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Increased Activity of Insulin-like Growth Factor-binding Protein in Human Thyroid Papillary Cancer Tissue

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It has been shown that both insulin-like growth factor-I (IGF-I) and IGF-binding proteins (IGFBPs) are produced by thyroid cells in culture and that the cells respond to IGF-I with increased DNA synthesis, suggesting an autocrine/paracrine role of IGF-I in the regulation of thyroid cell growth. We investigated the tissue contents of immunoreactive IGF-I (irIGF-I) and IGFBPs in human papillary carcinoma and compared them with those of normal thyroid tissue. When irIGF-I was measured after separation of the IGFBPs by gel-filtration, its content in carcinoma tissue was not different from that in adjacent normal tissue (566±58 vs. 424±75 pg/mg protein, N=10). Nor was there any difference in the abundance of IGF-I mRNA expression determined by slot blot analysis. On the other hand, IGFBP activity measured in terms of ^{125}I-IGF-I binding was significantly higher in cancer extracts. Western ligand blot analysis of IGFBPs revealed several species (24–42 kDa) of IGFBPs. The IGF-I-binding activity of 38–41 kDa species (corresponding to IGFBP-3) was not different between extracts of cancer tissue and those of normal tissue, whereas that of 28–32 kDa species was significantly higher in cancer tissue extracts. Since IGFBPs have been reported to modulate cellular responses to IGF-I, the present data suggest that higher IGFBP activity in cancer tissue is involved in regulating growth of thyroid papillary carcinoma cells.

Key words: Thyroid papillary cancer — IGF-I — IGFBP

Insulin-like growth factor-I (IGF-I) is a growth hormone-dependent serum growth factor with mitogenic activity in various types of cells.[1] Although the liver is the major site of serum IGF-I production, a wide variety of human tissues, both non-neoplastic and neoplastic, have been shown to express IGF-I mRNA and synthesize IGF-I.[2–5] These observations suggest that IGF-I may function as a paracrine/autocrine factor at or near its site of production in both normal and neoplastic cells. The biological effects of IGF-I are largely mediated by binding to type I IGF receptors with intrinsic tyrosine kinase activity. In addition to the type I receptors, both IGF-I and IGF-II can bind to a family of IGF-binding proteins (IGFBPs) which are structurally unrelated to IGF receptors. At least 6 classes of IGFBPs have been identified, and they are produced in a variety of tissues that are sites of IGF production.[6,7] Although the physiological functions of IGFBPs are still unknown, it has been proposed that these proteins modulate the biological effect of IGF-I or -II by complexing with the growth factor.[8,9]

We and others have previously shown that cultured thyroid cells possess receptors for IGF-I and respond to the growth factor with increased DNA synthesis and cell proliferation.[10,11] We have also identified IGF-I and -II receptors in human thyroid tissues, both normal and neoplastic.[12,13] In addition, IGF-I and IGFBPs have been identified in cultured medium conditioned by thyroid cells.[14–17] Thus, IGF-I appears to be involved in the regulation of thyroid cell growth.

Recent studies have shown that IGF-I is also an autocrine/paracrine factor in a number of neoplastic cells.[18] Minuto *et al.*[19] reported that the content of immunoreactive IGF-I (irIGF-I) in human thyroid nodular tissue is significantly higher than that in normal tissue. However, this increased irIGF-I may only reflect the different content of IGFBPs in thyroid tissue, since IGFBPs interfere with the radioimmunoassay (RIA) for IGF-I[20]; IGFBPs in the test samples bind radiolabeled IGF-I and thereby reduce the amount of labeled IGF-I available to the antibody to IGF-I, which would give falsely high IGF-I values when labeled IGF-I:antibody complex is separated from unbound IGF-I by the second antibody method or with polyethylene glycol (PEG). In this paper, we show that IGFBP activity is higher in human thyroid papillary carcinoma tissue than in adjacent normal tissue and that there is no difference in tissue content of irIGF-I or in expression of IGF-I mRNA between normal and tumor tissues.

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MATERIALS AND METHODS

Human thyroid tissue Thyroid tissue was obtained at the time of surgery from 28 patients who underwent thyroidectomy because of papillary carcinoma (8 patients) or follicular adenoma (14 patients). A fragment of normal tissue was obtained from the excised portion surrounding the tumor mass in every case. Tissue fragments were also obtained from 12 patients with Graves' disease. The surgical specimens were immediately weighed, frozen in liquid nitrogen and stored at −70°C until processed as described below. The diagnoses were established by histological examination.

Tissue extraction IGF-I was extracted from thyroid tissues with 1 *M* acetic acid according to the method described by D'Ercole *et al.*[3] Briefly, tissue fragments were minced with scissors on ice and then homogenized with a Polytron (Brinkman Instruments Inc., Westbury, NY) in chilled 1 *M* acetic acid (5 ml/g tissue) for 30 s at a dial setting of 6. The homogenate was shaken vigorously for 2 h at 4°C and centrifuged at 600*g* for 10 min at 4°C. The precipitate was re-extracted with fresh 1 *M* acetic acid in the same manner. The supernatant was pooled, lyophilized, resuspended in 50 m*M* Tris-HCl buffer, pH 7.4, in a ratio of 10 ml to 1 g of original tissue, and centrifuged to remove insoluble material. The resulting solution was stored at −20°C. Protein concentrations were determined by the method of Lowry *et al.*[21] using bovine serum albumin (BSA) as the standard. DNA content was measured as previously described.[22] Hemoglobin (Hb) content of tissue extract was determined by a modified benzidine assay as described by Conscience *et al.*[23] using human Hb (Ortho, Raritan, NJ) as a standard.

RIA for IGF-I Antibody to IGF-I was prepared by immunizing rabbits with recombinant human IGF-I (a gift from the Central Institute of Fujisawa Pharmaceuticals, Osaka) conjugated with bovine thyroglobulin. Recombinant human IGF-I (Fujisawa Pharmaceuticals) was radioiodinated by the Chloramine T method to a specific activity of 150 mCi (5.55 MBq)/mg. The assay buffer for the IGF-I RIA was 50 m*M* Tris-HCl buffer (pH 7.4), containing 0.3% BSA, 0.01 *M* EDTA and 0.02% NaN_3. Antiserum to IGF-I (final dilution 1:50,000) was incubated with ^{125}I-IGF-I in the presence of standard IGF-I solutions or test sample for 24 h at 4°C, and antibody-bound ^{125}I-IGF-I was separated from the unbound ^{125}I-IGF-I by adding ice-cold polyethylene glycol (final concentration: 12.5%) with 2% bovine gamma globulin as the carrier. The minimum detectable dose in the assay was 30 pg/tube, and the coefficients of intra- and inter-assay variation were 5.2% and 12%, respectively. Crossreaction of recombinant IGF-II (Eli Lilly, Indianapolis, IN) in the assay was less than 0.1%. Porcine insulin (Eli Lilly) did not crossreact at concentrations of up to 1 mg/tube. Tissue content was expressed as pg/mg protein of extract. Serum IGF-I concentrations were also measured by RIA after the samples had been extracted with acid-ethanol.[20]

Acid gel chromatography Both cancer tissue extracts and the corresponding normal tissue were gel-chromatographed on a Sephadex G-50 column (0.9×48 cm) equilibrated with 1 *M* acetic acid. This method was chosen because it allowed almost complete separation of IGF-I from its binding proteins. Fractions of 0.7 ml were collected, lyophilized, and reconstituted in 50 m*M* Tris-HCl buffer, pH 7.4, containing 0.2% BSA. They were then assayed for irIGF-I concentration and IGF-binding activity. IGF binding activity was measured using a modification of the method of Martin and Baxter.[24] Aliquots of 0.1 ml were incubated with approximately 20,000 cpm of ^{125}I-IGF-I at 4°C for 12 h in 50 m*M* Tris-HCl, pH 7.4, containing 10 m*M* $MgCl_2$ and 0.2% BSA in a final volume of 0.5 ml. To separate bound ^{125}I-IGF-I from free ^{125}I-IGF-I, 0.5 ml of an ice-cold mixture of 2% activated charcoal (Sigma) and 2% BSA in 50 m*M* Tris-HCl buffer, pH 7.4, was added to each tube. The mixture was allowed to stand for 10 min at 4°C, and the tubes were centrifuged at 2600*g* for 30 min at 4°C. Nonspecific binding was defined as binding obtained in the presence of an excess of unlabeled IGF-I (1 μg/tube), and specific binding was calculated by subtracting nonspecific binding from total binding. Percent specific binding was defined as the percentage of specific binding with respect to the total counts added.

Assay of IGF-I messenger RNA levels Total cellular RNA was isolated from thyroid tissue using the guanidium thiocyanate method.[25] RNA slot blot analysis was performed as suggested by the manufacturer of the apparatus (Schleicher and Schuell, Keen, NH). Briefly, the indicated amounts of RNA were denatured in the presence of 2.2 *M* formaldehyde, diluted in 10×SSC [20×SSC is 3 *M* NaCl and 0.3 *M* Na citrate, pH 6.9–7.0] and blotted onto a nylon membrane (Nytran, Schleicher and Schuell). The filters were hybridized to a human IGF-IA cDNA labeled with ^{32}P-dCTP by random-primed labeling (specific activity 1×10^9 dpm/μg DNA) and then subjected to autoradiography. Hybridization and washing were performed under highly stringent conditions, as described previously.[26, 27] The human IGF-IA cDNA[28] was kindly provided by Dr. Peter Rotwein (Washington University, St Louis, MO). The IGF-IA cDNA probe contains exons 1, 2, 3, and 5, which include the entire coding region of the IGF-IA precursor molecule.

Western ligand blotting To characterize further the IGFBP recovered from gel-chromatographic fractions, the active fractions were pooled, lyophilized and re-

constituted in 50 m*M* Tris/HCl buffer, pH 7.4. An aliquot was electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gel under non-reduced conditions. Electrophoresed proteins were electroblotted onto nylon membranes and the membranes were incubated with approximately 1×10^6 cpm of ^{125}I-IGF-I overnight at 4°C. The membrane was washed, and the IGFBPs were visualized by autoradiography according to the method of Hossenlopp *et al.*[29]

Statistical analysis Results are expressed as means±SE unless otherwise described. Statistical analysis was performed using Student's *t* test or Duncan's new multiple range test. Differences between groups were considered significant if the *P* value was less than 0.05.

RESULTS

irIGF-I content in unfractionated thyroid extracts Initial experiments using unfractionated extracts revealed an irIGF-I content of 5347±2281 pg/mg protein (mean± SE, N=8) in papillary thyroid carcinoma, which was significantly ($P<0.01$) higher than in surrounding normal tissue (450±57 pg/mg). The value for thyroid follicular adenoma (634±91 pg/mg; N=14) was also slightly higher than that in adjacent normal tissue (450± 51 pg/mg). The result is similar to that recently reported for nodular goiter.[19]

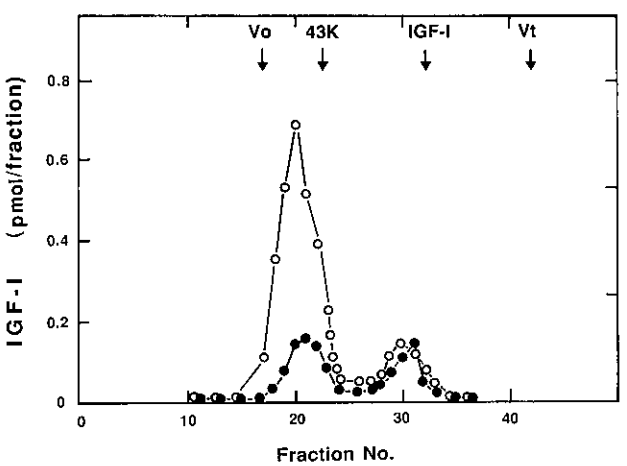

Fig. 1. Gel-filtration of thyroid extracts. Acid extracts (1 ml: 2 mg protein) of a human thyroid cancer tissue (open circles) and corresponding normal tissue (closed circles) were gel-filtered on a column equilibrated with 1 *M* acetic acid. The eluate was assayed for immunoreactive IGF-I. The elution positions of marker proteins are shown by arrows. Markers are blue daxtran (for void volume, Vo), ovalbumin (mol. wt. 43 kDa), IGF-I (mol. wt. 7.5 kDa) and NaI (for column volume, Vt).

Gel-chromatography of thyroid extracts When the extracts were fractionated on a Sephadex G-50 column in 1 *M* acetic acid, two peaks of irIGF-I activity were detected. In tumor extracts, approximately 10% of total activity was recovered in the fractions corresponding to the molecular weight of authentic IGF-I (mol. wt. 7,500), and most of the activity was eluted as species of molecular weight greater than 20,000 (Fig. 1). On the other hand, the proportion of the higher-molecular-weight species was much lower in extracts from the normal portion of the tissues. As described below, the higher-molecular-weight irIGF-I was due to non-specific interference by IGFBPs in IGF-I RIA, and irIGF-I values in unfractionated extracts did not reflect actual IGF-I content. Therefore, authentic irIGF-I values were calculated based on the amount of irIGF-I eluted in the fractions corresponding to mol. wt. 7,500 (fractions 25–33). The data thus obtained for thyroid papillary carcinoma, adenoma and normal tissue are summarized in Table I. Although the content of authentic IGF-I in papillary cancer tissue was higher than in the corresponding normal tissue, the difference was not significant. Nor was there any difference in the values in adenoma and surrounding normal tissue. When the values were corrected for tissue DNA, the irIGF-I content of thyroid cancer was 28% lower than in the surrounding normal tissue, but the difference was not significant. The values in Graves' disease (350±70 pg/mg; N=14) were not different from those in thyroid cancer or adenoma. There were no differences in the serum concentrations of IGF-I in patients with thyroid cancer, those with adenoma, and age-matched normal adults (212±23 ng/ml; N=23). Thus, the findings were inconsistent with those published in an earlier report.[19]

Slot blot analysis Using slot blot analysis we investigated whether the abundance of IGF-I mRNA expression

Table I. Immunoreactive IGF-I Content of Thyroid Tissue and Serum IGF-I Levels

	Age (years)	Tissue (pg/mg protein)	Serum (ng/ml)
Papillary cancer (N=8)	43±7		216±40
neoplastic tissue		566±58	
normal tissue		422±75	
Follicular adenoma (N=14)	38±3		188±27
neoplastic tissue		534±91	
normal tissue		304±51	

Thyroid extracts were chromatographed on a Sephadex G-50 column equilibrated with 1 *M* acetic acid as in Fig. 1. Fractions corresponding to the molecular weight of authentic IGF-I were pooled and assayed for irIGF-I. Serum concentrations of IGF-I were determined after the samples were extracted with acidic ethanol as described in the text.

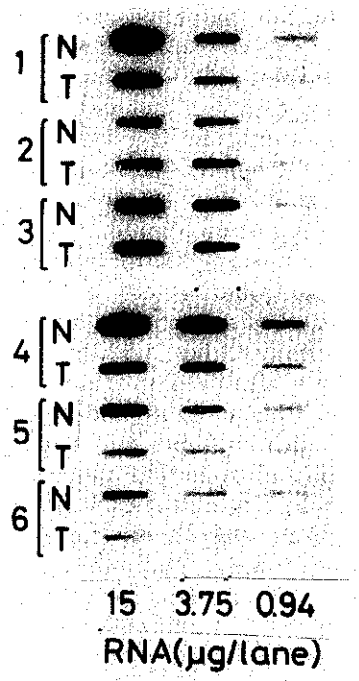

Fig. 2. RNA expression of IGF-I in thyroid tissue patients with papillary carcinoma. Samples of RNA in the amounts indicated were slot-blotted onto nitrocellulose membranes and hybridized to the probe as described in the text. Samples from 6 out of 8 patients with papillary carcinoma were analyzed. The remaining two samples could not be analyzed because of the limited amount of materials. T, neoplastic tissue; N, corresponding normal tissue.

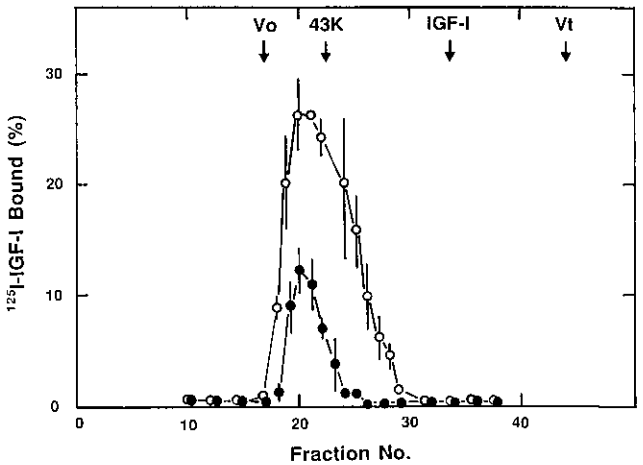

Fig. 3. Chromatographic profile of IGFBP activity of thyroid extracts. Extracts (1 ml:2 mg protein) of 8 thyroid papillary cancer (open circles) and adjacent normal tissues (closed circles) were gel-filtered as in Fig. 1. Fractions were lyophilized, reconstituted in assay buffer and assayed for ^{125}I-IGF-I binding activity as described in the text.

differs in cancer tissue and normal tissue. The quality of the mRNA was checked by Northern blot analysis (data not shown), which revealed multiple transcripts (a major band at 1–1.5 kb, and minor transcripts at 5.1 and 7.5 kb), as reported for a number of tissues. Fig. 2 shows the autoradiographs obtained in 6 out of 8 patients. It can be seen that the levels of IGF-I mRNA in tumor tissue are somewhat lower than, or almost identical to those in the corresponding normal thyroid tissue. Thus, we were unable to detect elevated levels of IGF-I mRNA in cancer tissue. These results strongly suggest that the apparently higher content of irIGF-I in unfractionated extracts from cancer tissues is attributable to the presence of IGFBPs in higher amounts, or with higher IGF-binding activity.

IGF-binding activity of thyroid extracts To demonstrate IGF-binding proteins in thyroid extracts, the column fractions containing apparent irIGF-I were tested for ^{125}I-IGF-I binding activity. As shown in Fig. 3, ^{125}I-IGF-I binding activity was detected in the fractions corresponding to those containing higher-molecular-weight IGF-I-like immunoreactivity (fractions 20–25). The figure also shows that the IGF-I-binding activity in papillary carcinoma is significantly ($P<0.01$) higher than in normal tissue in the majority of the fractions. IGF-I-binding activity was not detected in the fractions corresponding to authentic IGF-I.

It is well established that human serum contains several species of IGFBPs including IGFBP-3, which is the major species.[29] Thyroid extracts could be contaminated by blood, and the higher IGFBP activity in tumor extracts may be due to a larger amount of contaminating blood. This seems unlikely, however. There was no significant difference in Hb content between tumor tissue and surrounding normal tissue (175 ± 122 vs. 218 ± 99 μg/g tissue; mean$\pm$SD, N=8). Based on these data, contamination with serum in tissue extracts was estimated to be approximately 4%.

Western ligand blot analysis Thus far, 6 types of IGFBPs have been identified.[6] To characterize further the IGFBPs in thyroid extracts, the column fractions containing IGF-I binding activity were pooled, lyophilized and subjected to Western ligand blotting. Fig. 4 shows the results for three samples of thyroid cancer and surrounding normal tissue. The electrophoretic behavior of the IGFBPs in normal human serum (2 μl) is illustrated in the left two lanes; the major band was recognized at the 38–41 kDa position, which corresponds to IGFBP-3. Other bands of 34 kDa (IGFBP-2), 30 kDa (IGFBP-1) and 24 kDa (IGFBP-4), were also found, as

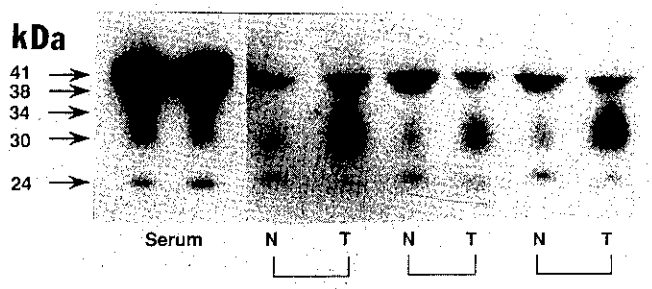

Fig. 4. Demonstration of IGFBPs in thyroid extracts by Western ligand blot analysis. Fractions of Sephadex G-50 containing IGFBP activities (fractions 19–25) were pooled and lyophilized, and 100 μg protein was subjected to SDS-PAGE. After transfer to nitrocellulose membranes, IGFBPs were identified by ligand blotting. The results obtained for 2 ml of 2 normal human sera are shown in the left two lanes. Samples from 6 out of 8 patients with papillary carcinoma were analyzed. The results shown are from three samples. Similar results were obtained for another two samples. T, neoplastic tissue; N, normal tissue.

previously described by Hossenlop *et al.*[29] The profiles of serum IGFBPs in the ligand blot were apparently the same in patients with thyroid papillary carcinoma and normal subjects (data not shown).

The distribution of IGFBPs in thyroid extracts was quite different from their distribution in serum. The proportion of 41/38 kDa species was much lower than in serum, and there was no difference in the density of this species in cancer tissue and normal tissue. This species has been identified as IGFBP-3 by Western blotting using antibody to IGFBP-3 (data not shown). In contrast, broad bands at 28–32 kDa were predominant in thyroid extracts, especially from cancer tissues. The density of 24 kDa band was also slightly higher in cancer tissue extracts. Similar data were obtained in two other samples, indicating that IGFBPs, especially the 28–32 kDa species, are prominent in thyroid papillary cancer tissues. There was no band reactive to antibody to IGF-I receptors.

DISCUSSION

IGFs are being increasingly recognized as important mitogens in a variety of neoplastic cells.[5, 18] We recently demonstrated that human papillary carcinoma cells have an increased number of IGF-I receptors,[12] and that a cell line derived from human papillary carcinoma responds to both exogenous and endogenous IGF-I.[30] Moreover, a number of studies have revealed production of IGF-I not only by normal[14, 15] but also by neoplastic thyroid cells.[30, 31] In addition, IGF-I mRNA has been expressed in all of the human thyroid cancers examined.[32] These results strongly suggest that IGF-I modulates the growth of neoplastic thyroid cells.

The data presented here demonstrate that neither the tissue content of authentic IGF-I nor the abundance of IGF-I mRNA was different between thyroid cancer and adjacent normal tissues. These results are inconsistent with an earlier report by Minuto *et al.*[19] Although they described the presence of IGFBP in thyroid extracts, the differences in the amount or activity of IGFBPs in nodular and normal tissue extracts had not been investigated.

The results obtained here both by ^{125}I-IGF-I binding and ligand blot analysis clearly demonstrate that IGFBP activity is much higher in thyroid papillary carcinoma tissue. The higher IGF-binding activity can not be ascribed to contamination with blood in the tumor extracts because the Hb content in the tumor extracts was not different from that in the corresponding normal tissue. It is most likely, therefore, that the apparently higher amount of irIGF-I in unfractionated extracts of thyroid cancer tissue is due to higher IGFBP activity, which interferes with the IGF-I radioligand assay.[20, 33] Since it has been reported that even acid-ethanol extraction is incapable of completely separating IGFBPs and IGF-I,[33] IGF-I values obtained without gel-filtration of the samples under acidic conditions are misleading.

Recent studies have identified six types of IGFBPs having 200–300 amino acids,[6] displaying different SDS-PAGE electrophoretic behavior.[29] Although the majority of circulating IGFBPs are derived from liver, a number of tissues or cell lines, including thyroid cells[14–17] can produce IGFBPs. The Western ligand blot in the present study showed that the IGFBP activity at 28–32 kDa is much higher in thyroid papillary carcinoma extracts. The 24 kDa species, which was also more abundant in thyroid cancer, appears to be IGFBP-4, as judged from its electrophoretic behavior in SDS-PAGE. It is unlikely that the 28–32 kDa species belongs to IGFBP-3, which is generally recognized as a 38/41 kDa doublet. Furthermore, unlike IGFBP-3, which is glycosylated, the 28–32 kDa IGFBPs were not adsorbed on a concanavalin A column (data not shown). Thus, the 28–32 kDa species may be IGFBP-2, or IGFBP-1. Since these bands were broad and ill-defined, they may represent a mixture of two or more species of IGFBPs. The possibility also remains that the 28–32 kDa species is derived from degradation of IGFBP-3. Further studies are required to clarify this point. The present data are very similar to those reported for human breast cancer by Pekonen *et al.*[34] They showed that the abundance of IGFBP-2, -3, -4, and -5 mRNAs was significantly higher in five tumors than in adjacent normal tissue, suggesting that increased IGFBPs content is typically associated with the malignant transformation of breast tissue.

The physiological significance of IGFBPs remains controversial. IGFBPs have been reported to be inhibitory or stimulatory with regard to the biological effect of IGF, depending on the type of IGFBP, the type of cells and the cell culture conditions. Purified IGFBP-1 has been shown to inhibit the biological effect of IGF-I in choriocarcinoma cells[35] and rat thyroid epithelial (FRTL-5) cells,[36] whereas it enhances the cellular response to IGF-I in human, mouse and chicken embryo fibroblasts.[37] IGFBP-2 is inhibitory to DNA synthesis stimulated by either IGF-I or IGF-II,[38] and IGFBP-3 has both inhibitory and stimulatory effects on DNA synthesis in fibroblasts, depending on the culture conditions.[39] Production of IGFBPs is regulated by a number of factors. For example, production of IGFBP-3 by cutured sheep thyroid cells is regulated by EGF or phorbol ester.[16] Moreover, TSH has an inhibitory effect on IGFBP production.[17] These findings are compatible with the concept that heterogenous IGFBPs modulate cellular responses to IGF-I and thereby affect IGF action in thyroid cells. However, it is unknown at present whether the higher IGFBP activity in thyroid papillary carcinoma cells is causally related to the uncontrolled cell growth.

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