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Advances in Brief

IGFBP-3 Gene Expression and Estrogen Receptor Status in Human Breast Carcinoma[1]

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Abstract

Insulin-like growth factors (IGF) I and II are potent mitogens for breast carcinoma proliferation. IGF-mediated proliferative activity can be markedly enhanced by the presence of specific IGF-binding proteins (IGFBPs). IGFBP-3 has been shown to enhance IGF-mediated growth in a number of systems. Studies have demonstrated IGFBP-3 secretion only in estrogen receptor (ER)-negative breast carcinoma cell lines while IGFBP-3 could not be detected in media conditioned by ER-positive cell lines. We investigated whether a relationship exists between ER status and IGFBP-3 mRNA expression in human breast carcinoma biopsy specimens. We have detected IGFBP-3 mRNA in breast carcinoma tissue obtained from patients utilizing *in situ* hybridization. Quantitation of IGFBP-3 mRNA levels was performed utilizing image cytometry. There was a significantly higher expression of IGFBP-3 mRNA in ER-negative breast carcinoma specimens when compared to the ER-positive specimens. Whether this higher expression of IGFBP-3 mRNA and presumed secretion of IGFBP-3 by ER-negative tumors play a role in the rapid proliferation and poor prognosis of these tumors remains to be determined.

Introduction

Breast carcinoma proliferation appears to be dependent on the presence of a variety of growth factors (1). IGF-I[3] and -II are powerful mitogens for breast carcinoma proliferation (2, 3). IGF-I and -II stimulation of breast carcinoma growth appears to occur predominantly in a paracrine fashion (4). These growth factors, secreted by the surrounding stromal cells, stimulate tumor growth through their binding and activation of the IGF-I receptor (2-4). In contrast to other peptide hormones, the majority of the IGFs in extracellular fluid are associated with specific IGFBPs. Six structurally distinct IGFBPs have been described; these IGFBPs share significant homology in their amino-terminal portion as well as sharing a common pattern of cysteine residues (5). These proteins are secreted into a wide variety of extracellular fluids. The type of IGFBP secreted is dependent on the specific cell type. The effect of these IGFBPs on IGF action is complex in that they may enhance or inhibit IGF-mediated cellular effects.

Breast carcinoma cell lines have been found to secrete various classes of IGFBPs (6-8). Several investigators have now reported that ER-negative breast carcinoma cell lines secrete predominantly IGFBP-3, while ER-positive cells secrete predominantly IGFBP-2 (8). ER-negative cells have been found to grow more rapidly than ER-positive cells and patients with these tumors usually have a worse prognosis than patients with their ER-positive counterparts (9). IGFBP-3 has been shown to enhance IGF-I-mediated cell proliferation in a number of systems and enhances IGF-I mediated breast carcinoma growth (10, 11).[4] In this report we investigated whether the previous relationship noted between IGFBP-3 secretion and ER negativity in cell lines was also found in patient breast carcinoma biopsy specimens.

Materials and Methods

***In Situ* Hybridization.** Twenty specimens of infiltrating ductal carcinoma obtained from patients at the Bowman-Gray Medical Center following an approved institutional review board protocol were immediately frozen and stored at −80°C; one half of each specimen was utilized for ER determination and flow cytometry analysis while the other half of the specimen was utilized to generate tissue sections. Six-μm sections were cut on a cryostat microtome and mounted on microscope slides coated with gelatin. *In situ* hybridization was performed according to the method of Wise *et al.* (12). A 207-base pair fragment representing most of exon 2 and the 3′ portion of exon 1 of the human IGFBP-3 gene, a region which is not homologous to regions of the IGFBP-1, -2, -4, -5, and -6 genes, was subcloned into the pBluescript Sk+ vector (Stratagene). T7 and T3 RNA polymerases were used to make antisense and sense RNA probes, respectively. The probes were labeled with ^{35}S-UTP (>1000 Ci/mmol; Amersham, Arlington Heights, IL). The specific activity of the probes was 10^8–10^9 cpm/μg RNA; approximately 5×10^6 cpm were applied to each section. All of the *in situ* hybridizations were performed with the same labeled probe and on the same day.

Image Cytometry. Hybridization signals obtained with sense and antisense probes were analyzed using a Perceptics Image analyzer equipped with an Axiovert 10 Zeiss microscope and a CCD video camera. For each slide, the following protocol was used to acquire images. First, one area of the slide without cells, but with remaining reagent derived opacity, was chosen as background to be used in a background subtraction step for all images captured from that slide. Next, each slide was scanned by the operator in order to identify tumor areas with highest amount of grain clusters. In a following step, 10 images were captured from 10 different areas of each slide, chosen among those areas showing maximum grain clusters; this was done with prior background subtraction using the background image mentioned above. All images were acquired with a resolution of 512 x 512 pixels and 16-bit precision and stored for subsequent analysis as the average of 16 frames. For densitometric analysis, the images were first converted from signed (which was the form the images were in after the background subtraction operation) to unsigned images suitable for further

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[3] The abbreviations used are: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; ER, estrogen receptor; PR, progesterone receptor.

[4] J. C. Chen, Z-M. Shao, C. T. Roberts, Jr., D. LeRoith, M. Sheikh, and J. Fontana, submitted for publication.

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Table 1 *Estrogen and progesterone receptor levels, S-phase fractions and IGFBP-3 mRNA levels in breast carcinoma biopsy specimens*

Patient	ER (fmol/mg of protein)	PR (fmol/mg of protein)	% S	% G_2 + M	IGFBP-3 mRNA (intensity) (arbitrary units)[a]
1	0	0	11	27	0.3127 ± 0.1145[a]
2	0	0	6	9	0.1979 ± 0.0713
3	0	0	3	6	0.0241 ± 0.0062
4	0	0	21	27	0.3459 ± 0.1457
5	0	0	13	17	0.0309 ± 0.0096
6	0	0	14	24	0.0263 ± 0.0109
7	0	0			0.1162 ± 0.0388
8	0	0	9	15	0.2679 ± 0.0960
9	0	0	9	23	0.0043 ± 0.0021
10	54	0	20	34	0.0284 ± 0.0107
11	58	20	7	12	0.0204 ± 0.0095
12	77	49	7	13	0.0307 ± 0.0120
13	74	187			0.0221 ± 0.0217
14	26	124			0.0437 ± 0.0164
15	100	40	3	6	0.0533 ± 0.0175
16	90	0	6	10	0.0418 ± 0.0155
17	77	108	4	6	0.0041 ± 0.0016
18	55	131	3	7	0.0267 ± 0.0096
19	39	120			0.1458 ± 0.0502
20	179	119	9	23	0.0523 ± 0.0211

[a] Mean of ten determinations (images) ± SD of the mean intensity $\left(M = \frac{I}{A}\right)$ obtained from each section.

analysis. Next, a threshold map was defined to include, as much as possible, only cell containing areas. This threshold map was then applied for analysis of all digitalized images captured from each slide. Mean intensity (*M*) was calculated by the formula

$$M = \frac{I}{A}$$

where *I* is the accumulated pixel intensity (2^{16} gray levels for a 16-bit image) and *A* is the total image area (512 x 512 pixels). The mean intensity values were then used for statistical analysis.

Steroid Receptor Assay. Steroid receptor assays were done by a modification of the dextran-coated charcoal procedure in which 1.0% gelatin was used instead of the dextran (13). Scatchard analysis were done and 10 fmol/mg of protein or greater was considered positive (13).

Flow Cytometry Assays. Frozen tissue (0.05 to 0.2 g) was mechanically treated with 2 to 3 ml of a propidium iodide (50 mg/ml) solution in a 3.4 M citrate buffer (pH 7.6) containing 10 mM NaCl, RNase (5.6 mg/ml), and 0.6% Nonidet P-40 as described previously (14). The resulting nuclei were syringed 3 times with a 26-gauge needle and filtered to remove clumps. The resulting cells were then analyzed on a FACStar. The ploidy was based on the ratio of peak channel of tumor cells to the peak channel of the diploid standard (stained human lymphocytes) in the DNA histogram and the cell kinetics was determined using the Modfit Analysis program (Verity Software) as described previously (14).

Results

Specimens were obtained from 20 patients (Table 1). Nine of the specimens were ER negative (ER = 0; PR = 0) and 11 samples were ER positive (ER >35 fmol/mg protein, PR >30 fmol/mg protein). The ER and PR status as well as the percentage of cells in S phase were determined as described in "Materials and Methods." There was no significant correlation between the ER status of the tumor and the percentage of cells in S phase as determined by DNA content when the data were analyzed utilizing the Wilcoxon rank sum test.

The level of IGFBP-3 mRNA in the various specimens were determined by *in situ* hybridization utilizing ^{35}S-labeled, 207-nucleotide sense or antisense complementary RNA probes (Fig. 1). *In situ* hybridization was performed as described in "Materials and Methods." A strong hybridization signal was observed more frequently in ER-negative samples when the antisense IGFBP-3 probe was utilized (Figs. 1*A* and 2*A*). A minimal to no hybridization signal was observed in many of the ER-positive samples (Figs. 1*C* and 2*C*) or when the probe oriented in the sense direction was utilized with either ER-negative or ER-positive samples (Figs. 1*B*, 2*B*, 1*D*, and 2*D*). That this reduced signal in many of the ER-positive samples was not due to degradation of IGFBP-3 mRNA was indicated by the detection of a hybridization signal in the stroma of the same section.

The IGFBP-3 mRNA hybridization signal was quantitated utilizing an image analyzer and quantitating the most positive areas of the section as described in "Materials and Methods."

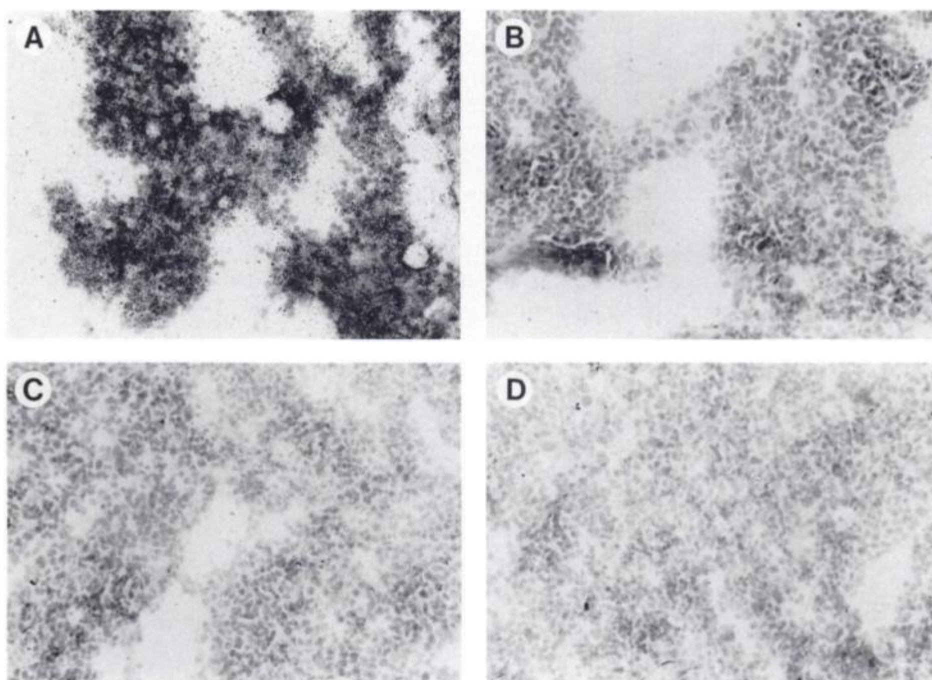

Fig. 1. Localization of IGFBP-3 mRNA in breast carcinoma biopsy specimens by *in situ* hybridization; brightfield photomicrographs, × 400. Biopsy specimens were cut into 6-μm sections, each section was incubated with 5 × 10^6 cpm ^{35}S-labeled antisense or sense IGFBP-3 probe, and hybridizations were performed as described in "Materials and Methods." *A*, ER-negative antisense; *B*, ER-negative sense; *C*, ER-positive antisense; *D*, ER-positive sense.

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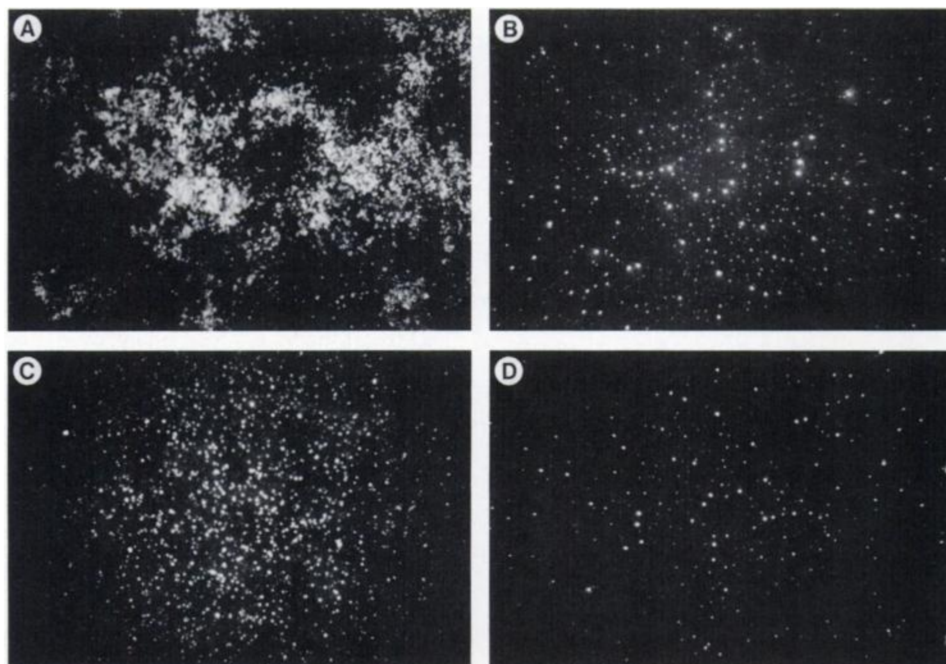

Fig. 2. Localization of IGFBP-3 mRNA in breast carcinoma biopsy specimens by *in situ* hybridization. Darkfield photomicrographs, × 400. Biopsy specimens were handled and hybridizations performed as described in the legend to Fig. 1 as well as "Materials and Methods." *A*, ER-negative antisense; *B*, ER-negative sense; *C*, ER-positive antisense; *D*, ER-positive sense.

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Ten fields from each section were analyzed and the mean was obtained (Table 1). Although there was some overlap in IGFBP mRNA levels between the ER-negative and ER-positive samples most likely due to tumor heterogeneity the means were significantly different with ER-negative samples expressing higher mRNA levels [ER-negative, 0.1473 ± 0.055 (SD); ER-positive, 0.0426 ± 0.019]. Utilizing the one tailed Student *t* test there was a statistically significant ($P < 0.05$) relationship between ER-negative status and the IGFBP-3 mRNA level.

Discussion

Numerous investigators have now demonstrated that purified IGFBPs may markedly enhance the activity of IGF on cells (10, 11, 15, 16). The conditions required for IGFBP stimulation of IGF-I-enhanced DNA synthesis appear to vary with either the specific cell type or class of IGFBP utilized. The mechanism by which IGFBP-3 enhances IGF-I action is unclear. Several studies have suggested that IGFBP-3 association with the cell membrane may be important for IGFBP stimulation of IGF-mediated cellular effects (10, 16, 17).

Cell-associated IGFBP-3 was often accompanied by an increase in membrane-bound IGF-I. It has been observed that in cells which synthesize and secrete IGFBP-3, the vast majority of IGF-I is bound to cell-associated IGFBP-3 (18). We have found that addition of IGFBP-3 to an ER-positive breast carcinoma cell line results in enhanced IGF-I stimulation of growth and enhanced IGF-I binding to the cell via binding to cell attached IGFBP-3.[4] IGFBP-3 may enhance IGF-I action by a number of mechanisms. Cell membrane-anchored IGFBP-3 may enhance IGF binding to its receptor, prevent down-regulation of the receptor, and perhaps prolong IGF transmembrane signaling. Purified IGFBP-3 has been shown to prevent IGF-I-triggered down-regulation of its own receptor in bovine fibroblasts (19). Whether any of these mechanisms play a role in IGFBP-3 mediation of IGF-I action in human breast carcinoma remains to be determined.

We have demonstrated for the first time in this study that the IGFBP-3 gene is expressed in human breast carcinoma biopsy specimens and that there is a significant correlation between ER negativity and increased IGFBP-3 mRNA levels as determined by *in situ* hybridization. Whether IGFBP-3 mRNA expression and presumed secretion play a role in the poor prognosis associated with this cell type and the greater propensity of these tumors to metastasize remains to be determined.

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