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Differential Expression of Insulin-like Growth Factor Binding Proteins in Human Non-small Cell Lung Cancer Cell Lines

Gabriele Jaques, Paul Kiefer, Hans J. Schöneberger, Barbara Wegmann, Ulrich Kaiser, Detlef Brandscheid and Klaus Havemann

The possible expression and secretion of insulin-like growth factor binding proteins (IGFBPs) by non-small cell lung cancer (NSCLC) cell lines was investigated and compared with possible IGFBP expression by primary NSCLC tumours. Cells growing under serum-free conditions released binding proteins with apparent molecular masses of 26–43 kD when analysed by a ligand blotting method under non-reducing conditions. Additionally, northern blot analysis of total RNA from NSCLC cell lines and tumours was performed using cDNAs coding for each of IGFBP-1, IGFBP-2, and IGFBP-3. This analysis revealed expression of all three mRNAs to varying degrees by all cell lines. In contrast all primary tumours analysed expressed predominantly IGFBP-2 and IGFBP-3 and none showed any evident expression of IGFBP-1. Both NSCLC cell lines and tumours synthesise IGFBPs but the pattern of expression differs significantly between cell lines and primary tumours.
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INTRODUCTION

INSULIN-LIKE GROWTH FACTORS (IGFs) are peptides which modulate growth in a variety of tissues and cell types [1]. IGFs are present in the circulation, in tissues, and in cell culture media and in all these locations are found to be bound to specific binding proteins [2]. These IGF-binding proteins (IGFBPs) bind both IGF-I and IGF-II with high affinity and specificity, and do not bind insulin [3]. There are at least four distinct forms of human IGFBPs which differ in terms of molecular mass, binding specificities and distribution in biological fluids and which have been cloned: IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4.

IGFBP-1 is the predominant IGFBP in amniotic fluid and IGFBP-1 mRNA is detected in decidua and secretory endometrium [4]. Elsewhere IGFBP-1 mRNA is found only in the liver (and in the hepatic HEP G2 cells) with concentrations higher in fetal compared to adult liver [5]. Analysis of mRNA in various tissues and cell lines reveals a different pattern of expression for IGFBP-1 and IGFBP-2. IGFBP-2 mRNA is detected in adult liver, brain, in Jurkat and kidney 293 cells, but not in HeLa, Namalwa and HEPG2 cells [6]. IGFBP-1 and IGFBP-2 are proteins with 40% sequence identity [6]. Both proteins contain regions of clustered Pro, Glu, Ser and Thr residues (PEST regions), a Arg–Gly–Asp sequence (RGD motif) and a cystein-rich amino-terminus [5, 6]. The major IGFBP in human adult serum is a 125–150 kD complex which dissociates under acidic conditions, releasing free IGFs and an acid stable IGFBP subunit. This binding subunit is referred to as IGFBP-3 (formerly IGFBP-53) with an apparent molecular mass of 53 kD under non-reduced and 43 kD under reduced conditions. Sequencing of cloned IGFBP-3 cDNA reveals a cysteine-rich primary structure of 264 residues and a predicted molecular mass of 28.7 kD [7]. The sequence has a 33% aminoacid identity including conservation of all 18 cysteine residues with IGFBP-1. Recently two additional IGFBPs have been described. One in conditioned medium from human bone cells and has limited aminoterminal sequence identity with IGFBP-1, IGFBP-2 and IGFBP-3 and appears to inhibit the action of IGF [8]. The other is a binding protein found in cerebrospinal fluid which specifically binds IGF-2 [9].

The biological functions of these binding proteins remain

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controversial. IGFBPs may function as providing a tissue depot for IGFs and/or in the humoral transport [10]. Additionally they may serve to modulate the biological activity of IGFs by enhancing or inhibiting the response of different cell types to these peptides as has been demonstrated *in vitro* [2, 8]. Therefore, IGFBPs may significantly influence autocrine or paracrine regulation of growth and metabolic effects induced by IGFs.

We and others have previously shown that small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) cell lines produce IGF-like proteins, express IGF-receptors and are IGF-responsive [11–16]. We could further demonstrate that SCLC cell lines express IGFBP-2 specific transcripts, but not IGFBP-1 or IGFBP-3 specific transcripts [17]. Here we compare the expression of IGFBP-1, IGFBP-2 and IGFBP-3 in a panel of NSCLC cell lines and compare it with results from tumour specimens.

MATERIALS AND METHODS

Cell lines

The cell lines used in this study were the NSCLC cell lines LCLC-97TM1, LCLC-103H, NCI-H157, and U-1810 (large cell carcinoma), ADLC-5M2, NCI-H23, NCI-H125, and A549 (adenocarcinoma), EPLC-32M1, EPLC-65H, EPLC-272H and U-1752 (squamous cell carcinoma), and MSTO-211H (mesothelioma), and the SCLC cell lines SCLC-22H, NCI-H69, NCI-H146, and FRE. They were either established in our laboratory or were donated by Drs A. F. Gazdar and J. Minna, NCI, Bethesda, Maryland [18, 19], by Dr J. Bergh, Departments of Pathology and Oncology, University of Uppsala, [20, 21], by Dr J. Reeve, MRC Centre, Cambridge, UK [22], or purchased from the American Type Culture Collection (Rockville). All cell lines, except SCLC cell lines, were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), designated R10 medium. The SCLC cell lines grew as floating cell aggregates whereas all other cell lines grew substrate-adherent. The SCLC cell lines were kept growing continuously serum-free for longer than one year as previously described [15]. Cultures were examined every month for mycoplasma contamination using *in situ* detection [23].

Preparation of conditioned media

Preparation of conditioned media (CM) from serum-free growing SCLC cell lines has been described previously [16]. Collection of CM from the other cell lines which grew substrate-adherent and not continuously serum-free was as follows: logarithmically growing cells from stock cultures were detached by trypsinisation, washed with R10 medium, and plated at 10^4 viable cells per ml in R10 medium. After 24 h, adherently growing cells were washed with RPMI 1640 and medium was changed to serum-free RPMI 1640 medium supplemented with 2 mmol/l L-glutamine, 30 nmol/l sodium selenite (Sigma T 9133), and 10 μg/ml 98% iron-free human transferrin (Sigma, T-2252). CM were harvested at the indicated times, clarified of debris by centrifugation at 10000 *g*, and stored at −20°C in aliquots until assayed.

Tumour specimens

Tumour specimens were obtained from 9 patients with NSCLC: seven from squamous carcinoma (tumours 2, 3, 4, 6, 7, 8, 9) and two from adenocarcinoma (tumours 1, 5). Immediately after excision the tissue was snap frozen in liquid nitrogen and stored at −80°C.

IGF-Binding capacity assay

The binding capacity of CM for IGF-I was determined by measuring aliquots of CM to bind [^{125}I]IGF-I [24]. 30 μl of CM was incubated in a final volume of 300 μl with 20000 cpm [^{125}I]IGF-I (Amersham, IM 172, specific activity 74 MBq/mmol) in 50 mmol/l sodium phosphate buffer, 0.25% bovine albumin, pH 6.5. After a 2-h room temperature incubation, bound and free [^{125}I]IGF-I were separated by adding 300 μl of a human γ-globulin solution (2 mg/ml, Pentaglobulin, Biotest Pharma, Dreieich, FRG) and 500 μl of 25% (wt/vol.) polyethylenglycol (6000–8000). The samples then were incubated on ice for 15 min and centrifuged at 3500 *g* for 20 min at 4°C. The supernatant was aspirated and the precipitated radioactivity quantitated. Results were corrected for non-specific binding (10–15% of total), i.e. the radioactivity precipitated from the same amount of radioactive ligand in plain medium.

Western ligand blot

Ligand blotting of CM was performed as described previously [25]. In brief, samples of CM were concentrated by precipitation with trichloroacetic acid (7% final concentration). The precipitates were redissolved in gel sample buffer and applied on the Mini-Protein II Dual Slab Cell electrophoresis unit (Biorad) using 12% discontinuous sodium dodecyl sulphate– polyacrylamide gels under nonreducing conditions. The transfer and radiolabelling detection buffers were as described by Hossenlopp *et al.* [25]. Following transfer of the proteins, the Immobilon-PVDF membrane (Millipore) was analysed for IGF-I binding by incubating with 200000 cpm [^{125}I]IGF-I for 4 h, washed, dried and autoradiographed (−70°C, 5–7 days).

Northern blot analysis

Total RNA was prepared from cells in logarithmic growth phase and tumour specimens using the guanidium thiocyanate method. For northern blot analysis 20 μg total RNA was denatured with dimethylsulphoxide and glyoxal, electrophoresed in 1.4% agarose and blotted onto Hybond-N membranes (Amersham). The resulting blots were hybridised with an IGFBP-1 cDNA clone (w85) [5], with an IGFBP-2 cDNA [6], and with the cDNA for IGFBP-3 [7]. The random primer extension method was used to label the cDNA probes with [γ-^{32}P] dCTP. Hybridisation was performed under stringent conditions according to standard protocols [26]. To control for variable RNA loading and transfer, the probes were removed from the Hybond membranes, and the blots were rehybridised with a 1.4 kb PstI/PstI fragment of a GAPDH cDNA probe, kindly provided by Dr Rodrigo Bravo.

RESULTS

Binding capacity of CM of NSCLC cell lines for IGF-1

The secretion of IGF-binding capacity into culture medium was investigated as a function of time. Six NSCLC cell lines were plated at a density of 5×10^4/ml in R-10 medium, cultivated for 24 h until the cells grew adherent, washed and cultivated for four days in serum-free ST-medium. As shown in Fig. 1, daily collected samples screened with the IGF-binding capacity assay showed linear increasing concentrations of the IGF-binding capacity of the CM. At day two cell line A549 reaches its binding plateau with about 80% [^{125}I]IGF-I of total radioactivity.

Ligand blotting of conditioned media

For the task of comparing the IGFBPs of 12 different NSCLC cell lines, serum-free supernatants were obtained as described

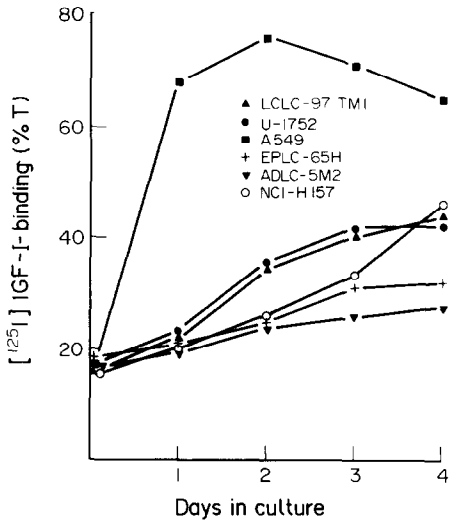

Fig. 1. Time course of IGF-binding capacity in CM of NSCLC cell lines. Culture medium was harvested at the indicated times and tested for IGF-I-binding capacity.

in Materials and Methods. As shown in Fig. 2 IGFBPs were determined by ligand blotting. All the conditioned media exhibited specific binding for IGF-I in the molecular mass range of 26–43 kD. The specificity of the binding was proven by the addition of 1.25 ng/ml unlabelled IGF-I in the incubation medium, which totally abolished the radioactivity from all bands (Fig. 3). Insulin, even at a concentration of 25 μg/ml in the incubation mixture did not affect the intensity of the labelled bands (data not shown). Insulin cross-reacts with IGF-I in binding to the type 1 receptor but does not bind to the IGFBPs.

Northern blot analysis

The expression of the different IGFBPs was measured by Northern blot analysis. Figure 4 shows a hybridisation experiment with the cDNA probes for IGFBP-1, IGFBP-2 and IGFBP-3 and the total RNA from 11 NSCLC, 3 SCLC, 1

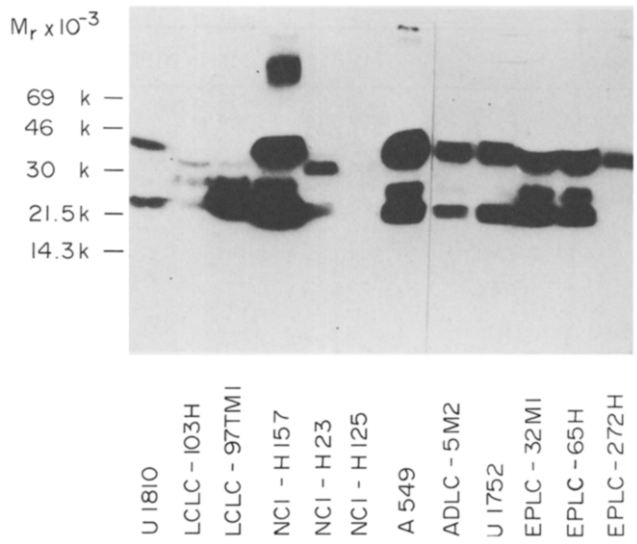

Fig. 2. Western ligand blot of IGFBPs in culture media from 12 NSCLC cell lines.

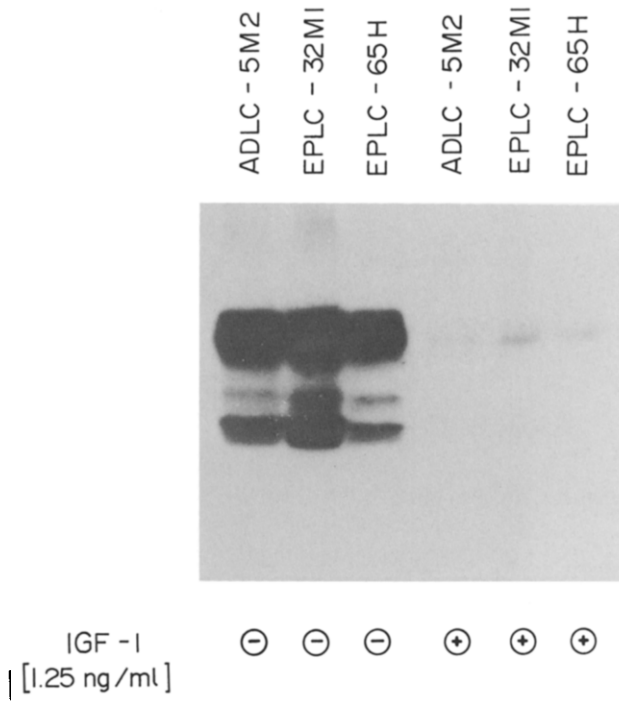

Fig. 3. Western ligand blot of IGFBPs in culture media from three NSCLC cell lines. The specificity of the reaction was proven by the addition of 1.25 ng/ml cold IGF-1.

hepatoma and from 1 human adult lung. The results with the IGFBP-1 specific cDNA probe indicate the presence of a 1.5 kb transcript in the RNA of the NSCLC cell lines ADLC-5M2, EPLC-32M1, EPLC-65H, LCLC-103H, MSTO-211H and A549. The NSCLC cell lines NCI-H23, LCLC-97TM1, NCI-H125, U-1752, U-1810 and the SCLC cell lines, NCI-H69, NCI-H82, and NCI-H146 were negative for IGFBP-1. When the same samples were hybridised with the IGFBP-2 cDNA probe a transcript of 1.6 kb was found only in the NSCLC cell lines, NCI-H23 and NCI-H125, but in all SCLC cell lines tested. A 2.5 kb transcript was found in the hybridisation experiment with the IGFBP-3 cDNA probe in the NSCLC cell lines ADLC-5M2, EPLC-32M1, EPLC-65H, LCLC-97TM1, NCI-H125, MSTO-211H, A549, U-1752 and U-1810. No hybridisation signal with the IGFBP-3 probe was detected in the NSCLC cell lines NCI-H23 and LCLC-103H, and the three SCLC cell lines.

The results of the northern blot analysis of the IGFBP-1, IGFBP-2 and IGFBP-3 mRNA levels in tumour tissues collected from 9 patients with NSCLC is presented in Fig. 5. In contrast to the results obtained from cell culture, no evidence of IGFBP-1 gene expression was obtained in any of tumour specimens. The IGFBP-2 transcript of 1.6 kb was detected, to varying degrees, in all the tumour probes and lung tissue tested, while IGFBP-3 RNA was detected in 7 of the 9 tumours, although the signal was sometimes weak. The NSCLC cell line A549, the SCLC cell line FRE and the hepatoma served as controls. A summary of the results from the northern blots is given in Tables 1 and 2.

DISCUSSION

These results demonstrate that lung cancer cell lines and tumour tissues can synthesise specific forms of IGFBPs. The results of ligand and northern blots reveal heterogeneity in the expression pattern of IGFBPs (IGFBP-1, IGFBP-2 and IGFBP-

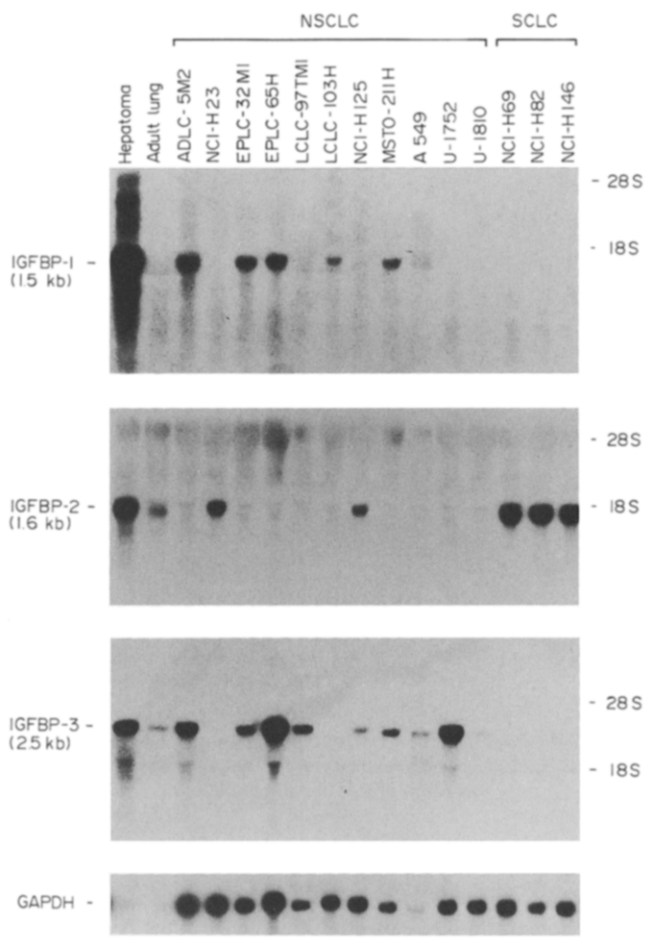

Fig. 4. Northern blot analysis of total mRNA isolated from 11 NSCLC cell lines, 3 SCLC cell lines, a hepatoma, and adult lung and hybridised with IGFBP-1, IGFBP-2 and IGFBP-3 cDNA as probes. Transcript sizes for IGFBP-1, IGFBP-2 and IGFBP-3 of 1.6 kb, 1.6 kb and 2.5 kb, respectively, are indicated. At the bottom, hybridisation of the same filter to a GAPDH cDNA probe is shown.

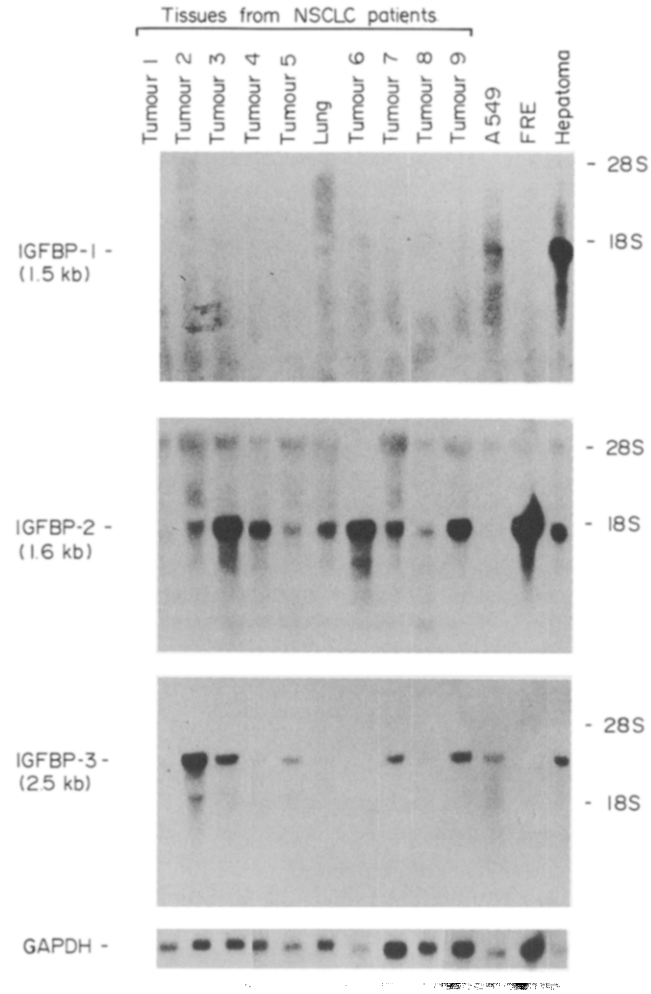

Fig. 5. Northern blot analysis of total RNA from 9 NSCLC tumour tissues (tumour 1 and 5 ADLC, all others EPLC), 1 lung, 1 NSCLC cell line, 1 SCLC cell line and hepatoma probed with IGFBP-1-, IGFBP-2- and IGFBP-3-cDNA.

3). Curiously the profile of expression of IGFBP-1, IGFBP-2, and IGFBP-3 differ between cell lines and tumour tissues from NSCLC patients: IGFBP-1 mRNA is detectable in 6/11 NSCLC cell lines, IGFBP-2 mRNA in 2/11 NSCLC cell lines, and IGFBP-3 mRNA in 9/11 NSCLC cell lines, while mRNA for IGFBP-1 is not detectable in tumour tissues, mRNA for IGFBP-2 in all tumour tissues and IGFBP-3 in 7/9 tumour tissues. Continuous cell cultures represent a convenient model, however, there are obvious limitations in extrapolating from *in vitro* studies of cultured cells to clinical tumours. One controversial aspect of this study is the fact, that mRNA for IGFBP-1 is not detected in primary tumours nor in normal lung but is detected in the majority of the NSCLC cell lines. Preliminary immunohistochemical studies on primary NSCLC tumours with a polyclonal antibody against IGFBP-1, however, showed granular staining patterns over nucleus and cytoplasma of tumour cells. It is possible, that the small fraction of positive cells would be diluted by negative cells and consequently could not be detected in northern blot analysis. It is also possible that the ability to secrete IGFBP-1 somehow confers an advantage to the NSCLC cells for growing in tissue culture.

Previously, we and others provided evidence that SCLC cell lines release low-molecular weight IGFBPs into serum-free conditioned media [16]. Northern blot hybridisation exper-

Table 1. *mRNA expression of IGFBP-1, IGFBP-2 and IGFBP-3 in lung cancer cell lines*

	IGFBP-1	IGFBP-2	IGFBP-3
NSCLC	++++	—	+++
ADLC-5M2	—	+++	—
NCI-H23	++++	—	+++
EPLC-32M1	++++	—	++++
EPLC-65H	—	—	+++
LCLC-97TM1	++	—	—
LCLC-103H	—	+++	++
NCI-H125	+++	—	+++
MSTO-211H	++++	—	++++
A549	—	—	++++
U-1752	—	—	+
U-1810			
SCLC			
NCI-H69	—	++++	—
NCI-H82	—	+++	—
NCI-H146	—	++	—

+–++++ = mRNA expression; — = no expression found.

Table 2. mRNA expression of IGFBP-1, IGFBP-2 and IGFBP-3 in primary tumours

	IGFBP-1	IGFBP-2	IGFBP-3
Tumours			
1 (A)	—	+	—
2 (Sq)	—	+	++++
3 (Sq)	—	++++	+
4 (Sq/A)	—	++	+
5 (A)	—	++	+
6 (Sq/SCLC)	—	++++	—
7 (Sq)	—	+	+
8 (Sq)	—	+	+
9 (A)	—	++	++
Lung	—	++	+

A: Adenocarcinoma, Sq: Squamous carcinoma; SCLC: small cell lung cancer; +–+++ = mRNA expression; — = no expression found.

iments, with cDNA probes for all three different IGFBPs, indicate that the only expressed IGFBP-gene being expressed was that for IGFBP-2 [17]. This is in contrast to the much more heterogenous pattern of IGFBP-gene expression exhibited by NSCLC cell lines as demonstrated in this study. IGFBP-1 is expressed in six of 11 NSCLC cell lines and all these cell lines reveal the typical transcript size of 1.5 kb, comparable to the positive control, human liver RNA. Northern blot analysis with IGFBP-3 specific cDNA showed a transcript size of 2.5 kb in all NSCLC cell lines except the cell lines NCI-H23 and LCLC-103H.

A simultaneous expression of two IGFBs is found in the NSCLC cell lines A549, MSTO-211H, NCI-H125, EPLC-65H, EPLC-32M1 and ADLC-5M2. In most cases a combination of IGFBP-1 and IGFBP-3 is found with the exception of cell line NCI-H125 which expresses IGFBP-2 and IGFBP-3.

In this study one particularly interesting observation is that all NSCLC cell lines tested expressed IGFBP-1 or IGFBP-3 and in some cases (5/11) both of them. This may prove to be one of the few common biological features of the histologically heterogenous NSCLCs. That IGFBP-1 and IGFBP-3 may function in different ways is suggested by comparison of their molecular structure. Unlike IGFBP-1 and IGFBP-2, IGFBP-3 does not possess the Arg–Gly–Asp (RGD) sequence. This motif is shared with several matrix proteins such as fibronectin, vitronectin and von Willebrand Factor and is considered essential for cell attachment [27]. Several lines of evidence indicate that the expression of IGF-BPs is tissue specific and developmentally regulated. In newborn rats the hepatic expression of IGFBP-2 decreases and IGFBP-3 increases with age [2]. IGF-BP mRNA of cell line BRL-3A, the rat homologue of IGFBP-2, is detectable in a wide range of fetal tissues, while transcripts of the IGFBP-1 gene were only found in fetal liver [5, 6]. Following reports from several laboratories, three new IGFBPs have been identified and designated IGFBPs -4, -5 and 6 [8, 9, 28]. It will be of interest to extend our studies to examine SCLC and NSCLC cell lines and corresponding tumours for the possible expression of these new members of IGFBP-family.

In lung cancer cell lines, IGF-1 and IGF-II are able to stimulate cell growth [29]. The role that IGFBPs play in modulating lung cancer cell response to the IGFs and how they function together with other growth factors needs further definition. Studies in progress examining the effect of purified IGF-BPs on IGF stimulation may elucidate their functional role in lung cancer cell lines and primary lung tumours.

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Autocrine Role of Insulin-like Growth Factor (IGF)-I in a Human Thyroid Cancer Cell Line

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An established cell line (TC-cell, clone 78) derived from human thyroid papillary cancer cells was investigated for production of peptide growth factors. The cells had specific binding sites for insulin-like growth factor-I (IGF-I) and responded to this growth factor with increased proliferation. Culture medium conditioned by TC cells was found to contain insulin-like growth factor (IGF)-I and IGF-binding protein(s). Furthermore, reverse transcription-polymerase chain reaction revealed expression of IGF-I mRNA. When monoclonal antibody to IGF-I receptors (αIR3) was added, the growth of TC cells cultured in serum-free medium was significantly reduced. The growth rate of the cells was restored when the antibody was removed from the medium. These results strongly suggest that TC cells produce IGF-I, which is involved in the regulation of their own growth.
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INTRODUCTION

RECENT STUDIES have shown that neoplastic cells are able to synthesise a number of peptide growth factors which may be involved in their own growth [1, 2]. Insulin-like growth factors (IGF-I and II) are among the growing list of such growth factors. IGF-I is a growth hormone-dependent plasma factor with potent cell growth promoting activity in a variety of cultured cells [3]. Although serum IGF-I is largely derived from the liver, many extrahepatic tissues or cells synthesise IGF-I, suggesting an autocrine or paracrine role in growth regulation [4]. Moreover, the involvement of IGF-I in cell growth has been suggested by the expression of IGF-I mRNA in several neoplastic cells [5–8].

Although pituitary thyrotropin (TSH) is the major regulator of thyroid growth and function, it has been shown that a number of growth factors affect the growth and function of thyroid epithelial cells. For example, epidermal growth factor (EGF) is a potent mitogen and inhibitor of TSH-induced iodide metabolism in thyroid cells of several species [9, 10]. We and others have recently shown that IGF-I have a stimulatory effect on thyroid cell growth [11, 12]. It appears that IGF-I is involved in the growth of human thyroid neoplastic cells. Minuto *et al.* showed higher concentrations of immunoreactive IGF-I (irIGF-I) in human thyroid tumorous tissues [13], and Williams *et al.* reported inhibition of growth of human thyroid adenoma cells by antibody to IGF-I [14]. Furthermore, we have recently shown that the number of IGF-I receptors is much higher in human

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