The insulin-like growth factors (IGFs), IGF-I and IGF-II, are potent mitogens for human lung and other epithelial cancer cell lines. Previous studies in defined medium lacking added IGF or insulin suggest that an IGF-related ligand can act as an autocrine growth factor for many cancer cell lines through action via the type I IGF receptor (IGF-R). Analysis of RNA isolated from human lung and breast cancer cell lines by reverse transcription of mRNA and polymerase chain reaction reveal that IGF-I and IGF-II mRNAs were co-expressed with IGF-R in the majority of cell lines. IGF-1 mRNA was detected in 11/12 small cell lung cancer cell lines (SCLC), 13/14 nonsmall cell lung cancer (NSCLC) cell lines, and 1/2 breast cancer cell lines. IGF-II mRNA was detected in 8/10 SCLC, 11/12 NSCLC cell lines, and 2/2 breast lines. All cell lines expressed IGF-R. For analysis of IGF peptide secretion, cell lines were adapted to growth in serum/hormone-free culture medium (R0), and to avoid interference by IGF-binding proteins, secreted IGF peptides were isolated under acidic conditions and analyzed by Western blotting. Based upon measurement of the sensitivity of the anti-IGF antibodies for detection of recombinant human IGFs, IGF peptides accumulated in conditioned medium at greater than picomolar concentrations should have been readily detected. In three cell lines (two lung and one breast) secreted IGF immunoreactivity was detected as three molecular mass species of 23, 14, and 6 kDa. Isolation and NH2-terminal sequencing of each of these species definitively identified them as differentially processed forms of the IGF-II prohormone. Despite the high frequency of IGF-I gene expression detected by reverse transcription-polymerase chain reaction analysis, only one lung cancer cell line, NCI-N417d, was found that unequivocally secreted IGF-I peptide. This direct sequence determination unambiguously identifies IGF-II as the predominant IGF involved in the autocrine growth stimulation of human lung and breast epithelial tumor cell lines and supports a growing body of literature that implicates IGF-II/IGF-R autocrine loops as a common growth mechanism in epithelial carcinogenesis.

Recent studies have identified the type I insulin-like growth factor receptor (IGF-R) as a potential control point for transformed cells (1–3). Using a variety of approaches, it has been demonstrated that the growth and tumorigenicity of transformed cells can be inhibited by the perturbation of IGF-R function (1, 4, 5), and IGF-R has been implicated in the protection of tumor cells from apoptosis (3). Human epithelial cancer cell lines express the IGF-R (6–9), proliferate in response to the IGFs (4, 6, 8), and exhibit reduced rates of growth when cultured in the presence of the monoclonal antibody a-I3, which inhibits the interaction of the IGF-R with its ligands (4, 8, 10). The ligands that bind IGF-R with high affinity are the insulin-like growth factors I (IGF-I) and II (IGF-II). These studies show that in vitro in defined medium that lacks IGFs or insulin, an IGF-related ligand can function as an autocrine growth factor for human epithelial cancer cell lines. Therefore, autocrine or paracrine loops involving the IGF-R and its ligand/s may be crucial determinants for the in vivo growth and tumorigenicity of transformed epithelial cells.

To date, few definitive data have characterized the synthesis and secretion of IGFs from human cancer cell lines. Most studies carried out on the mitogenic and autocrine effects of the IGFs have utilized exogenous addition of IGF-I (4, 8). For lung cancer cells, this approach was initially supported by work from several groups who reported small (milliunits/milliliter) amounts of “IGF-I-like immunoreactivity” in conditioned growth medium from lung cancer cell lines (11, 12). However, these original studies are now recognized to be confused by IGF-binding proteins (IGFBP), by IGF-II cross-reactivity with the antibodies used for the IGF-I measurements (13), and have been contradicted by later analysis of a small number of lung cancer cell lines from one of these groups (11), which suggests low and infrequent expression of IGF-I mRNA and a higher level of IGF-II mRNA expression. Comparing the data in these and other papers, it is apparent that IGF-R is frequently expressed (7, 14), but the identity of the relevant ligand is still unclear; the IGF-I-like immunoreactivity is not IGF-I (cell lines positive for immunoreactivity are negative for mRNA expression by RT-PCR), and there is post-transcriptional regulation of synthesis and/or secretion of the IGFs (cell lines positive by RT-PCR and Northern analysis are negative for secreted immunoreactivity) (11, 12, 15).

Elucidation of the identity of the critical IGF-R ligand is necessary for potential early tumor detection and therapeutic interventions in a wide range of human malignancies. In order to identify...
to determine the identity of the secreted IGF involved in cancer cell autocrine growth, we have carried out an extensive characterization of the expression of IGF mRNA, and the synthesis, processing, and release of IGF peptides by human lung and breast epithelial cancer cell lines. For these studies we used both Northern analysis and reverse transcription of mRNA followed by PCR (RT-PCR) to examine gene expression. We also purified to homogeneity several differentially processed and glycosylated IGF peptides secreted by human cancer cell lines, and we have definitively identified the peptides by amino-terminal Edman sequence analysis.

MATERIALS AND METHODS

Cell Lines—There are six main histologies of lung cancer: the neuroendocrine small cell lung cancers (SCLC) and the non-SCLC (NSCLC), which comprise squamous, adenocarcinoma, bronchoalveolar carcinoma, carcinoid, and large cell tumors. For these studies we analyzed 14 SCLC of classic and variant subtypes, 12 NSCLC cell lines, and 2 breast cancer cell lines obtained from the NCI-Navy Medical Oncology Branch, Bethesda, MD (listed in Table I). Reagents for cell culture, unless otherwise indicated, were obtained from Life Technologies, Inc. Cells were cultured at 37 °C in an atmosphere of 5% CO2 in RPMI 1640 with 5% heat-inactivated fetal bovine serum, or under serum- and hormone-free conditions (R0 medium: phenol red-free RPMI 1640 with 5 mM l-glutamine and 5 × 10−5 M sodium selenite (Sigma)). Cells and conditioned medium (R0CM) were harvested for analysis after the cells were adapted to the R0 medium and proliferated independently of serum components. Once adapted, the cell lines continue to grow in R0 medium and can undergo multiple passages (16).

Analysis of IGF Gene Expression—Cells from one 175-cm² tissue culture flask (approximately 5 × 10⁵ to 10⁶ cells) were washed twice with ice-cold Dulbecco’s phosphate-buffered saline, and poly(A)⁺ RNA was isolated directly from cells using a Micro-Fast Track mRNA isolation kit (Invitrogen Corp.), according to the manufacturer's instructions. IGF gene expression was initially assessed by Northern blotting, carried out according to standard procedures (17). For RT-PCR, poly(A)⁺ RNA (1 μg) was reverse-transcribed using a SuperScript™ kit (Life Technologies, Inc.), according to the manufacturer’s instructions. The single IGF-I gene comprises five exons, which are alternatively spliced to generate two mRNA transcripts (18). The two translation products, the IGF-IA and IGF-IB homologues, differ in the region that encodes the carboxy-terminal extension peptide (E-peptide), which is cleaved from pro-IGF-I during biosynthesis (18). Reactions for amplification of IGF-I cDNA were primed with an oligonucleotide complementary to the common mRNA region, which encodes the IGF-I NH₂-terminus, and antisense primers were complementary to sequences in the IGF-IA and IGF-IB splice variants. The primers had the following sequences: IGF-I sense, 5'-GGACCGGAGACGCTCTGGG-3'; IGF-I antisense, 5'-TCTGTTCTGCTGTCCGTTCC-3'; IGF-II sense, 5'-GATGCATGGCGTGTCCGTTCA-3'; IGF-II antisense, 5'-TTGGGCGGTGGTCTGCCCTTAGG-3'.

The integrity of the RNA and the efficiency of RT-PCR were monitored by amplifying the cDNA with primers specific for human type I insulin receptor (16). PCR reaction mixes consisted of 2 × PCR buffer (1.5 M MgCl₂, 200 μM dNTPs, 50 mM KCl, 10 mM Tris-HCl, pH 8.3). For amplification of IGF-I sequences, 35 cycles of PCR were programmed as follows: 94 °C, 15 s/50 °C, 15 s/72 °C, 15 s; followed by a final extension at 72 °C for 7 min. For amplification of IGF-I and II-IR primers, the annealing temperature was raised to 60 °C.

Gels were Southern blotted onto 0.2 μm nitrocellulose (Schleicher & Schuell) in 20 × SSC, baked at 80 °C for 2 h, and hybridized overnight at 42 °C with 10⁶ cpm/ml oligonucleotide probe complementary to sequences found between the PCR primers. The sequences of the antisense oligonucleotide probes were as follows: IGF-I probe, 5'-GGACCGGAGACGCTCTGGG-3'; IGF-II probe, 5'-GGACCGGAGACGCTCTGGG-3'; IGF-I probe, 5'-GATGCATGGCGTGTCCGTTCA-3'; IGF-II probe, 5'-TCTGTTCTGCTGTCCGTTCC-3'. The probe was end-labeled with [32P]ATP using T4 polynucleotide kinase (Life Technologies, Inc.) and diluted in hybridization buffer N (17). After hybridization the blots were washed at room temperature twice in 2 × SSC and once in 0.5 × SSC. Autoradiography was performed at −70 °C on Kodak X-AR film with an intensifying screen. PCR product derived from cell line H1385 was cloned into the TA cloning vector (Life Technologies, Inc.) according to the manufacturer’s instructions.

Anti-IGF Antibodies—For immunoblot studies investigating the secretion of IGF peptides from cultured cell lines, a commercial monoclonal anti-IGF-I (Upstate Biotechnology, Inc., Lake Placid, NY), reported to show 50% cross-reactivity with IGF-II, was used. An IGF-I-specific antibody was raised by immunizing rabbits with a synthetic antigen comprising amino acids 26–41 of the mature IGF-I peptide. The peptide was amided at the carboxyl terminal to enhance stability in vivo, and a tyrosine residue was included to allow iodination of the synthetic peptide. The sequence of the peptide IGF₁(26–41) was HN₁NYPKTYG5SSRARPQTH₁₉. Of this sequence only three amino acids (Ser₁⁸–Arg₁⁹–Pro) are shared with IGF-II. Serum from rabbits immunized with keyhole limpet hemocyanin-coupled peptide and the anti-IGF mAb were evaluated for specificity of IGF-I and IGF-II detection by Western immunoblot.

Immunoblot Analysis—Samples were separated electrophoretically on a 10–20% polyacrylamide gel (Novex, San Diego, CA) using the Tricine buffer system (20), electroblotted onto PVDF membrane (Millipore, Bedford, MA). After this step we incubated (1 h) non-denaturing (5% w/v) bovine serum albumin in phosphate-buffered saline and incubated overnight at 4 °C in 1 μg/ml anti-IGF monoclonal antibody or 1/100 dilution rabbit anti-IGF₁(26–41). For detection of bound antibody with 125I-protein A, blots probed with anti-IGF mAb were incubated with a secondary antibody (1/1000 rabbit anti-mouse antibody; DakoPatts, Glostrup, Denmark) for 1 h at room temperature. Washed blots were incubated with 10⁶ cpm 125I-protein A (1 h, room temperature), washed, and autoradiographed. The specificity of the immunodetection was established by preabsorbing the diluted antibodies with 1 μg rhIGF-I. For quantitation, blots were scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Analysis of IGF I, II, III, and IV in Conditioned Media from 1206 lung cancer cell lines was carried out using IGF-I and IGF-II conditioned medium from cells grown with R0 medium. Whole cell lysates and conditioned medium from cultured cell lines were screened for IGFs by immunoblot analysis. Whole cell lysates were prepared by lysing 10⁶ cells directly in SDS-PAGE sample buffer (without mercaptoethanol or bromophenol blue). Lysates were clarified by centrifugation at 100,000 g, and protein was estimated using the BCA assay (Pierce). Up to 100 μg of total cellular protein was electrophoresed for immunoblot assay of IGF-I, II, III, and IV. Four samples were run and screened for the secretion of IGF peptides by collection of 7-day conditioned medium (R0CM). The R0CM was centrifuged at 100,000 g at 4 °C and subjected to 100 μg rhIGF-I, II, III, and IV (R0CM) in the presence of the protease inhibitors bacitracin (1 μM; Bachor, Torrance, CA), phosphoramidon (1 μM, Sigma), and Pefabloc (1 μM) added to the R0CM after collection. Five hundred milliliters of R0CM was conditioned medium (R0CM) were collected and adjusted to pH 2.5 with trifluoroacetic acid (Pierce), mixed at 4 °C overnight, centrifuged at 4000 × g for 30 min, and filtered through a 0.45 μm filter. The R0CM was adjusted to 10% (v/v) acetonitrile and pumped at 15 ml/min onto a C₈ reverse phase column (30 mm × 30 cm DeltaPak 300R, Millipore-Waters) equilibrated in 10% acetonitrile, 0.1% trifluoroacetic acid (v/v). The column was washed with 100 μl 0.1% acetonitrile, 0.1% trifluoroacetic acid (v/v), and a linear gradient was applied with acetonitrile increased from 10% to 35% (v/v) over 30 min. Fractions corresponding to the IGF peptides were collected, freeze-dried, and resuspended in 200 μl of SDS-PAGE sample buffer. Before each R0CM was chromatographed, a blank gradient was run and screened for residual immunoreactivity from the previous run. IGF-I and IGF-II standards were chromatographed under identical conditions after all the R0CM samples were processed.

Purification of IGF-I in CM—R0CM (500 ml) from the cell line NCI-H287 was supplemented with 5 μg of rhIGF-I (final concentration 1.2 nm) and fractionated by C₈ HPLC as described above. Fractions were collected, freeze-dried, and analyzed by immunoblot for the presence of IGF-I and IGF-II. The IGF-positive fractions were pooled and treated in an identical fashion for the purification and NH₂-terminal sequencing of the IGF peptides (see below) as those isolated from unsupplemented R0CM.

Purification and NH₂-terminal Sequencing of Secreted IGFs—For isolation of anti-IGF immunoreactivity, R0CM (up to 10 liters) was
fractionated by preparative scale HPLC on a C₄ column. Fractions containing IGF immunoreactivity were applied to a phenyl reverse-phase HPLC column (5 μ; 2.1 mm × 25 cm, Vydac, The Separations Group, Hesperia, CA) equilibrated with 20% acetonitrile, 0.1% heptfluorobutyric acid (v/v) (HFBA, Pierce), and eluted with a gradient to 45% acetonitrile, 0.1% HFBA (v/v) over 150 min at 1 ml/min. One-minute fractions were collected. For amino acid sequencing, fractions containing the 6-and 14-kDa immunoreactive species were freeze-dried and resuspended in 50 μl of SDS-PAGE buffer and separated on 15-cm 12% Tricine SDS-PAGE gels. Samples for sequencing were electroblotted onto PVDF in 10 mM CAPS, pH 11, for sequencing were electrophoresed on a 15-cm 12% Tricine SDS-PAGE gel. Samples for sequencing were electroblotted onto PVDF in 10 mM CAPS, pH 11, for long exposure of the probed Southern blot to film.

IGF Gene Expression Shows Small Changes after Adaptation to Growth under R₀ Conditions—There was no change in the overall pattern of IGF gene expression when cells were adapted to growth under R₀ conditions, with the exception of three cell lines, NCI-H720, -H1092, and -H345. For the breast cancer cell line H2380 (data not shown). The pattern of IGF and IGF-R gene expression by RT-PCR in a subset of the cancer cell lines is shown in Fig. 1 (A and B). The majority of lung cancer cell lines (21/26) expressed IGF-I and IGF-R. For the breast cancer cell line H2380, derepressed the IGF-IB primers is single-stranded PCR product, when excised and reamplified, it yielded a product that migrated with the upper band. The figure shows a subset of the data used to generate Table I. Some bands not visible in the photograph were seen on longer exposure of the probed Southern blot to film.

FIG. 1. IGF and IGF-R gene expression in lung cancer cell lines as detected by RT-PCR (A) and IGF and IGF-R gene expression in the breast cancer cell lines (B). RT-PCR products were electrophoresed on agarose gels, Southern blotted, and probed with specific oligonucleotide probes complementary to an internal sequence to confirm the identity of the PCR products. The faster migrating band of the doublet visible with the IGF-IB primers is single-stranded PCR product; when excised and reamplified, it yielded a product that migrated with the upper band. The figure shows a subset of the data used to generate Table I. Some bands not visible in the photograph were seen on longer exposure of the probed Southern blot to film.

### RESULTS

IGF mRNA Is Widely Expressed in Human Cancer Cell Lines—Both IGF-I and IGF-II were co-expressed by the majority of cell lines tested, and all cell lines expressed mRNA for the IGF-R (summarized in Table I). Corroborating an earlier report on a small number of cell lines, the level of IGF-II mRNA in some lung cancer cell lines appeared higher than that of IGF-I.
Quantitative differences in the mRNA levels that were not detected using the current techniques.

IGF Peptides Are Secreted into CM—The sensitivity and specificity of the anti-IGF antibodies used to screen Western blots is shown in Fig. 2. No specific IGF immunoreactivity was detected in whole cell lysates of the cancer cell lines with either of the anti-IGF antibodies. As the commercial anti-IGF mAb proved to be sensitive for the detection of both IGF-I and -II, this antibody was used for the initial screening of HPLC-fractionated R0CM.

In the initial screening of 500 ml of R0CM, immunoreactivity was detected in medium conditioned by only 3/10 cell lines: NCI-H820, NCI-H2087, and NCI-H2380. Fig. 3A shows the chromatogram obtained when R0CM from the cell line NCI-H2087 was fractionated on a C4 column. IGF immunoreactivity (Fig. 3B) was present as three bands of 23, 14, and 6-kDa and eluted in fractions 34–36. When IGF-I and IGF-II standards were chromatographed on the preparative C4 column, they had the same retention time as the immunoreactivity from R0CM and co-migrated with Mr 6000 species on the mini-gels used for the immunoblotting (data not shown). The relative intensity and distribution of the three immunoreactive species varied between these cell lines (Fig. 3C). Electrophoresis under reducing conditions did not alter the apparent size of the three immunoreactive species, suggesting that they are monomeric species.

When larger volumes of R0CM (3–7.5 liters) from the cell lines NCI-H345 and NCI-N417 were concentrated on the C4 column, weak immunoreactive bands corresponding to Mr 6000 species on the mini-gels used for the immunoblotting (data not shown). R0CM from the cell lines NCI-H187 (2 liters), NCI-H1385 (1.5 liters), NCI-H510 (3 liters), MCF-7 (2 liters), and NCI-H460 (3 liters) tested negative for IGF immunoreactivity.

Specificity of Immunoreactivity—The specificity of the immunoreactivitity detected by the anti-IGF mAb in concentrated R0CM is shown in Fig. 4 (top panel). When the anti-IGF mAb was preincubated with 1 uM IGF-I, the immunoreactive material was no longer recognized, indicating that it is specific and IGF-related (Fig. 4, lanes B). The polyclonal anti-IGF-IY26–41 did not detect any immunoreactive material in the NCI-H2087 R0CM shown or in R0CM from any of the other cell lines investigated (Fig. 4, top panel, lane C). This antibody is highly specific for rhIGF-I and did not recognize 100 ng IGF-II on Western blot (Fig. 2, lower panel), although it is approximately as sensitive as the less specific commercial mAb for IGF-I. The Mr 6000 immunoreactive species co-migrates with rhIGF on SDS-PAGE and presumably represents mature IGF. The failure of the anti-IGF-IY26–41 to recognize this band suggested that it was IGF-II. The larger molecular size immunoreactive species presumably represent unprocessed or partially processed IGF prohormones. The ability of the anti-IGF-IY26–41 to recognize incompletely processed IGF-I prohormone is not known, and the failure of this antibody to recognize the 14- and 23-kDa immunoreactive species did not eliminate the possibility that these forms were unprocessed or partially processed IGF-I prohormones.
Stability of IGF-I in R0CM—The failure of the anti-IGF-1 antibody to detect fully processed 7.5-kDa IGF-I in the fractionated R0CM samples raised the possibility that it may have become degraded or was inefficiently recovered by the isolation procedures. To address this concern, rhIGF-I was added in a physiologically relevant concentration (2.5 μg/ml; 1.2 nm) into H2087 R0CM after collection, stored as usual, and fractionated on the C4 column in a process identical to the other R0CMs. The second panel of Fig. 4 demonstrates that the exogenous IGF-I was recovered and could be detected by the anti-IGF Y26–41, anti-serum. We also used Edman degradation to confirm that IGF-I sequence, at the level of 10 pmol, could be obtained from 1/20 of the pooled positive fractions purified by HPLC from NCI-H2087 R0CM into which 5 μg (660 pmol) of rhIGF-I had been added. This represents 30% recovery of the exogenously added rhIGF-I, determined from the initial and repetitive sequencer yield. However, rhIGF standards subjected to SDS-PAGE, electroblotted onto PVDF, and quantitated by NH2-terminal sequencing also resulted in a “recovery” of 25–50%. Given this yield at the blotting/sequencing step, 30% recovery of rhIGF-I from H2087 R0CM after storage and chromatographic isolation procedures followed by electroblotting and sequencing demonstrates that IGF-I is stable in R0CM and would have been recovered with minimal losses under the purification conditions used for these studies.

Purification and NH2-terminal Sequencing of Secreted Immunoreactivity—Given that the antibody studies did not confirm the identity of the IGF immunoreactivity secreted by the R0-adapted cell lines, we conducted a large scale purification of R0CM from the cell lines NCI-H2087 (10 liters), NCI-H820 (2 liters), and NCI-N417 (7.5 liters), and the breast line, NCI-H2380 (500 ml). The elution profile of the pooled fractions 34–36 from the preparative C4 column rechromatographed on a phenyl column with an acetonitrile/HFBA gradient is shown in Fig. 5. When standards were run on this column with the same gradient, IGF-II eluted at 73.4 min and IGF-I at 77.6 min. Ubiquitin, which had been identified as a contaminant in the IGF-positive preparative C4 HPLC fractions by amino acid sequencing of parallel fractions from the IGF-negative cell line H187, eluted in a broad peak from 92 min to 105 min. Coomassie staining of fractions after SDS-PAGE and electroblotting onto PVDF revealed protein bands that corresponded with the IGF immunoreactivity. The results of Edman sequence analysis of these bands are listed in Table II. All of the immunoreactive species secreted by the cell lines NCI-H820, NCI-H2087, and NCI-H2380 were identified as products of the IGF-II gene. IGF-I was identified by Edman sequence analysis of a peptide isolated from the variant SCLC line NCI-N417.

**DISCUSSION**

Previous in vitro studies have suggested that an IGF/IGF-R autocrine loop may be operating in many human epithelial cancer cell lines. The inhibitory effect of the anti-IGF-R antibody, α-IR3, on the growth of lung cancer cell lines has been documented (4, 10), and studies by others and ourselves have confirmed the inhibitory effect of this antibody on the clonal growth of the breast cancer cell line MCF-7 in semi-solid agar in the presence of serum (8) and the absence of added serum or insulin-like peptides.2 Our previous studies have shown that IGF stimulation of tumor cell growth is mediated via an active IGF-R (4). Recent work has confirmed the importance of IGF/IGF-R interactions in maintenance of the transformed phenotype and control of apoptosis (1–3). The capacity of the lung and breast cancer cell lines investigated in these studies to adapt to growth in unsupplemented basal medium with minimal changes in the pattern of IGF gene expression suggests that IGF/IGF-R autocrine loops may be constitutive elements which influence the growth characteristics of these tumors. As IGF-R is expressed by most actively growing cells, elucidation of the identity of the synthesized and secreted IGF ligand is crucial to our understanding of epithelial tumor biology.

The two mature 7.5-kDa IGF peptides exhibit close to 70% amino acid identity with each other and are 50% homologous with pro-insulin (22). Studies have implicated both IGFs in the

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2 K. A. Quinn, M.-j. Miller, and F. Cuttitta, unpublished observation.
growth and differentiation of normal epithelia. In the developing fetal lung, IGF-II appears to play a critical role, since mice lacking a functional IGF-II gene died at birth due to a failure of lung inflation (5). In situ hybridization studies have localized IGF-I expression to the mesenchymal compartment of adult and developing lung, suggesting that IGF-I may influence airway development via paracrine modes of action (23). Corroborating evidence has demonstrated the secretion of IGF-I by primary cultures of human lung fibroblasts (24). These findings suggest that the pattern of IGF expression in lung neoplasms may parallel that of the breast epithelium, where IGF-I is principally expressed by stromal tissue while IGF-II is expressed by epithelial tumor cells (25). Elucidation of the role of IGFs in autocrine growth is complicated by the synthesis of IGFBP which are expressed by many cancer cell lines, usually in large excess over the IGFs (14, 15, 26). The IGFBP therefore confound interpretation of early studies, which aimed to use anti-IGF antibodies to investigate the effect of IGF neutralization on cell growth. Using purification and assay methodologies that obviate interference by IGFBP, we have demonstrated conclusively the secretion by human lung and breast cancer cell lines of ligands that can bind and activate the IGF-R.

Purification, SDS-PAGE, and NH2-terminal Edman sequencing demonstrated IGF-II peptide secretion in the breast adenocarcinoma cell line (H2380), and two of the lung cancer cell lines investigated: an adenocarcinoma (NCI-H2087) and a bronchoalveolar carcinoma (NCI-H820). IGF-II peptide sequence was obtained from an M, 6000 species isolated from medium conditioned by a variant SCLC cell line, NCI-N417. IGF-II was secreted as multiple molecular weight forms, as has been described for IGF-II present in normal adult serum and serum from hypoglycemic patients bearing mesenchymal tumors (27). The post-translational processing of the primary IGF-II prepropeptide is complex and can yield multiple molecular weight forms of IGF-II, the nature of which is determined by differential post-translational cleavage and glycosylation events (19, 28). The 20-kDa preproprotein is sequentially processed by cleavage at a single lysine residue at position 21 of the prohormone to yield a 10.5-kDa peptide (pro-IGF-II E21), which has been isolated from normal human serum (29), and then by removal of the E-peptide to yield mature IGF-II. A 15-kDa form of pro-IGF-II E21 has also been isolated, which includes O-linked sialic acid residues attached to Thr8 of the E-peptide (28).

Studies are continuing to characterize fully the exact nature of the 14- and 23-kDa IGF-II species. The 6-kDa form identified here, which co-migrated on SDS-PAGE with the 7.5-kDa hIGF-I/II standards, was identified as fully processed mature IGF-II. The primary IGF-II translation product has been calculated to be a 20-kDa polypeptide (30). The 23-kDa form of IGF-II isolated here could represent the unprocessed precursor, which may be running with an artificially high molecular mass on SDS-PAGE gels, or could be due to glycosylation at Thr8 of the E-peptide region of the intact prepropeptide. Preliminary data indicates that the 14-kDa species, isolated from the H2087 adenocarcinoma, can be converted to a species migrating at 10 kDa on SDS-PAGE after incubation with O-glycosidase and neuraminidase (data not shown). It is therefore equivalent to the 15-kDa IGF-II characterized by Hudgens et al. (28). This glycosylated and sialated 15-kDa IGF-II has higher potency for growth stimulation and receptor activation than the 7.5-kDa peptide and altered interactions with IGFBP (27, 31). At least two of the three forms of IGF-II isolated from these lung and breast tumor cell lines are therefore capable of acting as autocrine growth factors.

From the incidence of IGF gene expression shown in Table I, it would appear that IGF autocrine loops are operating in the majority of the cell lines, i.e. IGF-R was co-expressed with one or both of the IGF peptide genes. IGF-II peptide secretion was detected in 3 of the 10 cancer cell lines intensively investigated. These three were cell lines with IGF-II mRNA levels detectable by the less sensitive Northern blot procedure. All of the cell lines investigated, with the exception of the SCLC cell line NCI-H187 and the breast line MCF-7, expressed one or both of the IGF-I transcripts. However, we were only able to definitively identify IGF-I peptide secretion by the SCLC line NCI-N417d. In the cell lines from which IGFs were not isolated, we calculate (based upon the sensitivity of the antibodies for the detection of the IGFs) that IGF peptides may only accumulate in CM at subpicomolar concentrations. As IGF-R inhibition studies with mAb α-IR3 demonstrate active IGF/IGF-R autocrine loops in many of the cell lines studied here (4), mechanisms could be operating by which a low level of synthesized IGF is preferentially delivered to the receptor and rapidly turned over, resulting in accumulation of synthesized IGF at levels too low for measurement using present techniques. Such mechanisms may be potentiated by the IGFBP, which we found to be secreted by human lung cancer cell lines in large excess of the endogenously secreted IGFs. Membrane-bound forms of IGFBP, which have been identified in lung cancer cell lines (14), may play a role in the delivery of IGFs to the receptor.
Addendum—Since this work was submitted for publication, a report on a single prostate cancer cell line has suggested that IGF-II/IGF-R is involved with autocrine growth regulation in that epithelial system (32), and a recent study demonstrated that an IGF-II/IGF-R autocrine loop mediates epidermal growth factor-induced proliferation in a cervical cancer epithelial cell line (9). Those findings, as well as the conclusive identification of multiple active forms of IGF-II synthesized and secreted by both lung and breast epithelial cancer cell lines reported here, point to the conservation of the IGF-II/IGF-R autocrine pathway as a central mechanism in the process of epithelial carcinogenesis.

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