The method of assay was essentially that outlined by Underwood and Van Wyk [7, 8] except a solid-phase second antibody was used (Sac-Cel; Cat. No. RD70; Wellcome Reagents Ltd.) and the procedure used was as outlined by the manufacturers. The

volume assayed was 5 μl for serum and 25 or 50 μl for cyst fluid. The final concentration of the antisera was 1 in 16,000. The IGF-I was measured in serum or cyst fluid in batches of about 50 samples and the inter- and intra-assay variation was less than 10%. IGF-I was obtained from Bachem Inc. (catalogue number PGR020).

The specificity of the antibody was such that it has 0.5% cross-reaction with IGF-II and minimal cross-reaction with insulin at 10^{-6} molar. It is also noteworthy that the concentration of insulin in cyst fluid has been reported to range from 0.3 to 2.3 ng/ml [9].

Acid extraction of IGF-I bound to blood-borne carrier protein was performed as described by Daughday *et al.* [10].

Epidermal growth factor assay

EGF concentrations were determined using a double-antibody radioimunoassay method described by Gregory *et al.* [11] except the antibody-bound EGF was separated from unbound EGF using a solid-phase second antibody (Sac-Cel; Wellcome Reagents Ltd.). EGF and its antiserum was generously donated by Dr. H. Gregory. The antibody has negligible cross-reaction with TGF-α, IGF-I or human growth hormone [12].

RESULTS

Specificity of assays

(a) *Insulin-like growth factor-I.* The specificity of the assay for IGF-I was based on several criteria. Firstly, there was the high quality of the antibody and its minimum cross-reaction with insulin and IGF-II already alluded to. Secondly, the inhibition curves obtained for varying amounts of cyst fluid were essentially parallel to that found for IGF-I standard (Fig. 1). Thirdly, immunoreactive IGF-I from cyst fluid behaved in a similar fashion to authentic IGF-I in two chromatographic systems. Thus immunoreactive IGF-I was absorbed and eluted from Sep-Pak C_{18} columns (Millipore Cat. No. 51910) and its elution pattern when subjected to gel-filtration chromatography (Sephacryl 200; 26 mm × 40 cm) was similar to that of authentic IGF-I (Fig. 2). The leading, middle and trailing fractions of this peak were pooled. Each pooled fraction gave an inhibition curve parallel to that of authentic IGF-I. To save repetition only one of these inhibition curves is shown in Fig. 3. Furthermore, the concentration of IGF-I in cyst fluid after chromatography in these two systems was similar to that found before chromatography when correction was made for procedural losses.

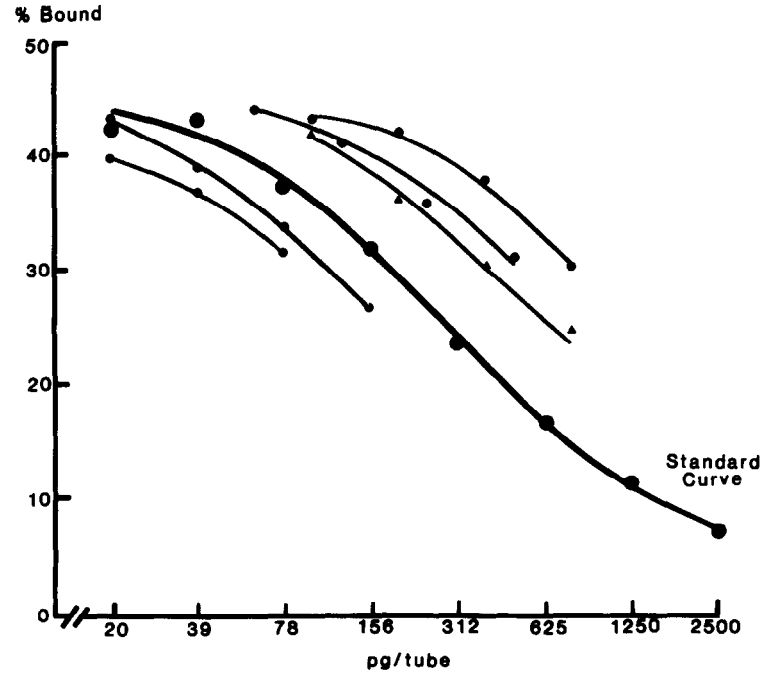

Fig. 1. Inhibition curves of immunoreactive IGF-I in cyst fluid. The inhibition curve of [^{125}I]IGF-I binding by increasing amounts of authentic IGF-I is denoted as 'standard curve'. The other curves show the inhibition of binding with serial dilution of five representative cyst fluids. The assay system was as described in the text and the volumes of cyst fluid used were 100, 50, 25 *and* 12.5 μl. *In some cases the titre of* 12.5 μl *of cyst fluid was too small to be sensibly recorded and therefore some curves exhibit only three points. The total radioactive counts was* 4000 *cpm.*

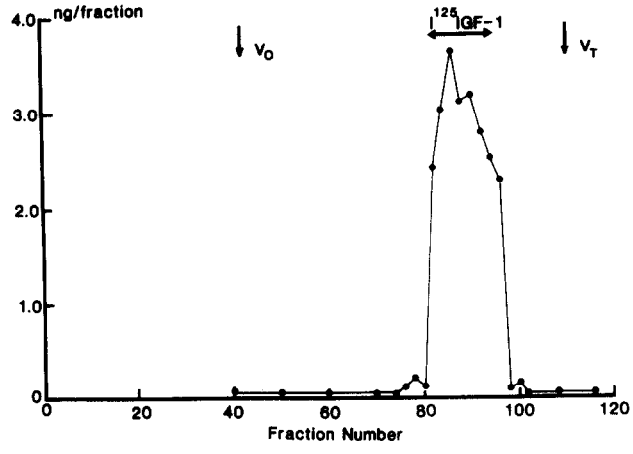

Fig. 2. Gel filtration of cyst fluid IGF-I. The elution pattern represents the immunoassayable IGF-I from 10 ml *of a cyst fluid pool prepared as described in the text. The column* (26 mm × 40 cm) *was Sephacryl* 200 *and the fraction size was* 2 ml. V_0 *and* V_t *denote the void volume (blue dextran) and total volume, respectively. The elution buffer was* 0.1 M *sodium phosphate* pH 7.4. *Also indicated is the elution position of authentic iodinated IGF-I.*

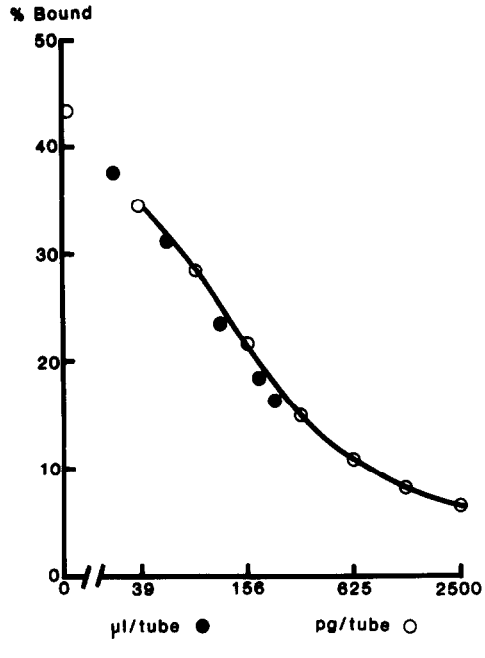

Fig. 3. Immunoassay inhibition curves of authentic IGF-I and cyst fluid 'IGF-I'. The figure shows a standard curve obtained using authentic IGF-I (○—○). ● *represents the percentage binding in the presence of serially diluted eluant of a fraction from the IGF-I peak after chromatography on Sephacryl* 200 *as shown in Fig.* 2.

(b) *Epidermal growth factor.* The specificity of the assay was deemed to be satisfactory on the basis of parallelism of the inhibition curve of immuno-EGF from cyst fluid compared with authentic EGF and also by the high specificity of the antibody to EGF. In addition, the antibody and ligand are similar to those used by Jaspar and Franchimont [6] who have demonstrated the specificity of the assay using gel filtration chromatography.

Levels of insulin-like growth factor-I and epidermal growth factor in cyst fluid

The levels of IGF-I and EGF in cyst fluid are shown in Fig. 4. For comparison the blood levels of IGF-I are also presented in Fig. 4. It can be seen that there is a wide scatter of results in all cases and a markedly skewed distribution for IGF-I in cyst fluid. The mean (median) concentrations for IGF-I in cyst fluid and blood and EGF in cyst fluid are 9.3 (5.9) ng/ml, 101 (87) ng/ml and 411 (416) ng/ml, respectively. The corresponding standard deviations were 8.2, 5.2 and 217 ng/ml.

Relationship between the levels of cyst fluid IGF-I and blood IGF-I or cyst fluid EGF

There was no significant relationship between the IGF-I levels in cyst and blood before (Fig. 5) or after acid extraction. However, there was a significant ($P < 0.01$) inverse correlation between the levels of cyst fluid EGF and IGF-I (Fig. 6). Calculations on log-transformed data gave the same levels of significance.

Protein binding of IGF-I

The level of immunoreactive IGF-I measured is appreciably influenced by the amount of its binding to carrier proteins. Thus acid extraction of serum IGF-I gave rise to a doubling of average IGF-I concentrations (Table 1) compared with a non-significant increase in cyst fluid IGF-I of about 5% (Table 1).

Ten millilitres of a cyst fluid pool was concentrated to 1 ml using a Diaflo ultrafiltration membrane (YM2, exclusion > 1000 mol. wt; Amicon Corp). The concentrate was incubated with tracer amounts of iodinated IGF-I and chromatographed

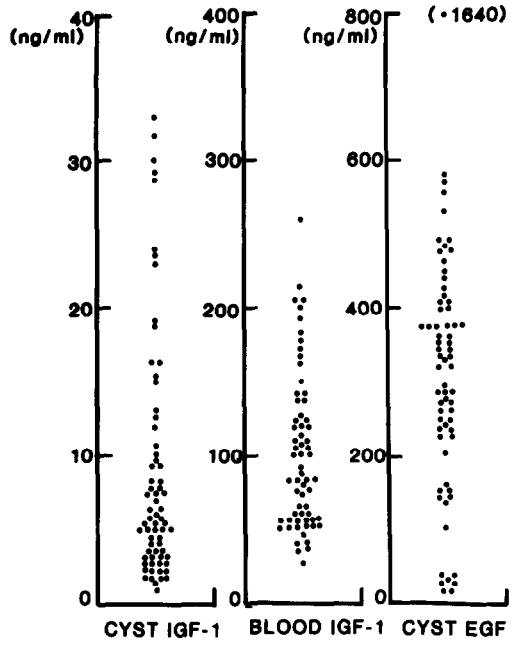

Fig. 4. Concentration of IGF-I and EGF in cyst fluid and IGF-I in serum. There were 68, 64 and 67 women for whom cyst IGF-I, serum IGF-I and cyst EGF, respectively, had been measured. In the case of multiple cysts the average value is shown.

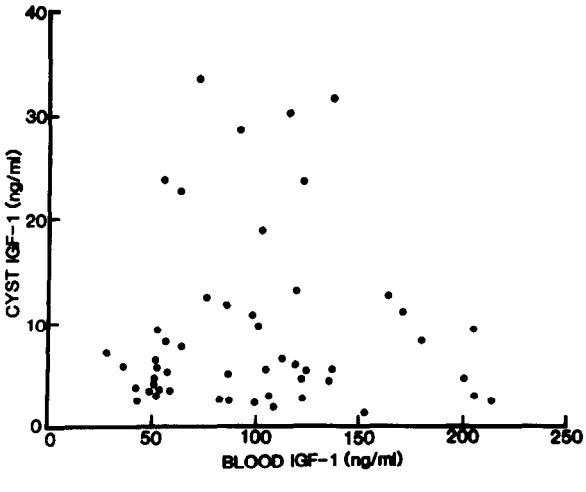

Fig. 5. Relationship between the levels of cyst fluid and serum IGF-I. There were 51 patients for whom blood and cyst fluid values were known. In the case of multiple cysts the average value was used.

on a Sephacryl 200 column (26 mm × 40 cm). The elution profile gave no indication of the presence of binding protein.

Growth factor concentration with multiple cysts

Some patients with gross cystic disease have multiple cysts which can occur in the same or opposite breasts. The levels of cyst IGF-I and EGF are shown in Figs 7 and 8. It can be seen that for IGF-I there is a high degree of concordance of levels whether the multiple cysts occur in one or both

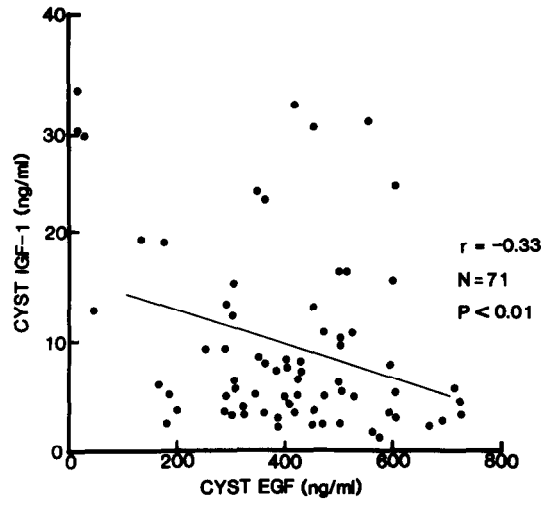

Fig. 6. Relationship between the levels of IGF-I and EGF in cyst fluids. There were 71 cyst fluid samples on which IGF-I and EGF was measured. Included in these data are 11 patients with multiple cysts; these comprised eight and three patients with two and three cyst fluid specimens, respectively. The best-fit line is indicated and has the equation y = 16.2 − 0.017x, where y and x are the concentrations (ng/ml) of IGF-I and EGF, respectively. The regression coefficient was −0.33 (P < 0.01).

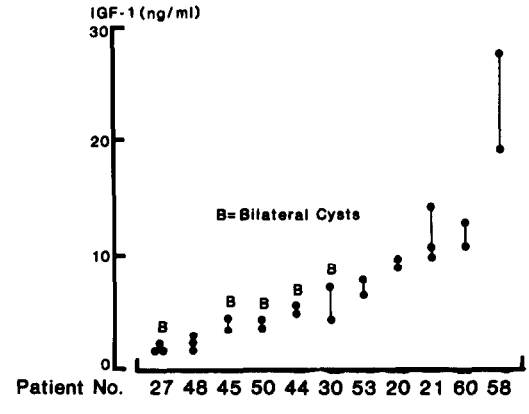

Fig. 7. IGF-I concentration in multiple cysts. The levels of IGF-I in inidividual cysts from women with multiple cysts are shown. Cysts which were aspirated from both breasts are indicated B. For convenience the women have been ranked in order of IGF-I concentration.

breasts. There was a similar trend for EGF levels although on the small number of patients studied this concordance was not as high.

DISCUSSION

The main findings of this study was that cyst fluid contains IGF-I at a level of about 5% of that found in blood, although there was no significant correlation between these amounts. The concentration of cyst IGF-I varied widely between patients and these amounts were log-normally distributed. There was also an inverse relationship between cyst IGF-I and EGF levels although the level of significance was not high.

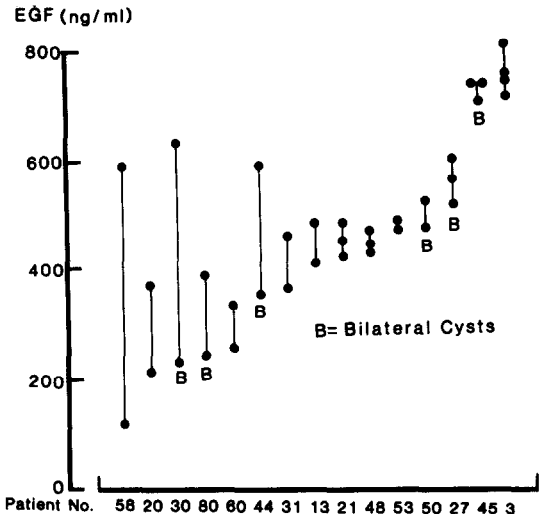

Fig. 8. EGF concentration in multiple cysts. The levels of EGF in individual cysts from women with multiple cysts are shown. Cysts which were aspirated from both breasts are indicated B. For convenience the women have been ranked in order of EGF concentration.

Table 1. Acid-extractable IGF-I in serum and cyst fluid

	Serum $n = 21$	Cyst fluid $n = 40$
Before extraction	87.4 ± 8.5	12.4 ± 1.5
After extraction	157.9 ± 8.2	12.4 ± 1.4
Ratio (± S.E.M.)	2.08 ± 0.19	1.05 ± 0.06

All results are expressed as mean ± S.E.M. and as ng/ml.
Acid extraction of IGF-I was as described by Daughday *et al.* [10].

The origins of cyst fluid IGF-I and EGF are unknown. The high concentrations of EGF could indicate local synthesis and would be in keeping with the report that EGF is expressed by human breast cancer cell lines, this synthesis being stimulated by oestradiol or progestins [13, 14]. On the other hand, the low concentration, relative to blood, of IGF-I would certainly render the peripheral circulation as being a reasonable source of cyst fluid IGF-I, even though IGF-I and its mRNA has been detected in human breast cancer cell lines [13, 15].

For women with multiple cysts there appeared to be a degree of concordance in the levels of IGF-I, or EGF, which was irrespective of whether the cysts were in one or both breasts. Whether this implies that the levels of growth factors are governed by the host is uncertain since we have yet to serially sample cysts or measure IGF-I in multiple cyst fluid specimens taken from patients with recurrent cystic disease. If growth factor levels were relatively constant it would be of interest to see whether the concentration of growth factor is related to breast cancer risk.

There is little or no evidence that cyst fluid contains IGF-I binding protein. Acid extraction of cyst fluid resulted in only a minor non-significant increase in IGF-I levels which contrasts with the doubling of assayable IGF-I in serum. Also, there was no indication of protein binding even after a 10-fold concentration of cyst fluid. One binding protein which has been found to occur in serum and other tissues is the placental protein binding PP12 [16, 17]. The amount of PP12 in cyst fluid was below the limit of sensitivity of the assay (0.9 ng/ml).

The relationship of IGF-I in cyst fluid and the increased risk of breast cancer is uncertain. IGF-I will increase the proliferation of both transformed and non-transformed breast cells [18, 19]. It is also noteworthy that the well-documented proliferative activity of insulin on mammary gland explants is mediated through the IGF-I receptors [18, 19].

The concentration of IGF-I in cyst fluid is similar to that found in human milk samples 3 or 4 days after parturition [20]. This parallels the situation with regard to EGF where the amounts of EGF in cyst fluid and human milk are also of the same magnitude [6, 21]. There is also a comparable change in EGF and IGF-I in that the levels of both are higher in colostrum than in milk [6, 20, 21].

We are at present investigating the relationship between cyst fluid concentrations of IGF-I and the levels of steroidal hormones in the hope that it will lead to a better understanding of the processes associated with gross cystic disease and possibly an insight into the aetiology of breast cancer.

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