The Expression and Function of IGFBP-3 in Normal and Malignant Breast Tissue

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Abstract. *The mitogenic and anti-apoptotic effects of insulin-like growth factor-I (IGF-I) are regulated by a family of insulin-like growth factor binding proteins (IGFBPs), particularly IGFBP-3. Little is known about the IGF-independent role of IGFBP-3 in breast cancer and the mechanisms regulating its production. The expression of IGFBP-3 in paired malignant and adjacent normal (n=53), and healthy normal (n=17) breast tissue samples was investigated using RT-PCR, immunohistochemistry and ELISA. We compared IGFBP-3 expression with other members of the IGF-I axis, other known tumorigenic genes and clinicopathological parameters. We also developed a novel tissue explant system using fresh normal and malignant breast tissue, with which we examined the in vitro effects of IGFBP-3 alone and in combination with known apoptotic agent, doxorubicin (n=6), on tissue viability and apoptosis. We demonstrated a high level of expression of IGFBP-3 mRNA in all samples. 96% of samples also expressed IGFBP-3 protein. No significant correlation was seen between IGFBP-3 expression and other clinicopathological parameters. The in vitro tissue explant system demonstrated that IGFBP-3 had little effect by itself on apoptosis. However, when used in combination with doxorubicin, increased apoptosis was seen in tumours. In contrast, less apoptosis was seen in normal tissue suggesting a protective effect. These divergent effects suggest a potential novel chemotherapeutic approach in the treatment of breast cancer. These findings suggest that IGFBP-3 may play a role in tumorigenesis, and that IGFBP-3 levels could be used in the future in cancer risk assessment/prevention or as markers of response to cancer treatments.*

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Insulin-like growth factor binding protein-3 (IGFBP-3) is a 40-44 kDa glycoprotein of hepatic origin that is the major binding protein for circulating insulin-like growth factor-1 (IGF-1). IGFBP-3 may inhibit the bioactivity of IGF-I by preventing it from binding to its own receptor, or conversely may enhance IGF-1's effects by increasing its bioavailability at its receptor (1-3). IGFBP-3 has been implicated in the pathogenesis of a number of different cancers, including breast (4, 5). Low circulating levels of IGFBP-3, and high serum IGF-I levels have been associated with an increased risk of developing breast cancer in pre- and post-menopausal women (6-8). However, studies are conflicting (9, 10).

Within the last decade it has become clear that IGFBP-3 is also able to regulate cell proliferation and apoptosis *via* IGF-1 independent mechanisms. Several studies have demonstrated IGFBP-3 to inhibit growth of breast cancer cells in an IGF-1 independent manner (11-13). It also directly potentiates the apoptosis induced by prior irradiation (14) and treatment with the apoptotic agents, ceramide and paclitaxel (15-17). In addition to its direct actions, alterations in IGFBP-3 expression may be responsible for the growth inhibitory effects of TGFβ, anti-oestrogens (18), retinoic acid (19, 20) and vitamin D analogues (21, 22). IGFBP-3, therefore, may be a major downstream effector of growth inhibitory and apoptosis-inducing agents in breast cancer. Its role in the normal breast, however, remains uncertain and *in vitro* effects on established cancer cell lines may not reflect *in vivo* physiology.

Previous studies of IGFBP-3 expression in breast cancer tissue are conflicting. Some have demonstrated that high levels of IGFBP-3 expression occurs early in breast tumorigenesis and may predict for a more aggressive outcome (23-26). Others show a decreased expression in cancers (27). Few *in vivo* studies have been performed. There have been no studies comparing the simultaneous expression of IGFBP-3 in normal and malignant breast tissue, an important issue as we have recently demonstrated differential expression of IGFBP-3 between normal and malignant colonic tissue (28), which may influence the local actions of IGF-1 in colorectal tumorigenesis and neovasculature (29).

The aims of this study were (i) to quantitate and localise expression of IGFBP-3 in normal and malignant breast tissue, (ii) to correlate this expression with IGF-1 and known tumorigenic genes; cyclo-oxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) (iii) to determine any association between this expression and the clinical phenotype of breast cancer, and (iv) to determine the IGF-1 independent effects of IGFBP-3 on apoptosis in normal and malignant breast tissue using a physiologically relevant explant model system.

Materials and Methods

Patient samples. The study was approved by the Local Research Ethics Committee of the East London and City Health Authority and all patients gave informed consent. Paired samples of normal (AN-adjacent normal tissue) and malignant (T-tumour) breast tissue were taken from 53 consecutive patients undergoing breast cancer surgery. Ten specimens were also collected from women with recurrent cancer that had developed resistance to Tamoxifen (RT=recurrent tumour, RN=recurrent normal) and 17 samples of normal breast tissue were collected from woman undergoing breast surgery for benign disease or breast reduction surgery (NN=healthy normal tissue). All tumours were clinically palpable (greater than 1cm in size) and samples of normal breast tissue were obtained as far away from the tumour site as possible (approximately 8-10 cm). After resection, all samples were placed immediately into 'RNA later' (Ambion, UK) snap frozen in liquid nitrogen and stored at –80˚C. Detailed clinico-patholigical information was obtained for each patient; tumour grade, size, lymph node status, presence of vascular invasion and ductal-carcinoma-*in-situ* (DCIS) and estrogen (ER)/progesterone receptor (PgR) status.

RT-PCR. RNA was extracted from the samples as previously described (30) except that the samples were homogenised in 2 ml cryotubes using a bead mill (Glen Creston Ltd, Stanmore, Middlesex, UK), with 3 steel beads per 0.5 ml lysis buffer. RNA quality and quantity was assessed, in triplicate, using RNA LabChips together an Agilent 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). Primers and probes were newly designed using Primer Express software (ABI, Warrington, UK) (Table I). Real-time RT-PCR assays were performed according to the criteria previously described (31). Reactions were carried out and results recorded and analysed using the ABI 7700 Prism Sequence detection system. mRNA levels were quantitated relative to amplicon specific standard curves (31). Copy numbers were normalised relative to total RNA concentration and expressed as copy numbers/μg RNA. All serial dilutions were carried out in duplicate. The reactions to generate standard curves were repeated twice, each time in triplicate. All clinical samples were tested in triplicate and the average value of the triplicates was used for quantification. Two no-template controls were included with every amplification run; one was prepared before opening all the tubes and dispensing the various reagents, the other at the end of the experiment.

Immunohistochemistry. Four-μm sections were cut from paraffin embedded blocks of the cancers and paired normal breast tissue, and deparaffinised by standard techniques. For antigen retrieval, sections were microwave treated under pressure for 4 minutes at 900 W in preheated 0.01M citrate buffer pH 6.0. Sections were then blocked with horse serum for 5 min to block non-specific binding before incubating with a rabbit anti-human affinity-purified polyclonal IGFBP-3 antibody (GroPep, Australia) at a 1/500 dilution for 90 min at 37˚C. After washing off the primary antibody in Tris-buffered saline, slides were incubated with a secondary biotinylated antibody (Vectastain Universal Elite ABC kit, Vector labs, Peterborough, UK) at room temperature for 30 min and rinsed again in Tris-buffered saline. A tertiary peroxidase-labelled avidin was subsequently applied at room temperature for 20 min before the sections were visualized with activated 39,39-diaminobenzidene-tetrahydrochloride solution (Kentec DAB tablets 4170, Biostat, Stockport, UK) for 10 min. Human liver sections were used as a positive control in each run. Specificity of the antibody was confirmed by absence of immunoreactivity after preincubation of the antibody with IGFBP-3. Assessment was performed in a blinded manner on two separate occasions by a single observer and a mean score determined in both the epithelial cells and the stroma of the intensity of staining (1=absent, 2=weak, 3=moderate, or 4=strong) and the percentage of positive staining cells (1=less than 5%, 2=5-50% or 3=more than 50%). A total score (max of 7) was then generated for the epithelial and stromal component of each section.

Primary explant system. Fresh normal and malignant breast tissue samples were obtained from 6 patients undergoing surgery for breast cancer. All cancers were clinically palpable (>1 cm in size). Tumour (T) and adjacent normal tissue (AN) samples were excised by a pathologist and dissected into 5-10 mg pieces under sterile conditions. Each piece was washed and then individually incubated in a 24 well plate at 37˚C and 5% CO_2 in 500 μl serum free RPMI medium containing: (i) medium only, (ii) 200 ng/ml IGFBP-3(Gropep, Australia), (iii) 0.3 mM doxorubicin (Sigma, Poole) and (iv) 200 ng/ml IGFBP-3 and 0.3 mM doxorubicin together. Explants

Table I. *Primers and probes for real time RT-PCR mRNA quantification of IGFBP-3, IGF-I, VEGF, COX-2, VDR, 1αOHase and 24OHase.*

Target gene	Primers and probes (5'-3')
IGFBP-3	F: AGA ACA GAT ACC CAG AAC TTC TTC
	R: CGC CCT CCG ACT GCT G
	P: TTT TAT TTC AAC AAG CCC ACA GGG TAT GGC
IGF-I	F: CTT CAG TTC GTG TGT GGA GAC AG
	R: CGC CCT CCG ACT GCT G
	P: TTT TAT TTC AAC AAG CCC ACA GGG TAT GGC
VEGF	F: TGT GAA TGC AGA CCA AAG AAA GA
	R: CGT TTT TGC CCC TTT CCC
	P: AGA GCA AGA CAA GAA AAT CCC TGT GGG C
COX-2	F: GAA TCA TTC ACC AGG CAA ATT G
	R: TTT CTG TAC TGC GGG TGG AAC CTG CCA
	P: TTC CTA CCA CCA GCA ACC CTG CCA
VDR	F: ATC TGC ATC GTC TCC CCA GAT
	R: AGC GGA TGT ACG TCT GCA GTG
	P: TGA TTG AGG CCA TCC AGG ACC GC
1αOHase	F: GCT ATT GGC GGG AGT GGA C
	R: GCC GGG AGA GCT CAT ACA GA
	P: CCC AAG AGA GCG TGT TGG ACA CCG
24 Ohase	F: TCC TTT GGG TAA AGC ATA TTC ACC
	R: GCC TGT CTG AAA GAA TCT ATG AGG
	P: AAC TGT TGC CTT GTC AAG AGT CCG

F=forward, R = reverse primers and P – Taqman probe.

were removed after 5 min and 24 hours of incubation, snap frozen in liquid nitrogen and stored at –80˚C. Apoptosis was quantified by (i) determining the caspase-3 proteolytic activity of the explant cell lysate (Caspase-3 protease assay, Apotarget, California) and (ii) immunohistochemistry against caspase-3 (ABC Kit, Vector Laboratories, USA) using a polyclonal anti-caspase-3 antibody and counting the positive staining cells.

Statistical analyses. Stats Direct (Ashwell, UK) was used to analyse all data. A *p*-value of less than <0.05 was considered significant. Mann-Whitney *U*-test was used for comparison of mRNA expression and the Chi-squared test used for comparison of number of samples with detectable expression in T and AN. Fisher's exact test was used where expected values were less than 5.

Results

Expression of IGFBP-3 mRNA in normal and malignant breast. IGFBP-3 mRNA was detected in all of the samples. There was no significant difference in expression levels between any of the groups (Table II). The median level of expression in the liver was 1.0×10^{10} copies/μg total RNA.

Expression of IGFBP-3 protein. Expression of IGFBP-3 protein was confirmed by immunohistochemistry with positive staining in 96% of samples (Table III). In tumours, 10 samples (42%) were strongly positive (total score of 10-14) and 13(46%) were weakly positive. In normal tissue, slightly more samples (58%) stained strongly for BP-3; however, this was not statistically significant. There was no statistically significant correlation between IGFBP-3 protein levels and mRNA copy number (R=–0.2, p=0.17).

Stromal versus epithelial expression of IGFBP-3. There was a universal increase in staining in the stroma of normal and malignant tissues whereas there was absent staining in 33% of normal and 54% of malignant epithelium (Figure 1). A similar pattern of staining was seen in both normal and malignant sections, although more tumours than normals had absent expression.

Comparison with clinico-pathological variables. No correlation was observed between IGFBP-3 mRNA/protein and tumour size, histological type, ER/PgR status and lymph node status.

Comparison with IGF-I, VEGF and COX-2 mRNA expression. A positive correlation was observed between IGFBP-3 and IGF-I mRNA levels in tumours (Spearman rank, R=0.38, p=0.01), but not in normal tissue. Overall, mean IGF-1 mRNA levels were significantly lower than IGFBP-3 levels (5.28×10^6 *versus* 1.6×10^8 mean copy number/μg total RNA, p<0.0001) and IGF-1 mRNA was not detectable in 5 tumours.

Primary explant culture. Incubation of breast tumour explants with doxorubicin alone caused a small increase in apoptosis. IGFBP-3 on its own had no effect. A marked increase in apoptosis was observed, however, when IGFBP-3 was combined with doxorubicin compared to either medium alone (p=0.004, Figure 2) or IGFBP-3 alone (p=0.01).

In normal breast tissue explants, apoptosis was only seen with the addition of doxorubicin (p=0.02). IGFBP-3 in combination with doxorubicin did not increase apoptosis.

Table II. *IGFBP-3 mRNA levels per μg total RNA in adjacent normal (AN), tumour (T), healthy normal (NN), recurrent tumour (RT) and recurrent normal (RN) breast tissue.*

	Median copy number	Range
AN	1.61×10^8	1.08×10^7 - 4.77×10^9
T	1.6×10^8	1.51×10^5 - 1.33×10^{10}
NN	2.6×10^8	2.24×10^6 - 1.63×10^9
RT	1.2×10^8	3.36×10^6 - 1.49×10^9
RN	1.2×10^8	5.19×10^7 - 3.27×10^8

Table III. *Immunostaining of IGFBP-3 in normal(N) and malignant(T) breast tissue demonstrating that 96% of samples stained positively for BP-3 and 50% of these samples were strongly positive.*

	N	T	Total
Negative (1-6)	1(4%)	1(4%)	2(4%)
Positive (7-9)	9(38%)	13(54%)	22(46%)
Strongly positive (10-14)	14(58%)	10(42%)	24(50%)
Total	24	24	48

Discussion

IGFBP-3 is emerging as an important regulator of cell proliferation and apoptosis through its effects on IGF-1 bioactivity and also through its potent IGF-I independent actions where it appears to function both by cell cycle blockade and the induction of apoptosis (32). This study has clarified its local expression within the breast: using quantitative RT-PCR analysis. Furthermore, expression is at a very high level, comparable to that of liver, the predominant source of circulating IGFBP-3.

Our finding of high IGFBP-3 expression in breast cancers is initially counter-intuitive given the reported anti-proliferative and pro-apoptotic effects of this protein. However, localisation of IGFBP-3 expression within the breast samples by immunohistochemistry and laser capture microdissection offers a solution to this paradox. The pattern of expression between normal and cancer tissue is clearly different. In normal tissue IGFBP-3 is expressed in both the stroma and the epithelial cells. This is in agreement with a proposed regulatory role for IGFBP-3 on cell proliferation, and its induction by wild type p53 (33). In marked contrast, expression of IGFBP-3 is significantly decreased in malignant epithelial cells, whereas it is expressed

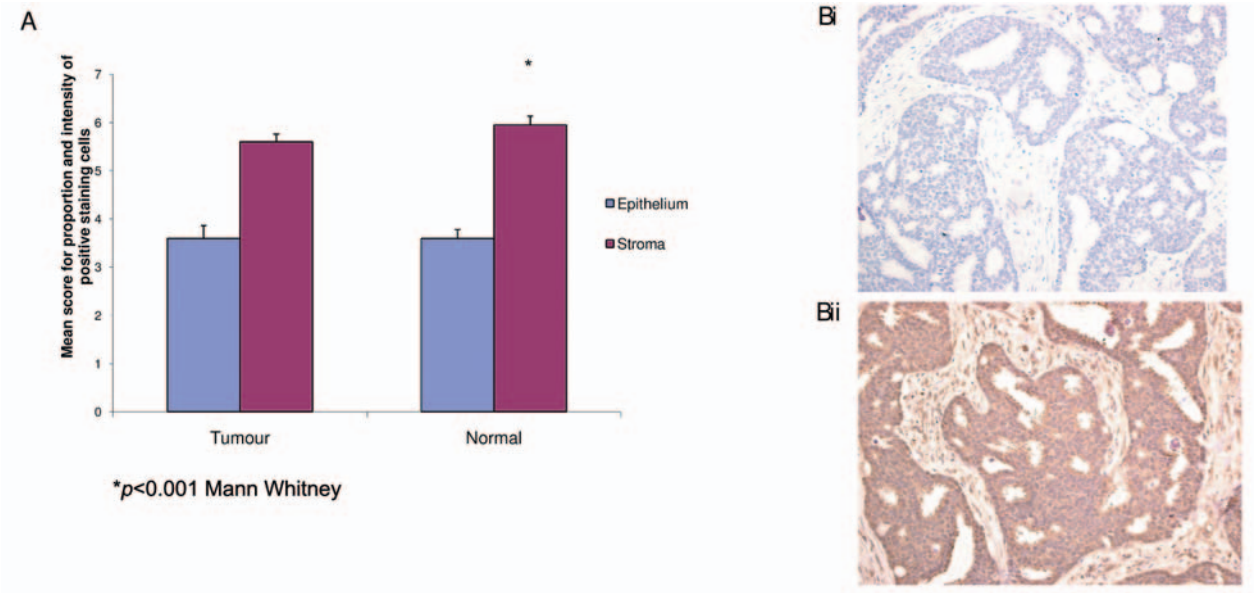

Figure 1. *Mean scores of all samples for proportion and intensity of positive staining cells in epithelium and stroma (A). Immunocytochemistry against IGFBP-3 expression in a breast tumour (Bi) and control (Bii).*

at high levels by the non-malignant stromal component of the tumours. It further adds to the epidemiological evidence from Renehan *et al*. that high serum levels of BP-3 are associated with an increased risk of breast cancer (8). It is possible that the high levels of BP-3 produced by the surrounding stroma are in response to a more malignant behaviour of the tumour. We have previously reported similar differential expression between normal and malignant colonic tissue(34) and hypothesise that the marked upregulation of IGFBP-3 expression in the stromal cells of cancers is the result of a physiological response to uncontrolled proliferation of the epithelial cancer cells. The malignant cells have switched off the synthesis of IGFBP-3, and have additionally developed a mechanism to prevent stromal IGFBP-3 protein from diffusing towards them. This suggests that they may be still responsive to its anti-proliferative and apoptotic effects. This theory is supported by previous reports of IGFBP-3 mRNA being absent from MCF-7 cancer cells (35), yet they respond appropriately to the anti-proliferative and apoptotic effects of exogenous IGFBP-3. The exact mechanisms responsible for the decreased expression in the malignant epithelial component of cancers remain undetermined but may involve differences in extra-cellular matrix, components of which clearly influence BP-3 expression, binding and apoptotic actions (36). Similarly, Vestey *et al*. detected constant stromal staining, however, they detected epithelial staining in 85% of samples (37) when compared to 46% in our study. They, however, only assessed tumours and ductal carcinoma-*in situ* and specifically set out to assess nuclear staining of tumours which was not detected in the study.

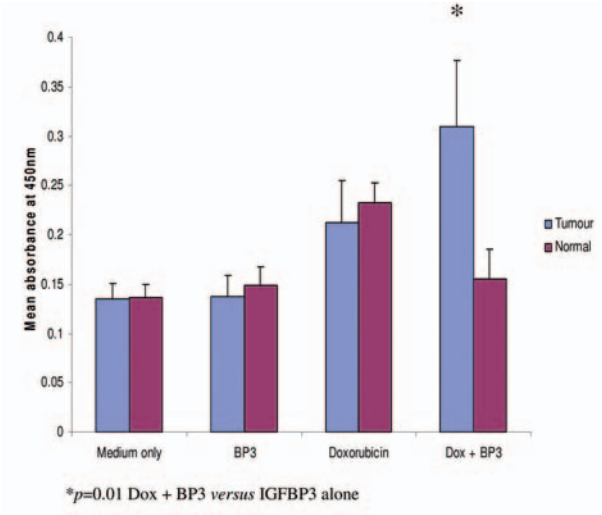

Figure 2. *Caspase-3 protease assay in normal and tumour explants at 24hr (n=6). An increase in caspase activity is seen in tumour explants incubated with doxorubicin and IGFBP3, however, a decrease is seen in normal explants. Values are expressed as mean±SEM.*

No studies have been previously performed comparing simultaneous IGFBP-3 levels in paired normal and malignant breast tissue and only one study found that BP-3 levels were higher in the cancers (38). Only 5 paired samples, however, were analysed. Previous studies have demonstrated a strong correlation between IGFBP-3 mRNA and IGFBP-3 detected by immunoradiometric assay (IRMA) but no statistically

significant correlation was seen between IGFBP-3 mRNA and IGFBP-3 detected by ligand blot or immunoblot (25). One previous study measured IGFBP-3 using ELISA in 195 node negative tumours (23). Their findings agreed with our data in that all samples expressed high levels of IGFBP-3. However, they did not correlate BP-3 levels in this group with patients who were lymph node positive. IGFBP-3 levels were, however, inversely correlated with age and ER/PgR status and found to be higher in tumours greater than 2 cm. Our data did not demonstrate any such association with hormone status or tumour size. This may be as the majority of our samples were from post-menopausal women with tumours that were 2 cm or greater and 85% of samples were ER positive. Our data, however, did reflect a general trend towards high levels of expression of IGFBP-3 mRNA in tumours which were histological grade I, ER positive, lymph node negative and without vascular invasion. A larger sample size with a wider variety of tumours would be needed to determine any statistical significance. This trend, however, clearly supports the anti-proliferative and pro-apoptotic role of IGFBP-3 protein in that these features confer a better prognostic outlook.

Our expression data is supported by our functional studies which have clarified the function of this local expression of IGFBP-3. It is now well established that IGFBP-3 exerts both IGF-1 dependent and independent effects on cell proliferation and apoptosis (39). It is likely that the local expression of IGFBP-3 in the breast is involved in modulating the local activity of IGF-1; although it's markedly higher expression compared to IGF-1 also suggests an additional significant IGF-1 independent role for breast IGFBP-3 gene product. This is also supported by its presence in samples that did not express IGF-I. Previous studies have demonstrated conflicting results on the IGF-1 independent effects of IGFBP-3 on cell function. Rajah *et al*. (13) reported that IGFBP-3 directly induced apoptosis of breast cancer cells, whereas Holly *et al*. (36) using Hs578T cells observed no effect. These differences may be explained by different mutations in the different cancer cell lines. Our use of the explant system avoids this problem and by maintaining *in vivo* tissue architecture allows a more physiologically relevant model with which to explore the effects of IGFBP-3. Our results indicate a clear difference in the response of normal and malignant breast tissue to IGFBP-3. Apoptosis induced by doxorubicin was similar in both normal and malignant breast tissue. However, the addition of IGFBP-3 markedly enhanced apoptosis in tumours, whereas it caused no effect in normal breast tissue suggesting that IGFBP-3 may have a protective effect on normal breast tissue. Such a differential effect has previously been reported in breast cancer cells (40) and thought to be due to the extracellular matrix(ECM). The ECM was intact in our study; therefore it is difficult to directly compare the studies.

Conclusion

Our findings suggest an important paracrine role of IGFBP-3 in mediating breast tissue homeostasis and a clear difference between normal and malignant tissue. The pro-apoptotic effects of IGFBP-3 also help explain some of the recent epidemiological studies linking low levels of IGFBP-3 coupled with high IGF-1 levels in the breast to a higher risk of breast cancer. Finally, strategies to increase cellular expression of IGFBP-3 may offer the opportunity of a novel powerful adjunctive therapy for breast cancer.

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