Insulin-like Growth Factor Binding Proteins in Human Breast Cancer Tissue

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ABSTRACT

Breast tumor cell lines have been shown to secrete at least five distinct insulin-like growth factor (IGF) binding proteins (IGFBPs), the secretion being related to the estrogen receptor (ER) content. In this study we investigated IGFBP mRNA expression and IGFBP content in relation to ER content in human breast tumors. Tissue specimens from 47 breast cancers were studied. In five cases the adjacent histologically normal tissue was also analyzed. IGFBP content in tissue homogenates was studied by Western ligand blot analysis, using [125I]-IGF-I as a label, and IGFBP mRNA expression by reverse transcriptase polymerase chain reaction. The results show that human breast tumors express mRNAs encoding IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-5. The pattern of IGFBPs in different tumors varies. No correlation exists between ER content and IGFBPs with molecular weights 24,000 Mr, 28,000 Mr, 34,000 Mr or 43,000 Mr, whereas the 49,000 Mr IGFBP was more abundant in ER negative tumors (P < 0.05). The IGFBP content was significantly (P < 0.05) higher in five tumors than in their adjacent normal tissues suggesting that increased content of IGFBPs is a feature typically associated with the malignant transformation of breast tissue.

INTRODUCTION

The IGFs I and II stimulate cell growth in multiple tissues and cell lines including malignant breast tumor cell lines (1). Both IGF-I and IGF-II mRNAs are expressed in breast cancer tissue and are suggested to be synthesized by stromal elements (2, 3). The expression of the IGF mRNAs in breast cancer cell lines is controversial, and recent studies indicate that breast cancer cell lines do not express authentic IGF-I mRNA, whereas IGF-II mRNA is expressed by some cell lines (2, 3). The IGF-I receptor content in breast cancer tissue has been correlated to ER status, whereas IGF-II receptor expression in breast cancer cell lines is not related to ER content (4, 5). The IGF action is modulated by IGFBPs. The IGFBPs act by either enhancing or by inhibiting the action of IGFs (6-8). In breast cancer cell lines a variety of different IGFBPs including IGFBP-1, IGFBP-2, and IGFBP-3 have been detected (9-11). IGFBP-1 has been shown to enhance the cell growth of breast cancer cell line MDA-231 in response to IGF-I (12), and a correlation of IGFBP secretion to ER content has been suggested (11). Thus, ER-positive cell lines secrete IGFBP-2 and a Mr 24,000 form, but not IGFBP-1 or IGFBP-3, whereas ER-negative cell lines secrete IGFBP-1 and IGFBP-3 in addition to the Mr 24,000 form. Normal bovine mammary cells have been shown to secrete IGFBP-2 and IGFBP-3 which secretion was stimulated by IGF (13). No information on IGFBP expression in normal human breast tissue or human breast cancer tissue is available. In view of the observed differences in IGF expression in breast cancer cell lines and breast cancer tissue, it was of interest to investigate IGFBP mRNA expression and IGFBP content in benign and malignant human breast tissue.

This study was undertaken to evaluate whether human breast cancer tissue expresses IGFBPs and whether the presence of IGFBPs in breast cancer tissue is related to ER receptor status.

MATERIALS AND METHODS

Patients and Tissue Samples. Patients who had breast cancer operations were included in the study with approval of the local ethical committee. Samples of tumor tissue and samples from macroscopically and histologically normal tissue, adjacent to tumor tissue, were immediately after excision dissected free from fat tissue, frozen in liquid nitrogen, and stored at -80°C until analyzed. Tissue samples were also fixed in formalin for histological evaluation. A total of 47 breast cancer (ductal carcinoma) samples were studied. In five cases both breast cancer tissue and adjacent histologically normal tissue were investigated. The distribution of epithelial and stromal cell components in these five pairs of tissues is given in Table 1.

RNA Extraction. Total RNA was extracted by a guanidinium thiocyanate method (14).

Chemicals and PCR Primers. Recombinant 59Thr-labeled IGF-I was purchased from Amgen Biologicals (Thousand Oaks, CA) and was iodinated by the chloramine-T method. IGFBP-1 MAb 6303 and MAb 6305 were from Medix Biochemica (Kauniainen, Finland). The blotting membrane for ligand blots was Hybond C-extra from Amersham International (Buckinghamshire, United Kingdom). BSA was from ICN Biomedicals, Inc. (Irvine, CA). All reagents used for RNA isolation were from Sigma Chemical Co. (St. Louis, MO). Maloney leukemia virus reverse transcriptase was from Gibco-BRL (Middlesex, United Kingdom). Ampli-Taq polymerase and deoxynucleotides were from Perkin-Elmer Cetus (Emeryville, CA), and human placenta DNA virus reverse transcriptase was from Gibco-BRL (Middlesex, United Kingdom). Ampli-Taq polymerase and deoxynucleotides were from Perkin-Elmer Cetus (Emeryville, CA), and human placental ribonuclease inhibitor was from USB (Cleveland, OH). The primers were 19-29 mer oligonucleotides, and the sequences were as follows: IGFBP-1: 5'S primer, nucleotides 328-347 and 3'AS, nucleotides 744-773 (15) (PCR product, 446 base pairs); IGFBP-2: 5'S primer, nucleotides 443-462 and 3'AS, nucleotides 920-939 (16) (PCR product, 497 base pairs); IGFBP-3: 5'S primer, nucleotides 36-56 and 3'AS, nucleotides 659-681 (17) (PCR product, 646 base pairs); IGFBP-4: 5'S primer, nucleotides 226-245 and 3'AS, nucleotides 793-819 (18) (PCR product, 594 base pairs); and IGFBP-5: 5'S primer, nucleotides 491-521 and 3'AS, nucleotides 834-860 (19) (PCR product, 370 base pairs).

RT-PCR. For generation of cDNA, 1.0 µg of total RNA template was incubated for 60 min at 37°C in a 20-µl reaction volume containing PCR buffer (50 µM KCl:10 mM Tris, pH 8.3), 3.5 mM MgCl2, 1 mM of each deoxynucleotide, 2.5 µM oligo (dT) primer, 20 units of human placental ribonuclease inhibitor, and 50 units of Maloney murine leukemia virus reverse transcriptase. The reaction was stopped by incubating at 95°C for 5 min and was then quick-chilled on ice. For cDNA amplification PCR was performed for 35 cycles. The concentration of specific primers was 0.5 µM and of Ampli-Taq polymerase, 1.25 units, in 50 µl of PCR buffer. The PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide. Attempted quantification of the RT-PCR product was performed according to Takeda et al. (20). In brief, 3 µl of a cDNA reaction mixture were used for amplification in the presence of 1 mmol each of ATP, GTP, and TTP; 0.2 mM CTP; and 0.1 µl of -32PdCTP (3000 mCi/mm; Amersham, Buckinghamshire, England). Thirty- and 35-cycle products

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3 The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; ER, estrogen receptor; RT, reverse transcriptase; PCR, polymerase chain reaction; MAb, monoclonal antibody; BSA, bovine serum albumin; cDNA, complementary DNA; IRMA, immunoradiometric assay.
were analyzed. Actin was used as an internal standard. The radioactivity expressed as cpm in the specific PCR band/cpm in the actin PCR band of hybridization mixture containing Sx standard saline citrate, 10xDenhardt's solution, 10% dextran sulfate, 7% sodium dodecyl sulfate, 5'-end-labeled 27-mer oligonucleotide used in the Southern blotting occurred between the two primers. The oligonucleotide used in the Southern blotting occurred between the two primers used in the PCR.

Southern Hybridization. The RT-PCR products were blotted by capillary transfer onto Zeta-Probe nylon membrane. The blotted membrane was probed according to the manufacturer's instructions in 20 ml of hybridization mixture containing 5X standard saline citrate, 10X Denhardt's solution, 10% dextran sulfate, 7% sodium dodecyl sulfate, 100 μg/ml of herring sperm DNA, and 0.75 × 10⁶ cpm/ml of 32P 5'-end-labeled 27-mer oligonucleotide, respectively. The oligonucleotide in the Southern blotting occurred between the two primers used in the PCR.

Northern Hybridization. Total RNAs (30 μg) were blotted by capillary transfer on Hybond-N membrane. The blots were dried at 80°C for 1 h, UV-cross-linked for 3 min, and hybridized as described earlier (21). The probes were the same 27-mer 32P-labeled probes as used in Southern blotting.

Tissue Homogenates. The frozen tissue was pulverized and was homogenized with an Ultra Turrax homogenizer in ice-cold 10 mm Tris: 1.5 mm EDTA: 5 mm Na₂MoO₄, pH 7.4. The homogenate was centrifuged at 40,000 × g for 1 h, the supernatant, called tissue cytosol fraction, was collected and used for determinations of IGFBPs and ERs. The blood contamination of tissue homogenates was less than 1% as determined by the hemoglobin content in the samples.

Western Ligand Blotting. Ligand blotting was performed as described by Holly et al. (22). In short, 120 μg of tissue cytosol protein (30 μl) were electrophoresed on 10% under sodium dodecyl sulfate-polyacrylamide gel electrophoresis nonreducing conditions. Electrophoresed proteins were electroblotted onto Hybond-C-extra nitrocellulose filters. The filters were blocked with 5% BSA and incubated for 2 h with 125I-labeled IGF-I (0.6 × 10⁶ cpm/ml), washed, and exposed to film for 7 days. The intensity of the bands was measured with LKB 2202 Ultroscan (LKB, Bromma, Sweden). The peak heights corresponding to Mₛ, 24,000, 29,000, 34,000, 43,000, and 49,000 IGFBPs were added to give the total amounts of IGFBPs. The ratio of IGFBPs in tumor and normal adjacent tissue was then calculated.

Estrogen Receptors. ER were determined with the Abbott ER-enzyme immunoassay monoclonal kit (Abbott Laboratories, North Chicago, IL) according to the manufacturer's instructions. An ER content >15 fmol/mg of cytosol protein was considered positive.

IGFBP-1 Immunoradiometric Assay. The IGFBP-1 content in cytosols was determined by a monoclonal IRMA as earlier described (23). MAB 6305 was used as catching antibody, and 125I-labeled MAB 6303 was used as detecting antibody. The sensitivity of the assay is 0.25 ng/ml, and the intra- and interassay variations are 6.2% and 9.8%, respectively. The IGFBP-1 content was expressed as ng/mg of protein.

RESULTS

Of the 47 tumors examined, 30 were ER positive and 17 were ER negative. In both ER-positive and ER-negative tumors, five distinct IGFBP species varying in size from Mₛ, 24,000 to Mₛ, 49,000 could be detected (Fig. 1). The Mₛ, 24,000 binding species was detected in 70% of ER-positive tumors and in 41% of ER-negative tumors. The Mₛ, 28,000 binding protein, which includes IGFBP-1, was present in 53% of ER-positive tumors and in 52% of ER-negative tumors (Table 2). When measured by the highly sensitive IGFBP-1 IRMA, 40% of ER-positive tumors contained measurable IGFBP-1 as did 18% of the ER-negative tumors. The concentration range was 0.15 to 0.85 ng of IGFBP per mg of protein, which concentrations are not detectable by Western ligand analysis. Only when concentrated breast cytosol samples were analyzed in Western blots using radiolabeled MAB 6303 as tracer could a faint band in the Mₛ, 28,000 region be detected (data not shown). This suggests that the main part of the Mₛ, 28,000 protein was binding proteins other than IGFBP-1. The Mₛ, 34,000 protein corresponding in size to IGFBP-2 was detected in 50% of ER-positive tumors and in 47% of ER-negative tumors, and the Mₛ, 49,000 protein corresponding to IGFBP-3 was detected in 40% of ER-positive tumors and in 76% of ER-negative tumors. Only the presence of Mₛ, 49,000 IGFBP differed significantly (P = 0.02) between ER negative and ER positive tumors, being more common in ER-negative tumors.

Using RT-PCR, mRNA for IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 was detected in all five breast tumors evaluated (two ER positive and three ER negative) (Figs. 2 and 3). The authenticity of the PCR products was verified by Southern analysis (data not shown). The amplification of IGFBP-1 mRNA cDNA was very low (Fig. 3). In Northern blot analysis of 5 breast tumors, IGFBP-1 mRNA could not be detected, whereas IGFBP-2 and IGFBP-4 mRNAs corresponding to mRNA sizes of 1.4, 2.4, and 2.1 kilobases, respectively, were visualized in all tumors (data not shown).

When comparing the binding protein pattern in 5 cancer tissues and normal adjacent tissues by Western ligand blot analysis, the IGFBP content was significantly higher (P < 0.05)

| Table 1 Distribution of stromal and epithelial cell components in five breast cancer tumors and in adjacent histologically normal tissue |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Patient | Cancer tissue (%) | Normal tissue (%) |
| | Epithelial | Stromal | Epithelial | Stromal |
| | | | | |
| 1 | 60 | 40 | 10 | 90 |
| 2 | 50 | 50 | 10 | 90 |
| 3 | 40 | 60 | 20 | 80 |
| 4 | 50 | 50 | 20 | 80 |
| 5 | 90 | 10 | 10 | 90 |

| Table 2 Presence of IGFBPs in 47 breast tumors in relation to ER content as determined by Western ligand blot analysis |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| IGFBPs (Mₛ, forms) |
| | 24,000 | 28,000 | 34,000 | 43,000 | 49,000 |
| ER positive (n = 30) | 21 (70%) | 16 (55%) | 15 (50%) | 6 (20%) | 12 (40%) |
| ER negative (n = 17) | 7 (41%) | 9 (52%) | 8 (47%) | 7 (41%) | 13 (76%) |
| Numbers in parentheses, percentage. | | | | | |
in cancer tissue than in normal tissue (Fig. 4). The ratios of IGFBP intensities (densitometry) in cancer tissues compared to normal adjacent tissues were 1.2, 1.6, 1.3, 2.0, and 8.2, respectively. Also when quantification of the RT-PCR reaction was performed, the cancer tissues contained more IGFBP than did adjacent normal tissue (Fig. 3). This was consistently observed in each tissue for IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5, whereas the message for IGFBP-1 was low.

DISCUSSION

In this study we have investigated the IGFBP content in human breast cancer tissue and IGFBP mRNA expression. Based on Western ligand blot analysis, the breast tumor tissues contain at least 5 different IGFBPs ranging in size from $M_r$ 24,000 to $M_r$ 49,000. Only the $M_r$ 49,000 IGFBP species correlate to ER status, being more common in ER-negative tumors. Expression of mRNAs encoding IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 was detected in both ER-positive and in ER-negative breast cancer tissues.

Clemmons et al. (11) reported that the IGFBP secretion from breast cancer tumor cell lines is correlated with ER status. Thus, ER-positive lines secreted IGFBP-2 and a $M_r$ 24,000 form but lacked IGFBP-1 and IGFBP-3, whereas ER-negative cell lines secreted IGFBP-1, IGFBP-3, and the $M_r$ 24,000 form but lacked IGFBP-2. In human breast tumors we were not able to find such a clear relationship between ER and IGFBP expression and production. Thus, all five IGFBP mRNAs were expressed in both ER-negative and ER-positive tumors. The fact that the $M_r$ 49,000 IGFBP, corresponding in size to IGFBP-3, was more abundant in ER-negative tumors supports a relation between this IGFBP and ER content in agreement with observations in breast tumor cell lines (11). Besides this, no other differences could be detected between ER negative and ER positive tumors, as analyzed by Western ligand blotting and IGFBP-1 IRMA. This is in clear contrast to observations in breast cancer cell lines (11). A more heterogeneous population of cells in tumors than in cell lines may account for this difference. Furthermore, cells in continuous culture often have selective properties which make them suitable for continuous culturing.

We have reported earlier that only 2 of 72 breast cancer tissues were positive for IGFBP-1 as measured by a radioimmunoassay with a sensitivity of 9.8 ng/ml (4). Our present data showing that 15 of 47 breast tumors contained IGFBP-1 as measured by the more sensitive IRMA are consistent with previous findings, since only small amounts of IGFBP-1 could be found. Actually, all tissue cytosols contained IGFBP-1 concentrations below the detection limit of the radioimmunoassay used earlier. These low amounts of IGFBP-1 are also below the detection limit in Western ligand blot analysis. Further support for the low IGFBP-1 content in breast tissue and tumors is the low quantity of IGFBP-1 mRNA measured by quantitative RT-PCR and our inability to detect IGFBP-1 mRNA by Northern analysis. Thus, a physiological role for IGFBP-1 in normal breast tissue and breast cancer seems unlikely.

Approximately half of the tumors contained $M_r$ 24,000, 28,000, 34,000 or 49,000 IGFBPs, whereas the $M_r$ 43,000 IGFBP was detected in one fourth of the tumors. It is of interest that breast tumors, like breast cancer cell lines, express such a broad variety of IGFBPs consisting of at least 5 IGFBP species. This is of special interest in view of the more abundant content of IGFBPs in tumors than in adjacent normal tissue. Similarly, we have earlier reported that the IGF-1 receptor is more abundant in breast tumors than in adjacent normal tissue. The role
of these alterations in IGF-related proteins in the development of breast tumors is not clear. A possible role for the IGF system in breast cancer cell growth has, however, been implied (2-4, 11, 12). Thus, all breast tumors studied have been shown to express IGF-I mRNA in their stromal cells, and both IGF-I and IGF-II stimulate breast cancer cell growth, acting through both the IGF-I and IGF-II receptors (2, 3, 24). On the other hand, IGFBPs can either inhibit or stimulate IGF action (12, 25). In breast tumor cell line, IGFBP-1 was reported to stimulate the IGF-I induced cell growth. Due to the very low content of IGFBP-1 found in human breast tumors, a biological role for IGF-I induced cell growth. Due to the very low content of IGFBP-1 found in human breast tumors, a biological role for IGF-I seems unlikely. On the other hand, the abundant and variable presence of IGFBPs in human breast tumors suggest that IGFBPs may play a physiological role as modulators of IGF action in breast cancer. This effect may be different in different tumors, depending on which IGFBPs are expressed.

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