Journal of Cellular Biochemistry 52:196–205 (1993)

Expression of Insulin-Like Growth Factor Binding Proteins in Human Breast Cancer Correlates With Estrogen Receptor Status

Jose A. Figueroa, James G. Jackson, William L. McGuire, Robert F. Krywicki, and Douglas Yee

Department of Medicine, Division of Medical Oncology, University of Texas Health Science Center, San Antonio, Texas 78284-7884 (J.A.F., J.G.J, W.L.M., D.Y.); Oncology Service, Brooke Army Medical Center, Fort Sam Houston, San Antonio, Texas 78234 (R.F.K.)

Abstract The insulin-like growth factors (IGFs) have been implicated in the growth regulation of human breast cancer. Since the IGFs are associated with specific binding proteins (IGFBPs) which may modulate receptor/ligand interactions, production of IGFBPs by breast cancer cells could alter their IGF-dependent growth. This study examined the expression of IGFBPs 4, 5, and 6 in eight breast cancer cell lines (BCCLs) using ribonuclease (RNase) protection assays. IGFBP-4 mRNA was detected in all BCCLs studied. IGFBP-5 expression was higher in estrogen receptor (ER) positive cells, while IGFBP-6 mRNA was detected in only two ER negative BCCLs. We also found that E_2 treatment enhanced the expression of IGFBPs 2, 4, and 5 in T47-D cells. We next studied IGFBP mRNA expression in 40 primary breast tumors. All tumors expressed mRNA for IGFBPs 2–6 but none expressed IGFBP-1 message. IGFBP-3 expression was higher in ER negative tumors, while that of IGFBP-4 and -5 was higher in ER positive specimens. These differences were statistically significant ($P < .05$). Ligand blot analysis of tumor extracts confirmed the presence of IGFBPs in breast cancer tissues. Thus, differential IGFBP expression in ER positive and negative tumors suggests an important role for this protein in breast cancer biology. © 1993 Wiley-Liss, Inc.

Key words: IGFs, cell growth, breast cancer cell lines, tumor biology, hormonal regulation

Polypeptide growth factors are important mediators of normal and neoplastic cell growth in vitro. The observation that several human tumors express high affinity receptors for specific growth factors has led to the assumption that these peptides operate in an autocrine or paracrine fashion [Aaronson, 1991]. Therefore, it has been proposed that growth factor–mediated cell proliferation may account, at least in part, for the unregulated growth of human cancer.

The insulin-like growth factors (IGFs) are mitogenic polypeptides that have been implicated in the autocrine and paracrine growth of human breast cancer [Yee et al., 1989a; Osborne et al., 1989; Cullen et al., 1992]. In contrast to other peptide hormones, the IGFs are associated with high affinity binding proteins in all extracellular fluids [Baxter and Martin, 1989]. To date, six distinct IGF binding proteins (IGFBPs) have been cloned and structurally characterized [Shimasaki and Ling, 1991]. A great interest in elucidating the physiologic functions of the IGFBPs has been stimulated by the observation that some of these proteins can modulate IGF action in different experimental systems. For example, IGFBPs can both potentiate and inhibit IGF-mediated DNA synthesis in cultured fibroblasts [De Mellow and Baxter, 1988; Blum et al., 1989; Liu et al., 1991] and can interfere with IGF effects in transformed cells [Ritvos et al., 1988; Campbell and Novak, 1991; Culouscou and Shoyab, 1991]. Thus, interactions between IGFBPs, ligands, and receptors suggest a complex mechanism for the regulation of IGF-stimulated cell proliferation.

We and others have shown that IGFBP production by human breast cancer cell lines is heterogeneous and that their pattern of expression is related to the cell's estrogen receptor (ER) status [Yee et al., 1989b, 1991; Clemmons et al., 1990; Figueroa and Yee, 1992]. For example, our previous study showed that IGFBP-1 was secreted by only two ER negative cell lines

Received October 27, 1992; accepted December 20, 1992.

Address reprint requests to Douglas Yee, Department of Medicine, Division of Medical Oncology, University of Texas Health Science Center, San Antonio, TX 78284-7884.

© 1993 Wiley-Liss, Inc.

(MDA-MB-231 and Hs578T), while IGFBPs 2 and 3 were predominantly expressed by ER positive and ER negative cells, respectively. The differential expression of IGFBPs by breast cancer cells and its relation to ER status suggest the possibility that some of these binding proteins may be under hormonal regulation. Furthermore, since the IGFs are mitogenic for breast cancer cells, alterations in IGFBP synthesis by breast tumors could influence malignant growth by modulating the actions of locally produced IGFs. In support of this view, we have shown that at least one IGFBP (IGFBP-1) can neutralize IGF-I dependent growth in the ER positive breast cancer cell line MCF-7 [McGuire et al., 1992].

Based on the potential biological importance of these binding proteins and the observation that several unidentified IGFBPs are secreted by BCCLs in culture, we examined the expression of all the known IGFBPs in several breast cancer cell lines and human breast cancer tissues. In this study we have characterized mRNA expression of IGFBPs 4, 5, and 6 in cultured breast cancer cells and showed that estradiol (E_2) is capable of regulating the expression of certain IGFBPs in the ER positive cell line T47-D. We also demonstrated that IGFBPs are produced by human breast cancer tissues and that a correlation exists between the level of mRNA expression of certain IGFBPs and tumor ER status.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. All electrophoresis equipment was obtained from Biorad (Richmond, CA). [^{32}P] UTP and [^{32}P] CTP were purchased from Dupont/New England Nuclear Corp. (Boston, MA). Radiolabelled IGF-I and molecular weight protein standards were obtained from Amersham (Arlington Heights, IL). Protein concentration assay was purchased from Pierce (Rockford, IL). Centricon-3 microconcentrators were obtained from Amicon (Danvers, MA). Restriction enzymes and RNA polymerases were purchased from Promega (Madison, WI). Improved Minimal Essential Medium (IMEM) and fetal calf serum (FCS) were obtained from GIBCO/BRL (Bethesda MD) and from Innovar (Gaithersberg, MD), respectively. Radiographic film and Quanta II intensifying screens were purchased from Kodak (Rochester, NY). Antibodies to IGFBP-2 and -3 were purchased from UBI, Inc. (Lake Placid, NY).

Cell Lines and Breast Cancer Tissues

MCF-7, ZR75-B, Hs758T, and CAMA I were originally obtained as previously described [Yee et al., 1991]. All other cell lines were obtained from the American Type Culture Collection (Rockville, MD). Breast cancer cell lines were maintained in IMEM plus insulin and 10% FCS in a humidified 5% CO_2 atmosphere. OVCAR-3 was maintained in RPMI 1640 plus 10% FCS. Breast cancer tissues were obtained from the San Antonio Tumor Bank, a large repository of tumor specimens from throughout the country, and stored at −70°C until used. Estrogen receptor content was routinely measured in these specimens.

cDNA Probes

pG4BP-25 and pSP73BP-53, containing IGFBPs 1 and 3 cDNAs, were kindly provided by Dr. D.R. Powell (Baylor College of Medicine, Houston, TX). pHBP-2, pHBP-4, pHBP-5, and pHBP-6 were a gift from Dr. S. Shimasaki (Whittier Institute for Diabetes and Endocrinology, La Jolla, CA). 36B4 was a gift from Dr. P. Chambon (INSERM, Strasbourg, France). The cRNA probes used to detect IGFBP mRNA were transcribed from different portions of each cDNA; probes for IGFBP-1 and IGFBP-3 have previously been described [Yee et al., 1991]. Other cDNAs used for templates were a 382bp StyI-EcoRV IGFBP-2 fragment [Binkert et al., 1989], a 505bp EcoRI-HindIII IGFBP-4 fragment [Shimasaki et al., 1990], a 317 bp SacII-SacI IGFBP-5 fragment [Shimasaki et al., 1991a], and a 267bp PstI-PstI IGFBP-6 [Shimasaki et al., 1991b].

RNA Extraction and Ribonuclease (RNase) Protection Assay

Total RNA was isolated from cell lines and tumor specimens using phenol-chloroform extraction [Chomczynski and Sacchi, 1987]. RNA concentration was determined spectrophotometrically and its integrity examined by agarose gel electrophoresis. The human IGFBP probes were used to synthesize radiolabelled antisense RNA probes using a transcription kit (Promega). The probe sizes are noted above. Radiolabelled 36B4 RNA probe was transcribed from a 200 bp PstI-PstI 36B4 cDNA. The expression of this ubiqui-

tous, non-estrogen-regulated gene was used as a RNA loading control [Bassett et al., 1990]. For each determination 20 μg of total RNA was used, and RNase protection assays were performed as previously described [Yee et al., 1988]. Samples were then centrifuged and pellets resuspended in 5 μl of 80% formamide buffer, denatured at 85°C for 5 min, and electrophoretically fractionated in a 6% polyacrylamide/8 M urea gel. Size markers (New England Biolabs) were end-labelled with [^{32}P] CTP. After electrophoresis, the gel was dried, and exposed to X-ray film in the presence of an intensifying screen for 12–96 h.

Estradiol (E_2) Treatment of T47-D Cells

Cells were plated in 100 cm^2 dishes and incubated with IMEM plus insulin and 10% FCS and growth to subconfluence. They were then washed 3 times with phosphate buffered saline (PBS) and placed in phenol-free IMEM plus 5% double charcoal stripped calf serum (CSS). After an additional 24 h, cells were washed with PBS and placed in serum-free media (IMEM plus 292 mg/l glutamine, 20 mM 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid buffer, 2 mg/l fibronectin, 2 mg/l transferrin, and trace elements) with or without 1 nM estradiol for 24 h. Cells were washed again with ice-cold PBS and harvested directly in guanidinium thiocyanate for RNA extraction as described above.

Western Ligand (WLB) and Immunoblot Analysis

MCF-7 cells and 30 mg of breast tumor powder were homogenized in 300 μl of 5% SDS, boiled for 5 min, and centrifuged at 25,000*g* for 3 min. The supernatant was removed and total protein concentration determined using a copper-bicinchoninic acid kit (Pierce). Then 200 μg of total breast tumor protein was mixed with an equal volume of sample buffer (.1 M Tris HCl, pH 6.8, 4% SDS, .2% bromophenol blue, and 20% glycerol) and separated by electrophoresis on a 12% SDS-PAGE gel along with pre-stained molecular weight standards (Amersham, Arlington Heights, IL). Proteins were then transferred to nitrocellulose and hybridized with radiolabelled IGF-I as previously described [Hossenlopp et al., 1986]. The blot was exposed to X-ray film for 2 weeks at −70°C. Conditioned media (CM) from OVCAR-3 cells was obtained under conditions identical to those described for E_2 treatment of breast cancer cell lines (BCCLs) except that estrogens were not used. CM was concentrated twentyfold using Centricon filters with a 10,000 molecular weight cut-off (Amicon) and 30 μl aliquots were analyzed using the WLB technique described above. The blot was exposed to X-ray film for 24 h at −70°C.

Some conditioned media samples were also measured by Western immunoblot after ligand blotting. Membranes were blocked in a Tris buffered saline–Tween 20 solution (.15 M NaCl, .01 M Tris HCL, pH 7.4, .05% Tween 20). IGFBP-2 and -3 antibodies were diluted according to the instructions of the manufacturer and detected using an electrochemiluminescent kit (Amersham).

Statistical Analysis

Protected fragments were quantified by directly measuring the radioactivity in each band using a radioanalytical isotope scanner (Ambis, San Diego, CA). Net counts were divided by 36B4 values and reported as a corrected net count. This correction was performed to account for loading differences and to compare results between groups. Differences between group means were analyzed using Wilcoxon 2-Sample Test. P values $< .05$ were considered significant.

RESULTS

Expression of IGFBPs 4, 5, and 6 in BCCLs

We utilized RNase protection assays to examine the expression of IGFBP-4, -5, and -6 in several human BCCLs. All the cell lines studied expressed significant levels of IGFBP-4 mRNA irrespective of their ER status (Fig. 1A and data not shown). IGFBP-5 mRNA expression, although heterogeneous, was higher among ER positive cell lines (MCF-7, T47D, and ZR75-B). However, its association with ER status is not clear since at least two ER negative cell lines (Hs578T, MDA-MB-468) expressed significant IGFBP-5 mRNA levels (Fig. 1B). IGFBP-6 expression was more restricted than that of IGFBP-4 and -5, with low levels detected only in the ER negative cell lines MDA-MB-231 and Hs578-T (Fig. 1C). These results confirmed the heterogeneous expression of IGFBPs by human BCCLs. Table I summarizes the expression pattern of all IGFBPs in the BCCLs studied.

E_2 Regulation of IGFBP Expression in T47-D Cells

It is well established that estrogens can be mitogenic for certain breast cancer cells in cul-

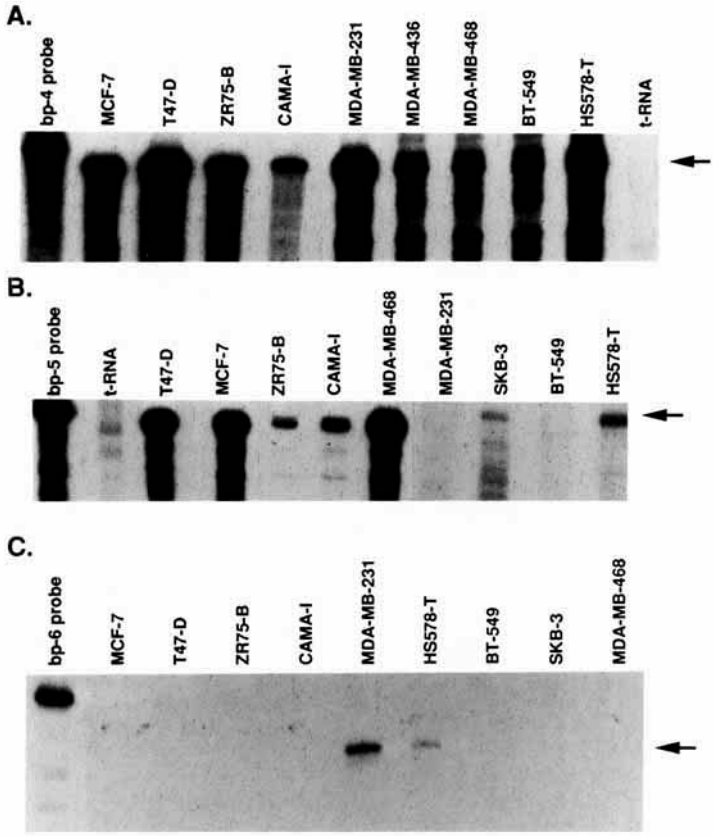

Fig. 1. RNase protection assays showing expression of (**A**) IGFBP-4, (**B**) IGFBP-5, and (**C**) IGFBP-6 in BCCLs. Each lane contains 20 μg of total RNA. Lanes labelled *probe* contain undigested riboprobe. Arrows show protected mRNA fragments. MCF-7, T47-D, and ZR75-B are estrogen receptor (ER) positive cell lines, while CAMA I, MDA-MB-468, MDA-MB-436, MDA-MB-231, BT-549, Hs578-T, and SKB-3 are ER negative. tRNA was used as a negative control.

TABLE 1. IGFBP Expression in Human Breast Cancer Cell Lines*

Cell line	BP-1	BP-2	BP-3	BP-4	BP-5	BP-6	ER
MCF-7	−	++	±	+	++	−	+
ZR75B	−	+	+	+	+	−	+
T47D	−	++	−	++	++	−	+
SKBR3	−	−	−	+	+	−	−
MDA-MB-231	+	−	++	+	−	+	−
MDA-MB-468	−	+	N/A	+	++	−	−
MDA-MB-436	−	+	−	+	−	N/A	−
Hs578T	+	−	++	+	−	+	−
CAMA I	−	+	+	+	+	−	−

*Expression of IGFBPs 1, 2, and 3 was determined in earlier studies [Yee et al., 1989b, 1991]. mRNA expression was graded as absent (−), detectable (+), and high (++). ER represents estrogen receptor status and N/A means the information was not available.

ture. Moreover, E_2 has been shown to stimulate the autocrine production of IGF-II in T47-D cells, suggesting its proliferative effects are in part mediated by growth factor actions [Yee et al., 1988]. Since certain IGFBP species are capable of modulating IGF actions in different experimental systems, it is possible that some E_2 effects on breast cancer proliferation may be related to its regulation of IGFBP production. With this in mind, we examined the ability of E_2 to affect IGFBP expression in T47-D cells. Total RNA was extracted after cells were exposed to

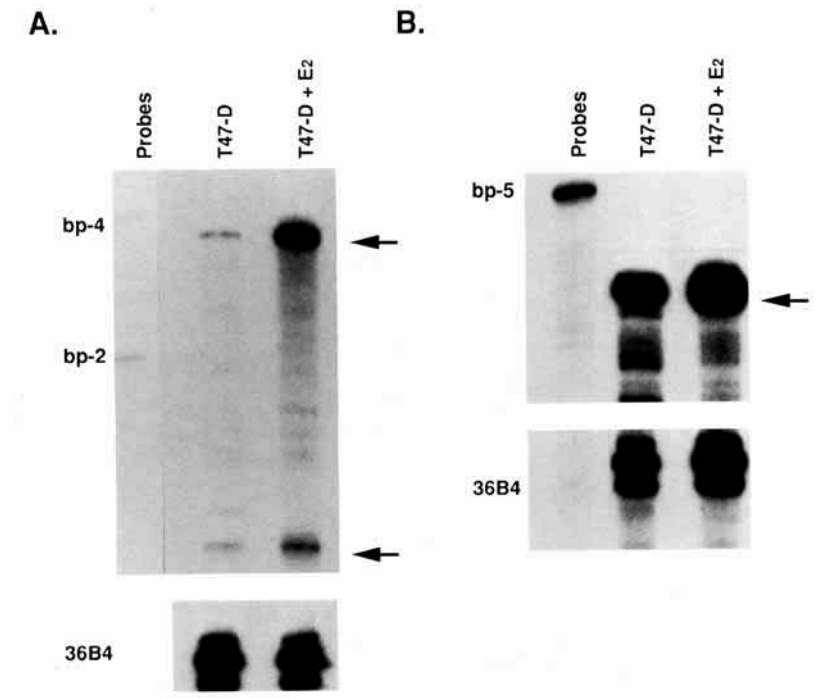

Fig. 2. Effects of E_2 on the expression of IGFBPs 2, 4, and 5 in T47-D cells. RNase protection assay showing expression of IGFBPs 2 and 4 (**A**) and IGFBP-5 (**B**) in control and E_2-treated T47-D cells. Cells were treated with E_2 for 24 h and 20 μg of total RNA were hybridized per lane. The protected fragments migrate below their respective probes (arrows). Expression of 36B4 was measured in the same specimen and used as a loading control (see Materials and Methods).

E_2 for 24 h and RNase protection assays performed to document alterations in IGFBP mRNA expression. We found that estrogen treatment of T47-D resulted in cell proliferation (data not shown) and enhanced expression of IGFBP-2, -4, and -5 (Fig. 2). These results, together with our previous work demonstrating E_2 regulation of these same IGFBPs in MCF-7 cells [McGuire et al., 1992], indicate that certain IGFBP species are under hormonal control in BCCLs.

IGFBP mRNA Expression in Human Breast Cancer Tissues

To see whether the relationship between ER status and IGFBP expression was also maintained in vivo, we examined the expression of all known IGFBPs in 40 breast cancer specimens. IGFBP mRNA levels were directly quantified by scanning the gel with a sensitive radioanalytical system. We found that none of the specimens expressed IGFBP-1 mRNA (Fig. 3 and data not shown). All tumor samples expressed specific mRNA transcripts for IGFBPs 2–6 although the level of expression varied significantly between specimens (Figs. 3, 4). IGFBP-5 was highly expressed by most tumors, while IGFBPs 3 and 6 were expressed at low levels in the specimens studied. Figure 5 depicts the relative expression of the different IGFBPs in breast cancer tissues after correction for total RNA expression (see Materials and Methods).

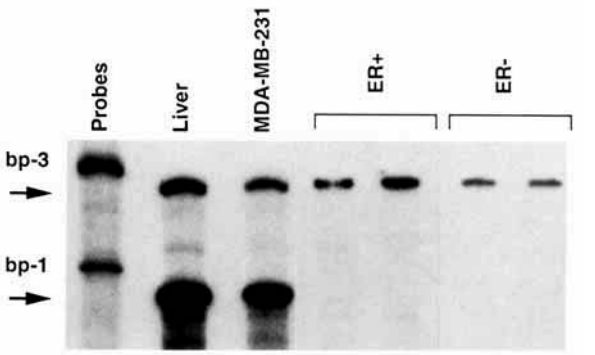

Fig. 3. Expression of IGFBPs 1 and 3 RNA in human breast cancer tissues. RNase protection assay showing the expression of IGFBPs 1 and 3 in human liver, MDA-MB-231, and ER positive and ER negative human breast tumors. Arrows show the migration position of RNA protected fragments.

Since E_2 can regulate IGFBP expression in BCCLs in vitro, we looked for differences in

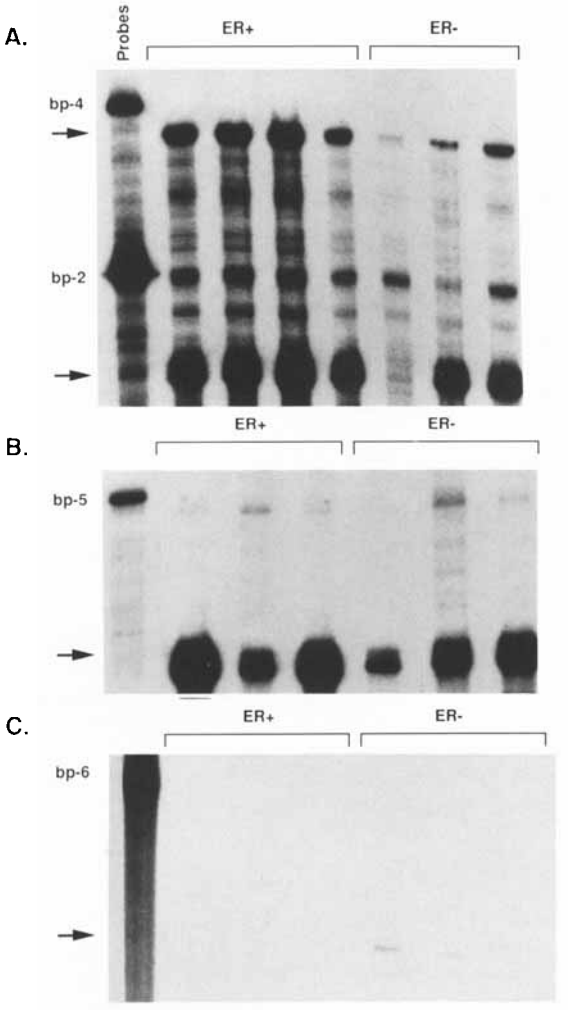

Fig. 4. IGFBP expression in human breast cancer tissues. RNase protection assays showing expression of (A) IGFBPs 2 and 4, (B) IGFBP-5, and (C) IGFBP-6 in ER positive and ER negative breast cancer tissues. In each lane 20μg of total RNA was used. Bands representing protected fragments migrate below their respective probes (arrows). Bands migrating parallel (A, B) to riboprobe represent undigested probe.

IGFBP mRNA expression between ER positive (>10 femtomoles/mg protein) and ER negative (<10 femtomoles/mg protein) breast tumors. Our results showed that IGFBP-3 transcripts were more abundant in ER negative tumors, while mRNA levels of IGFBPs 4 and 5 were higher in ER positive specimens. Differences in IGFBP-3 and -4 mRNA levels between ER positive and negative tumors were highly significant ($P < .01$) while that of IGFBP-5 was of borderline significance ($P < .048$) (Fig. 6). On the other hand, expression of IGFBP-2 and -6 was not significantly different between ER positive and negative specimens (Fig. 6).

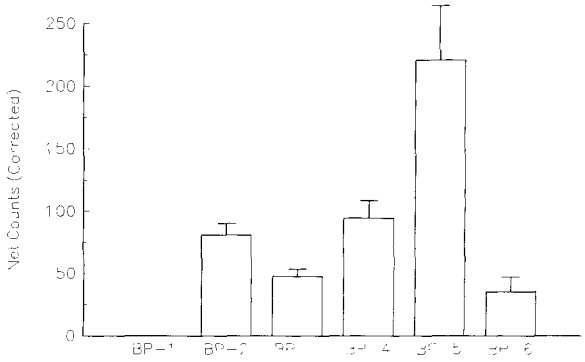

Fig. 5. Relative expression of IGFBPs in breast cancer tissues. Radioactivity of IGFBP protected fragments was quantified and corrected for total RNA using 36B4 as a control (see Materials and Methods). Results were expressed as the mean net counts obtained for each IGFBP in a group of 40 tumor specimens. Error bars represent standard error of the mean.

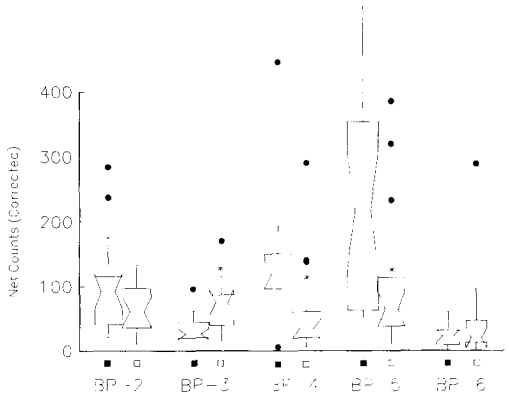

Fig. 6. Differences in IGFBP expression between ER positive and ER negative breast cancer tissues. This box plot graph represents IGFBP expression in 40 breast tumors specimens (20 ER positive and 20 ER negative). The dotted line represents the sample median and the box contains 50% of the values. The notches show the 95% confidence interval. The complete range of values are contained within the whiskers, although some outlying values are represented by a dot. Solid squares mark the position of ER positive tumors; open squares are ER negative. Asterisks show significant differences between ER positive and ER negative tumor IGFBP expression ($P < .05$).

WLB and Immunoblot of Tumor Extracts

We examined 20 tumors using WLB and found detectable bands of 48, 44, 34, 28, and 24 kDa in the tumor extracts examined (Fig. 7). The 34 kDa band is compatible with IGFBP-2. Figure 8A (lanes 1–4) shows that this binding protein species is detected by a specific IGFBP-2 antisera in cancer cell conditioned media. The doublet at 48 and 44 kDa detected on ligand blotting is compatible with IGFBP-3 expression (Fig. 8B, lanes 1,2). The smaller band at 30 kDa detected

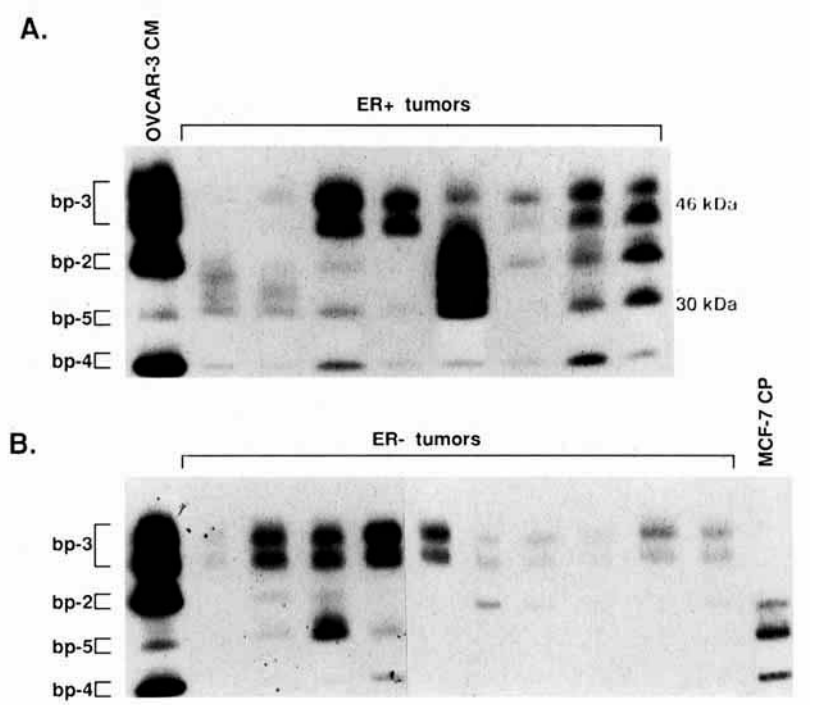

Fig. 7. Western ligand blot of breast tumor extracts. Ligand blot analysis of (A) ER positive and (B) ER negative tumor extracts. Migration position of IGFBPs are marked based on reported values and immunoblot studies (see text). Conditioned media (CM) from the ovarian cancer cell line OVCAR-3 and MCF-7 cell pellets (CP) were used as positive controls. Excess cold IGF-I eliminated binding of radiolabelled IGF-I (data not shown).

by this antisera corresponds to an N-terminal fragment of IGFBP-3. This fragment does not bind IGF-I and is not likely to be detected by ligand blotting [Zapf et al., 1990]. These findings are compatible with previous work using specific antisera against IGFBP-2 and -3 in BCCL CM [Clemmons et al., 1990; De Leon et al., 1990].

The only abundant binding protein species mRNA expressed in common between MCF-7 and MDA-MB-231 was IGFBP-4 (Table I). Since both cells express a 24 kDa protein in their CM, these data suggest this is indeed IGFBP-4 [Yee et al., 1991]. Other investigators have also identified the 24 kDa protein as IGFBP-4 [Camacho-Hubner et al., 1992]. The 28 kDA band most likely represents IGFBP-5 since it is found only in the CM of cells that express IGFBP-5 mRNA (MCF-7); MDA-MB-231 cells do not express IGFBP-5 mRNA and do not secrete a 28 kDa binding protein species. Therefore, based on these observations, the 28 kDa band most likely represents IGFBP-5.

IGFBP-1, which migrates between IGFBPs 4 and 5, was not detected in any of the specimens studied. IGFBP-6 does not bind IGF-I with high affinity [Bach et al., 1992; Zapf et al., 1990] and its production by tumors could not be evaluated using IGF-I WLB.

Thus, protein species compatible with IGFBP-2, -3, -4, and -5 can be detected in extracts from breast cancer cells. We are currently examining a larger group of tumors to determine if the relationships between ER and IGFBP expression can also be detected by ligand blotting.

DISCUSSION

Several lines of evidence suggest the IGFs are important in the autocrine and paracrine growth regulation of breast cancer cells. In contrast to other polypeptide growth factors, regulation of cell proliferation by the IGF system may depend on interactions between ligands, receptors, and high affinity IGFBPs. Although six distinct IGFBPs have been described, their exact physiologic role is not well understood. However, several studies have demonstrated that some IGFBP species can inhibit or facilitate IGF action depending on the experimental conditions employed. For example, IGFBP-3 has been shown to potentiate IGF-I–induced DNA synthesis in

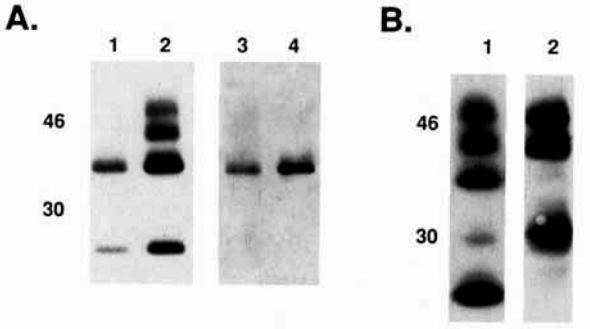

Fig. 8. Comparison between ligand blotting and immunoblotting for IGFBP-2 and -3. **A:** Conditioned media from MCF-7 (lanes 1,3) and OVCAR-3 (lanes 2, 4) were examined by ligand blotting (lanes 1,2). The blot was then used for IGFBP-2 immunoblotting showing the species migrating at 34 kDa is likely to be IGFBP-2. An electrochemiluminescent detection system was utilized; exposure in lanes 3 and 4 was 30 seconds. **B:** OVCAR-3 conditioned media was examined in ligand blotting (lane 1) followed by immunoblotting (lane 2) with an IGFBP-3 antibody. The IGFBP doublet species migrating at 48 and 44kDa is likely to be IGFBP-3; the species at 30 kDa is a truncated form of IGFBP-3 (see text).

human fibroblasts [De Mellow and Baxter, 1988; Blum et al., 1989], while IGFBP-1 inhibited IGF-I–mediated thymidine incorporation in chick embryo fibroblasts [Liu et al., 1991].

We have previously shown that human BCCLs express IGFBP-1, -2, and -3. In the present study we have characterized the expression of IGFBP-4, -5, and -6 in several human BCCLs. Our results, and those of other investigators [Clemmons et al., 1990; De Leon et al., 1990; Sheikh et al., 1992], indicate that the pattern of IGFBP expression in BCCLs is heterogeneous and in part related to ER status. Additionally, we have shown that E_2 treatment increased the expression of IGFBP-2, -4, and -5 in the ER positive BCCL T47-D. The fact that similar results were previously obtained using MCF-7 cells [McGuire et al., 1992] confirm the association between ER and IGFBP expression in vitro.

To determine if our in vitro observations were relevant to breast cancer in vivo, we examined the expression of all the known IGFBPs in 40 primary breast tumors. mRNA levels for IGFBPs 2–6 were detected in all the tumors examined, while IGFBP-1 message was not found in any of the specimens. We observed variations in the relative expression of IGFBPs among the specimens analyzed and, more importantly, found that mRNA levels of IGFBP-3, -4, and -5 correlated with tumor ER status. Two groups have recently examined IGFBP expression in human breast cancer tissues. Using in situ hybridization in 20 breast tumors, Shao et al. [1992] reported higher expression of IGFBP-3 mRNA in ER negative specimens. Although some overlap existed between ER positive and negative tumors, the difference between groups was statistically significant. More recently, Pekonen et al. [1992] used WLB to study 47 breast tumors and found that IGFBP-3 protein levels were higher in ER negative specimens. At least four different IGFBPs were identified in tumor extracts. As in our study, the authors could not detect IGFBP-1 using ligand blotting although very low levels of this binding protein (< 1 ng/ml) could be detected using a sensitive immunoradiometric assay. This level of IGFBP-1 could arise from blood contained within the extracted tumor specimens. We agree with Pekonen et al. [1992] that little or no expression of IGFBP-1 is seen in breast cancer; thus it seems unlikely that IGFBP-1 has a significant physiologic function in the regulation of tumor cell growth.

Pekonen et al. [1992] also demonstrated IGFBP mRNA expression in five tumors using reverse transcription polymerase chain reaction. Unfortunately, no attempt was made to correlate mRNA levels with ER status. In this study we have independently confirmed the presence of at least four distinct IGFBPs in breast cancer tissues as well as the relationship between ER negativity and IGFBP-3 expression in vivo. Furthermore, our results are the first to demonstrate a correlation between IGFBP-4 and -5 mRNA levels and ER positivity in human breast tumors.

The observation that expression of IGFBP-4 and -5 is induced by estrogen in vitro and is also higher in ER positive tumors points to an association between ER functionality and binding protein expression in vivo. Thus, local endocrine factors may play an important regulatory role in the expression of certain IGFBPs by breast tumors. Since it has been proposed that estrogen's mitogenic actions are mediated through stimulation of growth factor synthesis by breast cancer cells [Dickson and Lippman, 1987; Yee et al., 1988; Osborne et al., 1989; Stewart et al., 1990], it is tempting to speculate that estrogenic regulation of IGFBP production may result in augmented cell proliferation by modulating cellular responsiveness to locally produced IGFs. Further study is needed to define the precise functions of each IGFBP species and their importance in estrogen and IGF-dependent breast cancer growth. Alternatively, if expression of

certain IGFBPs serves as a marker for ER function, it is possible that IGFBP expression may convey prognostic information as is the case with other estrogen-regulated genes (progesterone receptor, cathepsin D, pS2) [McGuire and Clark, 1992]. We are currently examining this hypothesis by examining IGFBP expression in a larger tumor population.

ACKNOWLEDGMENTS

D.Y. is a PEW Scholar in the Biomedical Sciences and is supported by grant R29CA52592. J.A.F. is supported by NRSA training grant CA09434. We regret the untimely death of our friend and teacher Dr. William L. McGuire prior to the publication of this work.

REFERENCES

Aaronson SA (1991) Growth factors and cancer Science 254 1146–1153

Bach LA, Thotakura NR, Rechler MM (1992) Human insulin-like growth factor binding protein-6 is O-glycosylated Biochem Biophys Res Commun 186 301–307

Bassett P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajcer OL, Chenard MP, Rio MC, Chambon P (1990) A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas Nature 348 699–704

Baxter RC, Martin JL (1989) Binding proteins for the insulin-like growth factors Structure, regulation and function Prog Growth Factor Res 24(1) 49–68

Binkert C, Landwehr J, Marg J-L, Schwander J, Heinrich G (1989) Cloning, sequence analysis and expression of cDNA encoding a novel insulin-like growth factor binding protein (IGFBP-2) EMBO J 8 2497–2502

Blum WF, Jenne EW, Reppin F, Kietzmann K, Ranke MB, Bierich JR (1989) Insulin-like growth factor I (IGF-I)-binding protein complex is a better mitogen than free IGF-I Endocrinology 125 766–772

Camacho-Hubner C, Busby WH, McCusker RH, Wright G, Clemmons DR (1992) Identification of the forms of insulin-like growth factor-binding proteins produced by human fibroblasts and the mechanisms that regulate their secretion J Biol Chem 267 11949–11956

Campbell P, Novak JF (1991) Insulin-like growth factor binding protein (IGFBP) inhibits IGF action on human osteosarcoma cells J Cell Physiol 149 293–300

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction Anal Biochem 162 156–159

Clemmons DR, Camacho-Hubner C, Coronado E, Osborne CK (1990) Insulin-like growth factor binding protein secretion by breast cancer cell lines Correlation with estrogen receptor status Endocrinology 127 2679–2686

Cullen KJ, Lippman ME, Chow D, Hill S, Rosen N, Zwiebel JA (1992) Insulin-like growth factor-II overexpression in MCF-7 cells induces phenotypic changes associated with malignant progression Mol Endocrinol 6 91–100

Culouscou JM, Shoyab M (1991) Purification of a colon cancer cell growth inhibitor and its identification as an insulin-like growth factor binding protein Cancer Res 51 2813–2819

De Leon DD, Bakker B, Wilson DM, Lamson G, Rosenfeld RG (1990) Insulin-like growth factor binding proteins in human breast cancer cells Relationship to hIGFBP-2 and hIGFBP-3 J Clin Endocrinol Metab 71 530–532

De Mellow JS, Baxter RC (1988) Growth hormone-dependent insulin-like growth factor (IGF) binding protein both inhibits and potentiates IGF-stimulated DNA synthesis in human skin fibroblasts Biochem Biophys Res Commun 156 199–204

Dickson RB, Lippman ME (1987) Estrogenic regulation growth and polypeptide growth factor secretion in human breast carcinoma Endocr Rev 8 29–43

Figueroa JA, Yee D (1992) The insulin-like growth factor binding proteins (IGFBPs) in human breast cancer Breast Cancer Res Treat 22 81–90

Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S, Binoux M (1986) Analysis of insulin-like growth factor binding proteins using Western blotting Use of the method for titration of the binding proteins and competitive binding studies Anal Biochem 154 138–143

Liu L, Brinkman A, Blat C, Harel L (1991) IGFBP-1, an insulin-like growth factor binding protein, is a cell growth inhibitor Biochem Biophys Res Commun 174 673–679

McGuire WL, Clark GM (1992) Prognostic factors and treatment decisions in axillary node-negative breast cancer N Engl J Med 326 1756–1761

McGuire WL Jr, Jackson JG, Figueroa JA, Shimasaki S, Powell DR, Yee D (1992) Regulation of insulin-like growth factor binding protein expression by breast cancer cells Use of IGFBP-1 as an inhibitor of IGF action J Natl Cancer Inst 84 1336–1341

Osborne CK, Coronado EB, Kitten LJ, Arteaga CL, Fuqua SAW, Ramasharma K, Marshal M, Li CH (1989) Insulin-like growth factor II (IGF-II) A potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor Mol Endocrinol 3 1701–1709

Pekonen F, Nyman T, Ilvesmaki V, Partanen S (1992) Insulin-like growth factor binding proteins in human breast cancer tissues Cancer Res 52 5204–5207

Ritvos O, Ranta T, Jalkanen J, Suikkari AM, Voutilainen R, Bohn H, Rutanen EM (1988) Insulin-like (IGF) growth factor binding protein from human decidua inhibits the binding and biological action of IGF-I in cultured choriocarcinoma cells Endocrinology 122 2150–2157

Shao Z-M, Sheikh MS, Ordonez JV, Feng P, Kute T, Chen J-C, Aisner S, Schnaper L, LeRoith D, Roberts CT Jr, Fontana J (1992) IGFBP-3 expression and estrogen receptor status in human breast carcinoma Cancer Res 52 5100–5103

Sheikh MS, Shao Z-M, Clemmons DR, LeRoith D, Roberts CT Jr, Fontana JA (1992) Identification of the insulin-like growth factor binding proteins 5 and 6 (IGFBP-5 and 6) in human breast cancer cells Biochem Biophys Res Commun 183 1003–1010

Shimasaki S, Gao L, Shimonaka M, Ling N (1991b) Isolation and molecular cloning of insulin-like growth factor-binding protein-6 Mol Endocrinol , 5 938–948

Shimasaki S, Ling N (1991) Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5, -6) Prog Growth Factor Res 3 243–266

Shimasaki S, Shimonaka M, Zhang HP, Ling N (1991a) Identification of five different insulin-like growth factor binding proteins (IGFBPs) from adult rat serum and molecular cloning of a novel IGFBP-5 in rat and human J Biol Chem 266 10646–10653

Shimasaki S, Uchiyama F, Shimonaka M, Ling N (1990) Molecular cloning of the cDNAs encoding a novel insulin-like growth factor-binding protein from rat and human Mol Endocrinol 4 1451–1458

Stewart AJ, Johnson MD, May FEB, Westley BR (1990) Role of insulin-like growth factors and the type I insulin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells J Biol Chem 2165 21172–21178

Yee D, Cullen KJ, Paik S, Perdue JF, Hampton B, Schwartz A, Lippman ME, Rosen N (1988) Insulin-like growth factor II mRNA expression in human breast cancer Cancer Res 48 6691–6696

Yee D, Favoni RE, Lippman ME, Powell DR (1991) Identification of insulin-like growth factor binding proteins in breast cancer cells Breast Cancer Res Treat 18 3–10

Yee D, Favoni RE, Lupu R, Cullen KJ, Lebovic GS, Huff KK, Lee PDK, Lee YL, Powell DR, Dickson RB, Rosen N, Lippman ME (1989b) The insulin-like growth factor binding protein BP-25 is expressed by human breast cancer cells Biochem Biophys Res Commun 158 38–44

Yee D, Paik S, Lebovic GS, Marcus RR, Favoni RE, Cullen KJ, Lippman ME, Rosen N (1989a) Analysis of IGF-I gene expression in malignancy, evidence for a paracrine role in human breast cancer Mol Endocrinol 3 509–517

Zapf J, Kiefer M, Merryweather J, Masiarz F, Bauer D, Born W, Fischer JA, Froesch ER (1990) Isolation from adult human serum of four insulin-like growth factor (IGF) binding proteins and molecular cloning of one of them that is increased by IGF I administration in extrapancreatic tumor hypoglycemia J Biol Chem 265 14892–14898