CLINICAL STUDY

IGF-I in human breast cancer: low differentiation stage is associated with decreased IGF-I content

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Abstract

Objective: Few investigations on the potential role of IGF-I in human breast cancer have used morphological criteria, and the data presented on the localisation of IGF-I are controversial. Moreover, little information exists on a potential correlation between local IGF-I and the grade of malignancy or prognostic factors. Therefore, we investigated the immunohistochemical localisation of IGF-I in specimens of human breast cancer tumours of the ductal type, graded as G1/G2 (well-/moderately differentiated, $n = 115$) and G3 (poorly differentiated, $n = 28$).

Methods: IGF-I immunoreactivity was quantified using a scaling from no (–) to numerous (+++) IGF-I-immunoreactive cells. From 29 of the G1/G2 and 17 of the G3 tumours IGF-I was also measured by RIA. Cytosolic oestrogen receptor (ER) and progesterone receptor (PR) levels, and S-phase fraction were established and related to the number of IGF-I-immunoreactive cells.

Results: IGF-I immunoreactivity occurred predominantly in ductal epithelial cells. Of G3 tumours, 57% exhibited IGF-I immunoreactivity as compared with 84% of G1/G2 tumours. Correspondingly, the amount of IGF-I measured by RIA was significantly lower in G3 tumours (6.9 ± 0.9 ng/g wet weight) than in G1/G2 tumours (10.5 ± 1.1 ng/g wet weight) ($P = 0.031$). G1/G2 tumours exhibited a higher percentage of IGF-I-immunoreactive cells (16% –, 23% +, 41% ++, 20% +++) than G3 tumours (43% –, 37% +, 12% ++, 8% +++). When comparing the – with the +++ G1/G2 tumours, the frequency of IGF-I-immunoreactive cells was related significantly to the ER ($P < 0.016$) and the PR ($P < 0.008$) levels. In G1/G2 and G3 tumours, the ER and PR levels increased with the amount of IGF immunoreactivity while the S-phase fraction increased with decreasing IGF-I content. In 25% of the specimens, IGF-I immunoreactivity occurred in stromal cells, but there was no obvious difference between the different types of tumours. The survival of the G1/G2 tumour patients increased with increasing numbers of IGF-I-immunoreactive cells.

Conclusions: It is concluded that IGF-I is associated with the more-differentiated type of epithelial cells and that increasing dedifferentiation goes along with decreased IGF-I content. Thus, the presence of IGF-I immunoreactivity in breast cancer epithelial cells indicates a lower degree of malignancy than the lack of IGF-I.

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Introduction

The insulin-like growth factors (IGFs) constitute a family of polypeptides which exert insulin-like metabolic and mitogenic/growth-promoting effects on a variety of target cells (1–3). This family consists of two major forms, IGF-I and IGF-II (4). According to the 'classical' somatomedin hypothesis (5), growth hormone (GH) exerts its stimulatory effects on growth via IGF-I produced in the liver under the influence of GH. After secretion into the circulation IGF-I is considered to act on target tissues in an endocrine manner (5) via interaction with specific IGF-I receptors

(type 1 IGF receptors). However, IGF-I is not only produced in the liver but also in numerous extrahepatic tissues where production is probably also under the control of GH (6). There is, indeed, increasing evidence that this locally produced IGF-I mediates GH-stimulated growth in a paracrine/autocrine manner (1, 7–9).

It has been suggested that IGF-I enhances the development of breast cancer. Human breast tumours and human breast cancer cell lines express the type 1 IGF receptor (10–13), and its blockade suppresses the mitogenic and proliferative action of IGF-I on MCF-7 breast cancer cells (14). However, it is still under debate whether IGF-I exerts its effects by the endocrine

(15, 16) and/or the paracrine/autocrine route (17, 18). A preferential endocrine action of IGF-I would have to be assumed to explain the finding that circulating IGF-I levels correlate with breast cancer risk in premenopausal women (19) and with the size of the breast tumour (20). However, others have not observed significant differences in IGF-I or IGF-binding protein (IGFBP) serum levels between breast cancer patients and healthy control groups matched for age and menopausal status (21, 22). Thus, the role of circulating IGF-I in breast cancer development remains uncertain, and a paracrine/autocrine IGF-I action must also be seriously considered: Radioimmunological and molecular biological studies have shown that IGF-I is produced in human breast tumour parenchyma. The cellular sites of IGF-I synthesis in breast cancer tissue have not yet been clearly defined because few studies have used morphological methods to localise IGF-I, and contradictory results have been obtained. According to the prevalent present opinion IGF-I is expressed in stromal cells (17, 23–25) from which it is released to act on ductal cells. However, RT-PCR studies have shown that IGF-I mRNA occurs in breast cancer cell lines (26, 27), and IGF immunoreactivity has been detected in tumour cells *in situ* (28–30), leading to the suggestion that IGF-I may have independent prognostic significance in the early phase of disease (30). Furthermore, the expression pattern of local IGF-I during the progression of breast cancer has not yet been studied.

Therefore, we investigated the cellular localisation of IGF-I in human breast cancer using immunofluorescence on surgical specimens and compared tumours of histopathological differentiation grades G1/G2 (well-/moderately differentiated) and G3 (poorly differentiated). In addition, IGF-I was extracted and measured by RIA in many of the morphologically studied tumours. Using a quantitative scaling from – (no IGF-I-immunoreactive cells) to +++ (high number of IGF-I-immunoreactive cells), IGF-I immunoreactivity in tumour cells was quantitatively evaluated. We also focused on the potential correlation between the number of IGF-I-immunoreactive cells and well-known indicators of malignancy, such as S-phase fraction, oestrogen receptor (ER) and progesterone receptor (PR) status.

Materials and methods

Tissue preparation

The relevant data of the 143 patients studied are summarised in Table 1. Breast cancer specimens were obtained by surgery during the period 1985 to 1992 when two of us (U G F and S F) were working at the Karolinska Hospital in Stockholm (Sweden). In 46 of the specimens, sufficient material was available to allow extraction and radioimmunological determination

Table 1 Characteristics of the patients.

Number of patients	143
Age	
Mean (years±s.e.)	62.4±1.3
Range	31–84
Follow-up time (years)	> 6
Number of premenopausal women	30
Number of postmenopausal women	113
Number of G1 tumours	5
Number of G2 tumours	110
Number of G3 tumours	28
Age G1/G2	
Mean (years±s.e.)	63±1.5
Range	38–84
Age G3	
Mean (years±s.e.)	61±2.5
Range	31–80
TNM stage	
I	63
IIA	31
IIB	32
IIIA	17
Percentage of G1/G2 tumours with TNM stage	
I	45.5
IIA	22.1
IIB	23.4
IIIA	9.0
Percentage of G3 tumours with TNM stage	
I	33.2
IIA	28.0
IIB	16.7
IIIA	22.1

of IGF-I. Immediately after dissection, these specimens were divided into two portions one of which was extracted (see below) for the IGF-I RIA, while the other portion and the remaining 97 specimens were fixed in 4% neutral formalin. After an overnight rinse in 70% ethanol, specimens were dehydrated in a graded ethanol series and embedded in Paraplast (Mediate, Nunningen, Switzerland).

Tumour grading

Conventional histopathological grading was used to assess the degree of malignancy of the tumours applying the three factors, 'tubule formation', 'nuclear pleomorphism' and 'mitotic counts' (31). For each factor, the recommended numerical scoring system on a scale of 1–3 was used in order to ensure that each variable was assessed individually. The well-known sources of error inherent in this standardised semiquantitative method (31) were considered. The procedure is known to have a good reproducibility and has been adopted for use in the minimum histopathological dataset by the European Breast Screening Pathology Group (31). Our grading resulted in five well-differentiated (G1), 110 moderately differentiated (G2) and 28 poorly differentiated (G3) tumours. Because of the small number of G1 tumours the G1 and G2 tumours were pooled for the further analyses (Table 1).

Immunohistochemical protocol

Sections were cut at 4 µm and processed for immunofluorescence. To reduce non-specific binding, sections were pretreated with PBS containing 2% BSA and 2% normal goat serum. Sections were incubated at 4 °C for 12 h with antiserum 116 (dilution 1:500) raised in rabbit against human IGF-I. This antiserum reacts specifically with IGF-I in several organs and species (9, 32), including man (33). After repetitive washing in PBS (pH 7.4) 1:100 biotinylated goat anti-rabbit IgG (Bioscience Products, Emmenbrücke, Switzerland) was added for 30 min at room temperature. Thereafter, sections were washed in PBS and incubated with 1:50 streptavidin-fluorescein-isothiocyanate (FITC) (Bioscience Products) for 30 min at room temperature.

The specificity of the reactions was tested using the following controls: (i) replacement of the primary antiserum by non-immune rabbit serum and (ii) preabsorption of the IGF-I antiserum with recombinant human (rh) IGF-I, rhIGF-II (both from Novartis, Basel, Switzerland) or bovine insulin (Peninsula, Heidelberg, Germany) (40 µg and 400 µg peptide/ml antiserum diluted 1:500). Sections of human pancreas known to contain IGF-I-immunoreactive islet cells (33) were also processed in every incubation series and served as positive controls. Photomicrographs were taken with a Zeiss Axiophot (Zeiss, Zürich, Switzerland). The fluorochromes were visualised with a fluorescence module for FITC (BP 450–490 nm, FT 510, LP 515–565 nm).

Quantitative grading of IGF-I-immunoreactive cells

Five random sections through each tumour investigated were cut and processed for IGF-I immunofluorescence. In five different areas of the tumour tissue, 30 adjacent epithelial cells were inspected. If no IGF-I-immunoreactive cells were observed, the tumour was graded as –. Between one and ten IGF-I-immunoreactive cells resulted in grading as +, 11–20 IGF-I-immunoreactive cells as ++, and 21–30 IGF-I-immunoreactive cells as +++. Two independent observers performed the quantitative grading of IGF-I-immunoreactive cells. The few cases of difference were discussed and a third investigator was asked for his opinion. The final value was set by consensus.

Extraction of IGF-I

The tumour tissues were homogenised in five volumes of 1 mol/l acetic acid containing 0.02% human serum albumin (HSA), 1 µmol/l pepstatin A, 0.6 TIU/ml aprotinin, 10 µmol/l leupeptin and 1 mmol/l phenylmethylsulphonylfluoride and centrifuged at 10 000 *g* for 10 min. Supernatants were lyophilised and redissolved in 250 µl PBS/HSA. After addition of 1 ml 0.5 mol/l

HCl, IGF-I was eluted on SEP-PAK C18 cartridges (Waters, Milford, MA, USA) according to the protocol supplied by Immunonuclear (Stillwater, MN, USA). This procedure removes IGFBPs. After reconstitution with 500 µl PBS/0.2% HSA, samples were assayed at two dilutions (undiluted and 1:2) in the IGF-I RIA using rhIGF-I as a standard.

RIA

The IGF-I RIA was performed in PBS/0.2% HSA buffer (pH 7.4) at 4 °C as described (34). One hundred microlitres of the sample were added to 200 µl IGF-I antiserum (diluted at 1:1 000). After 24 h, 100 µl (about 30 000 c.p.m.) of ¹²⁵I-IGF-I (specific radioactivity 300–400 µCi/µg; Anawa, Wangen, Switzerland) were added and the mixture incubated for another 24 h. Two hundred microlitres of PBS/HSA buffer containing 40 µg rabbit IgG and 50 µl goat anti-rabbit gamma globulin antiserum (diluted at 1:2.5 with PBS) were added. After 60 min at 4 °C the mixture was centrifuged at 3500 *g* for 20 min, the supernatant removed and the precipitate counted in a gamma counter.

Steroid receptor assays

Cytosolic ER and PR analyses were performed with the isoelectric focusing technique (35). The receptor values were expressed as fmol/µg DNA.

S-phase fraction

DNA ploidy was assessed by both flow (FCM) and image (ICM) cytometric methods (36, 37). According to the DNA indices obtained, the tumours were divided into diploid, tetraploid and aneuploid groups. The percentage of cells in S-phase was assessed from both the FCM and ICM DNA histograms, except for diploid cases, where the S-phase fraction was obtained from ICM histograms only (37).

Statistical analysis

ER and PR status as well as S-phase fraction were compared with the quantitative grading of IGF-I immunoreactivity. Statistical analysis of the data was performed with a StatView 4.5 program. This included an ANOVA *t*-test (unpaired) for the RIA measurements and a Bonferoni/Dunn analysis with a significance level of 5% for the evaluation of the potential correlation of the amount of IGF-I-immunoreactive cells and ER, PR and S-phase fraction. All data are expressed as means ± S.E.M.

Results

Immunofluorescence

Preabsorption of antiserum 116 with 40 µg rhIGF-I/ml antiserum diluted 1:500 completely blocked the IGF-I immunoreactions (Fig. 1a and b), but these were not affected by preabsorption with IGF-II or insulin at concentrations up to 400 µg/ml.

IGF-I immunoreactivity was mainly observed in ductal cells (Figs 1–3). The numbers of IGF-I-immunoreactive cells, however, differed essentially between the different tumour grade categories (Figs 4 and 5). In moderately (G2) to well- (G1) differentiated tumours more ductal cells (84%) displayed IGF-I immunoreactivity than in poorly differentiated (G3) tumours (57%). Moreover, G1/G2 tumours exhibited a higher

Figure 1 Two consecutive sections of a G3 tumour scaled ++. The first section (a) was incubated with antiserum 116 (dilution 1:500) against human IGF-I and the second (b) with the antiserum (dilution 1:500) preabsorbed with 40 µg rhIGF-I/ml. Preabsorption of the antiserum completely abolished the immunoreactions in numerous slightly stained and few intensely stained epithelial cells. Bar = 25 µm.

Figure 2 Localisation of IGF-I immunoreactivity in representative G1 (a) and G2 (b, c) human breast cancer specimens. In all tumours, some stromal cells (arrows) are IGF-I immunoreactive. (a) The majority of ductal cells in this G1 tumour (scaling +++) contain IGF-I immunoreactivity. Bar = 45 µm. (b, c) G2 ductal tumours scaled ++ (b) and + (c) exhibit IGF-I-immunoreactive epithelial cells. In (b) both number and staining intensity of the IGF-I-immunoreactive cells are higher than in (c). Bars = 25 µm.

Figure 3 Distribution of IGF-I immunoreactivity in representative G3 tumours. (a) Several tumour epithelial cells display IGF-I immunoreactivity of different intensity (scaling ++). In the stroma, IGF-I-immunoreactive cells are present (arrow). (b) G3 tumour scaled + with some slightly stained and few intensively stained epithelial cells. (c) G3 tumour which displays no IGF-I-immunoreactive epithelial cells but numerous and intensively stained IGF-I-immunoreactive cells (arrows) in the surrounding stroma. Bars = 20 µm.

Figure 4 Semiquantitative grading of IGF-I immunoreactivity in G1/G2 ($n = 115$) breast cancer tumour epithelium related to prognostic factors. Means $\pm$ S.E.M.

Figure 5 Semiquantitative grading of IGF-I immunoreactivity in G3 ($n = 28$) breast cancer tumour epithelium related to prognostic factors. Means $\pm$ S.E.M.

percentage of IGF-I-immunoreactive cells (16% -, 23% +, 41% ++, 20% +++) than G3 tumours (43% -, 37% +, 12% ++, 8% +++) (Figs 4 and 5).

In about 25% of the tumour specimens investigated, stromal cells were IGF-I immunoreactive. IGF-I-immunoreactive stromal cells (Figs 2a-c and 3a) occurred singly or in clusters. In most cases, the staining intensity of the stromal cells was lower than that of the ductal cells. With the exception of four G3 tumours there was no obvious difference in the percentage of IGF-I-immunoreactive stromal cells between the different tumour grade categories. These G3 tumours exhibited no IGF-I-immunoreactive epithelial cells but numerous and intensely stained IGF-I-immunoreactive cells in the surrounding stroma (Fig. 3c).

Tissue IGF-I content

The radioimmunological measurements revealed that G3 tumours ($n = 17$) contained 6.9 ± 0.9 ng IGF-I/g tumour wet weight whereas G1/G2 tumours ($n = 29$) contained 10.5 ± 1.1 ng IGF-I/g tumour wet weight. The difference was statistically significant in the unpaired t -test ($P = 0.031$).

ER status, PR status and S-phase fraction

The results obtained for ER and PR levels and for the S-phase fraction in relation to the number of IGF-I-immunoreactive cells are summarised in Figs 4 and 5. The comparison of - vs +++ IGF-I-immunoreactive cells within the group of G1/G2 tumours showed that the frequency of IGF-I-immunoreactive cells was weakly related to the S-phase fraction ($P < 0.126$), but significantly to the ER ($P < 0.016$) and the PR ($P < 0.008$) levels. Furthermore, ER and PR levels showed a tendency to increase with the number of IGF-immunoreactive cells while the S-phase fraction increased with a decreasing number of IGF-I-immunoreactive cells (Fig. 4). Analysis of the G3 tumour data resulted in a weak correlation between the frequency of the IGF-I-immunoreactive cells and the ER level ($P < 0.173$). The corresponding values amounted to $P < 0.218$ for the PR level and $P < 0.294$ for the S-phase fraction. In addition, the ER and PR levels increased with the frequency of IGF-I-immunoreactive cells while the S-phase fraction increased with decreasing numbers of IGF-I-immunoreactive cells (Fig. 5). The subgroup of G1/G2 and G3 tumours with ER-/PR- status exhibited extremely low (- to +) IGF-I immunostaining.

Follow-up

When the percentage of patients with a 5 year survival was related to the number of IGF-I-immunoreactive cells the values amounted to 62.5% -, 95.7% +,

97.4% ++ and 100% +++ for the G1/G2 tumours, and 94.9% –, 97.6% +, 91.7% ++ and 100% +++ for the G3 tumours.

Discussion

In our study, IGF-I immunoreactivity was mainly confined to ductal cells of human breast cancer. Only about one-quarter of the specimens investigated showed IGF-I-immunoreactive stromal cells. Furthermore, IGF-I immunoreactivity in stromal cells was generally low when compared with that in epithelial cells. Only few studies have dealt with the cellular localisation of IGF-I in human breast cancer tissue, and the reports on the localisations of IGF-I immunoreactivity or IGF-I mRNA are quite controversial. IGF-I mRNA has only been found in stromal cells (38, 39) whereas others detected IGF-I immunoreactivity exclusively in epithelial cells (28) or in both epithelial and stromal cells (29). In our study, ductal tumour parenchyma displayed intense IGF immunoreactivity while stromal cells exhibited only weak immunoreactivity. Similarly, Toropainen *et al.* (30) found IGF-I immunoreactivity predominantly in tumour epithelial cells (91% of the preparations) and less (29% of the preparations) in stromal cells with weaker staining intensity. The percentage of IGF-I-immunoreactive tumour cells reported in the latter study is in a similar range to that found in our study (IGF-I-immunoreactive tumour cells: G1/G2 84%, G3 57%, stromal cells 25%).

Based on the earlier studies where IGF-I had not been detected in epithelial cells (17, 23, 38, 39), it has been claimed that IGF-I is released from stromal cells and acts on ductal cells to enhance the development of breast cancer (18, 25). However, IGF-I gene expression has recently been shown by RT-PCR in epithelial breast cancer cells and in primary breast cancer cell cultures (27). IGF-I mRNA expression has also been detected in H2380 breast cancer cells (26) whereas MCF-7 breast cancer cells, commonly used to study the effect of IGF-I on breast cancer cells, do not express IGF-I (24, 27).

The present study compares for the first time the frequency of IGF-I-immunoreactive cells with the tumour stage. In moderately to well-differentiated tumours (G1/G2), the majority (84%) of ductal cells displayed IGF-I immunoreactivity, whereas in poorly differentiated tumours (G3) this percentage was only 57%. These immunohistochemical data are in agreement with our RIA measurements, which show that less-differentiated G3 tumours contain less IGF-I than the more-differentiated G1/G2 tumours.

The results of numerous biochemical and/or immunohistochemical ER and PR analyses of the neoplastic parenchymal cells in breast cancer seem to give, in addition to the data of the lymph node status, valid prognostic and predictive information for the individual

patient (e.g. 40–42). Positive receptor status is widely accepted to correlate with favourable prognostic features, such as a lower rate of cell proliferation and histological evidence of tumour differentiation (40). In daily clinical work, such data form the basis for the individual selection between various kinds of hormonal therapy. Thus, the different hormonal treatment options are mainly based on the receptor status (ER and PR) of the tumour cell nuclei (40, 41). Such 'endocrine treatment' is considered as a realistic and effective alternative to the more-conventional cytotoxic chemotherapy in many women (42).

Few and contradictory results have been presented on the potential correlation between the ER or PR status and the local IGF-I content in breast cancer. A positive correlation between ER content and IGF-I immunoreactivity has been reported by Mizukami *et al.* (28), whereas no correlation between ER or PR content and the intensity of IGF-I immunoreactivity has been found by Toropainen *et al.* (30). In the present study, the frequency of epithelial IGF-I-immunoreactive cells in G1/G2 tumours significantly correlated with both the ER ($P < 0.016$) and the PR content ($P < 0.008$) when comparing the tumour populations containing no (–) and high (+++) numbers of IGF-I-immunoreactive cells. In G3 tumours there was only a tendency but no significant correlation of the ER and PR status to increase with the frequency of IGF-I cells. The fact that no statistical significance was obtained for the G3 tumours may be due to the smaller number of samples as compared with the G1/G2 tumours. Nevertheless, our results indicate that a positive correlation exists between the ER and PR status and the IGF-I content in ductal epithelial cells.

The cell proliferation potency of the neoplastic parenchymal cells in breast carcinoma is another well-known marker of prognosis and prediction of the course of the disease and, hence, for the choice of therapy (43). In addition to conventional histopathological grading (31) as also used in the present study, DNA-cytometric S-phase fraction determination (43, 44), which is thought to be the strongest cell kinetics marker to assess prognosis in breast cancers, has been applied (45, 46).

In our study, the comparison of – vs +++ IGF-I-immunoreactive cells within the groups of the G1/G2 and the G3 tumours showed that the frequency of IGF-I-immunoreactive cells was only weakly related to the S-phase fraction (G1/G2 $P < 0.126$, G3 $P < 0.294$). However, in both G1/G2 and G3 tumours, the S-phase fraction increased with decreasing IGF-I content. Thus, our data may indicate a negative correlation between the amount of IGF-I immunoreactivity in the tumour cells and the S-phase fraction. The lack of statistical significance is probably due to the limited number of patients investigated.

In the human breast cancer cell line H2380, IGF-I and the type 1 IGF receptor were co-expressed,

suggesting an autocrine function of IGF-I (26). Type 1 IGF receptor levels were found significantly higher in low-risk than in high-risk patients (47). In agreement, the type 1 IGF receptor immunoreactivity was expressed at high levels in breast tissues of normal controls and in well- and moderately differentiated but at low levels in poorly differentiated human breast cancers (48). This led to the proposal that an elevated type 1 IGF receptor content may be a favourable prognostic indicator for tumour progression. In analogy, our results suggest that the presence of IGF-I immunoreactivity in breast cancer epithelial cells indicates a lower degree of malignancy than its absence and, thus, corroborates the proposal by Toropainen *et al.* (30). These findings somehow contradict the hypothesis that enhanced cellular growth (hyperplasia and malignant growth) results from enhanced IGF-I signalling (49) by 'upregulation of type 1 IGF receptor and IGF-I peptide'. In contrast, our results suggest that IGF-I may maintain a more-differentiated tumour stage and a lower proliferation rate of breast epithelial cells, and that increasing dedifferentiation may be associated with decreased IGF-I content in the epithelial cells. The observed 100% 5 year survival of the G1/G2 and G3 tumour patients showing a +++ scaling of immunoreactivity and the fact that the survival rate in the G1/G2 tumour patients increased with increasing IGF-I immunoreactivity support this proposal. Therefore, not only the content of the type 1 IGF receptor (47) but also that of its ligand IGF-I may serve as (favourable) prognostic factors. Furthermore, the observed significant positive correlation between ER and PR content and IGF-I immunoreactivity underlines this contention.

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