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Partial Characterization of Insulin-like Growth Factor I in Primary Human Lung Cancers Using Immunohistochemical and Receptor Autoradiographic Techniques[1]

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ABSTRACT

We investigated primary human lung cancers resected surgically or obtained at autopsy. Included were squamous cell carcinoma (SQC) (five cases), adenocarcinoma (ADC) (six cases), large cell carcinoma (LCC) (four cases), and small cell carcinoma (SCC) (two cases). The objective of the study was to search for the presence of insulin-like growth factor I (IGF-I)-like immunoreactivity using immunohistochemical staining and for the localization of IGF-I binding sites, using *in vitro* quantitative receptor autoradiographic techniques.

IGF-I-like immunostaining was present in all cases of SQC, ADC, and LCC, but not in cases of SCC. Strong immunostaining was observed in cases of SQC. On the other hand, ADC and LCC tissues showed a moderate or weak staining. Specific binding sites for IGF-I were present in all cases of SQC, ADC, LCC, and SCC examined. High densities of ^{125}I-IGF-I binding sites were localized in cases of SQC and SCC. Low to high densities of the binding sites were found in LCC. Cases of ADC showed low densities of ^{125}I-IGF-I binding sites. Specific binding obtained at a concentration of 80 pM ^{125}I-IGF-I was competitively displaced by unlabeled IGF-I, with a 50% inhibitory concentration value of 1.84 ± 0.31 × 10^{-10} mol, whereas human insulin was much less potent in displacing the binding. This specificity profile is consistent with characteristics of IGF-I receptors. Scatchard analysis showed the presence of a single class of high affinity binding sites for IGF-I, with a K_d of approximately 1 nmol.

Thus, the possibility that IGF-I may play a role in the growth of human lung cancers would have to be considered.

INTRODUCTION

Growth factors are involved in the transformation and/or proliferation of neoplastic cells (1–4), and neoplastic cells can produce the endogenous growth factors which act on the their own cells (1–5). Among growth factors, the production or gene expression of IGFs,[3] related to the functions of differentiation and growth of cells and tissues (6), was demonstrated in several types of human neoplastic cells (1, 3, 4). IGFs are homologous to amino acid sequences of proinsulin (7), and at least two forms of IGFs, IGF-I and IGF-II, are present in human serum (7). IGF-I receptors on cell surface membranes (8) are structurally similar to insulin receptors (9). Additionally, IGF-I and insulin bind to specific receptors with a high affinity and to each other with a lesser affinity (9). IGF-II receptors have no apparent affinity for insulin (9).

Human lung cancers can be mainly classified into four types: SQC; ADC; LCC; and SCC (10). IGF-I is secreted or released from lung cancers (SQC and ACC) resected surgically and some established SCC cell lines (4, 11–13). Moreover, high affinity receptors for IGF-I were observed in SCC cell lines (4, 12, 13). These findings suggest that IGF-I may be an important growth factor in human lung cancers, acting in an autocrine or a paracrine fashion (6, 14). The bindings for IGF-I in SQC, ADC, and LCC have apparently not been reported.

We made use of *in vitro* quantitative receptor autoradiographic techniques (15, 16) to investigate whether or not IGF-I binding sites could be demonstrated in four types of human lung cancers. Distributions of IGF-I-like immunoreactivity in human lung cancers were examined, using immunohistochemical techniques.

MATERIALS AND METHODS

Tissue Samples. Seventeen lung cancers resected surgically or obtained at autopsy at Nagasaki Municipal Hospital were microscopically classified into SQC (5 cases), ADC (6 cases), LCC (4 cases), and SCC (2 cases), according to the rules of the Japanese Lung Cancer Society (14) (Table 1). The tumors obtained were immediately fixed in 15% neutral buffered formalin for immunohistochemical staining, and part of the fresh tumors was immersed in isopentane at −30°C for quantitative receptor autoradiography.

Immunohistochemical Staining of Tissues. Polyclonal human IGF-I antibody (amino acids 1 to 70, IGF-I) was purchased from KabiGen AB, Stockholm, Sweden, and was used at a 1:4000 dilution. Formalin-fixed, paraffin-embedded tissues were sectioned at a 5-μm thickness. After deparaffinization of the sections, endogenous perioxidase activity was abolished with 0.3% H_2O_2 solution. Immunostaining was carried out by the avidin-biotin peroxidase complex method using Vectastain ABC kits (Vector Lab., Inc.). To determine the specific binding, immunostaining was abolished by preincubation of the primary antibody with an excess of unlabeled IGF-I (Peninsula Lab., Inc.) prior to immunostaining.

Quantitative Receptor Autoradiography. Frozen 16-μm-thick sections were cut in a cryostat at −16°C, thaw mounted onto gelatin-coated glass slides, and stored overnight under vacuum at 4°C. The sections were preincubated for 15 min in 25 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 0.1% bovine serum albumin, and 1 mg/ml of bacitracin (Sigma), followed by incubation for 120 min in fresh buffer with 3-[^{125}I]iodotyrosyl-IGF-I (^{125}I-IGF-I) (specific activity, 1837 Ci/mmol; Amersham International plc., United Kingdom), with or without unlabeled 10^{-7} M IGF-I (Amersham) (17). In the kinetic experiment, consecutive tissue sections were incubated with 80 pmol of ^{125}I-IGF-I in the presence of increasing concentrations of unlabeled IGF-I, ranging from 10^{-7} to 10^{-13} mol. Human insulin (Peptide Institute, Japan) was also used as a displacer for ^{125}I-IGF-I binding, ranging from 10^{-5} to 10^{-9} mol. The tissue sections were washed 3 times (1 min each) with 25 mM Tris-HCl buffer (pH 7.4) at 4°C. After drying under a stream of cool air, the sections and ^{125}I standards (Amersham) were opposed to ^{3}H-hyperfilm (Amersham) at room temperature for 7 days. Absorbances were quantified by computerized microdensitometry (UHG-101; Unique Medical Co., Japan). ^{125}I-IGF-I binding kinetic values were calculated from data on competition studies, using the LIGAND computer program (18).

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[3] The abbreviations used are: IGF, insulin-like growth factor; SQC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma; SCC, small cell carcinoma; IC_{50}, 50% inhibitory concentration.

Table 1 *Apparent ^{125}I-IGF-I binding site concentrations in human lung cancers resected surgically or obtained at autopsy*

Tissue sections were incubated with 80 pM ^{125}I-IGF-I. Absorbance measurements were converted into fmol/mg of protein.

Pathology	Case	Age (yr)	Sex	Surgery or autopsy	Apparent density (fmol/mg of protein)
Squamous cell carcinoma	1	74	M	Surgery	11.92
	2	59	M	Autopsy	11.17
	3	53	M	Surgery	11.78
	4	67	M	Surgery	7.22
	5	56	M	Surgery	9.68
Adenocarcinoma	6	74	F	Surgery	3.04
	7	56	F	Autopsy	3.66
	8	58	F	Surgery	2.77
	9	67	M	Autopsy	3.23
	10	51	F	Surgery	3.44
	11	55	M	Surgery	2.38
Large cell carcinoma	12	63	M	Autopsy	4.59
	13	46	M	Surgery	2.54
	14	63	M	Autopsy	7.06
	15	57	M	Autopsy	1.61
Small cell carcinoma	16	45	M	Surgery	9.80
	17	55	M	Autopsy	8.00

RESULTS

IGF-I-like immunoreactive cells were detected in SQC, ADC, and LCC (Fig. 1, *a* to *c*). The SQC cells gave a strongly positive reaction, while the moderately or poorly differentiated cells tended to show a weaker reaction than did the well-differentiated cells. In cases of ADC and LCC, immunostaining was observed in a few areas. The ADC cells invading the surrounding tissue and not secreting mucin mainly gave a positive reaction. The stained LCC cells were scattered, and most were present in the periphery of the tumor mass. Immunostaining was nil in cases of SCC (Fig. 1*d*). Immunostained cells were also present in the columnar epithelium of the primitive airways, lung fluid within the airway, cartilage, and pulmonary vessel walls (data not shown). When the primary antibody was immunoabsorbed with an excess of unlabeled IGF-I, there was no evidence of immunostaining (data not shown).

Binding sites for ^{125}I-IGF-I were distributed in all the human lung cancers examined. The apparent concentrations of specific binding sites calculated from the total binding (Fig. 2, *a* to *d*) and nonspecific binding (Fig. 2, *e* to *h*) amounts are shown in Table 1. Nonspecific binding accounted for about 10% of the total binding. High densities of ^{125}I-IGF-I binding sites were present in cases of SQC and SCC. Densities of the binding sites in cases of LCC ranged from high to low. On the other hand, cases of ADC showed low concentrations of IGF-I binding sites.

The kinetic experiment was done using SQC (Cases 1 to 5), LCC (Case 14), and SCC (Case 16) showing high densities of ^{125}I-IGF-I binding. IGF-I binding sites were characterized by competitive experiments in the presence of increasing concentrations of unlabeled IGF-I. Specific binding of ^{125}I-IGF-I obtained at the concentration of 80 pM radiolabeled ligand was competitively inhibited by unlabeled IGF-I, with an IC_{50} of $1.84 \pm 0.31 \times 10^{-10}$ mol (Figs. 3 and 4). The same effect was seen with unlabeled human insulin, with an IC_{50} of $5.26 \pm 0.82 \times 10^{-7}$ mol. As shown in Table 2 and Fig. 4, Scatchard analysis of data obtained from a LIGAND computer program indicated a single class of high affinity IGF-I binding sites, with an K_d of approximately 1 nmol. The maximum binding capacity (B_{max}) ranged from 191.61 to 461.49 fmol/mg of protein in the case of SQC, 178.25 fmol/mg of protein in the case of LCC, and 298.50 fmol/mg of protein in the case of SCC.

DISCUSSION

We obtained evidence for the localization of IGF-I-like immunostaining and high affinity IGF-I binding sites in four types of human lung cancers. Strong immunostaining was observed in cases of SQC, and moderate or weak staining was seen in cases of ADC and LCC. On the other hand, no immunostaining was observed in cases of SCC. Minuto *et al.* (11) reported that tissue extracts from SQC and ADC obtained surgically contained a high amount of IGF-I-like immunoreactivity. Some, but not all, of the established SCC cell lines release a high amount of IGF-I into the culture medium (4, 12, 13). However, expression of the IGF-I gene was not detected in four types of lung cancer cell lines (5). This discrepancy may contribute to the difference in the biological behavior observed *in vivo* and *in*

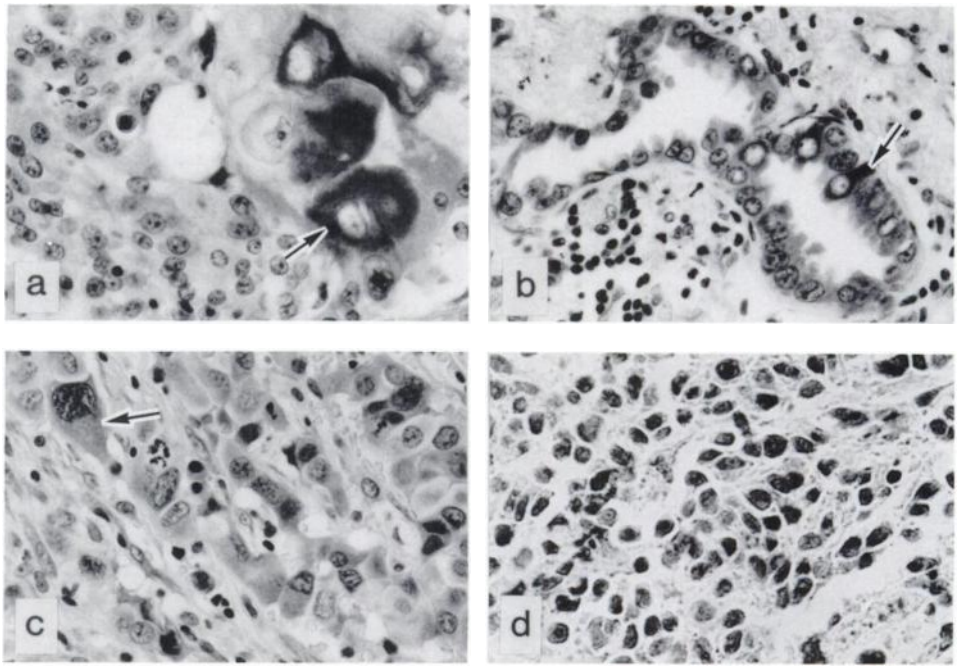

Fig. 1. Immunohistochemical staining with hematoxylin counterstaining in SQC (*a*), ADC (*b*), LCC (*c*), and SCC (*d*). *Arrows* indicate one positive cell in each cancer. Magnification: × 280 (*a* to *c*); × 760 (*d*).

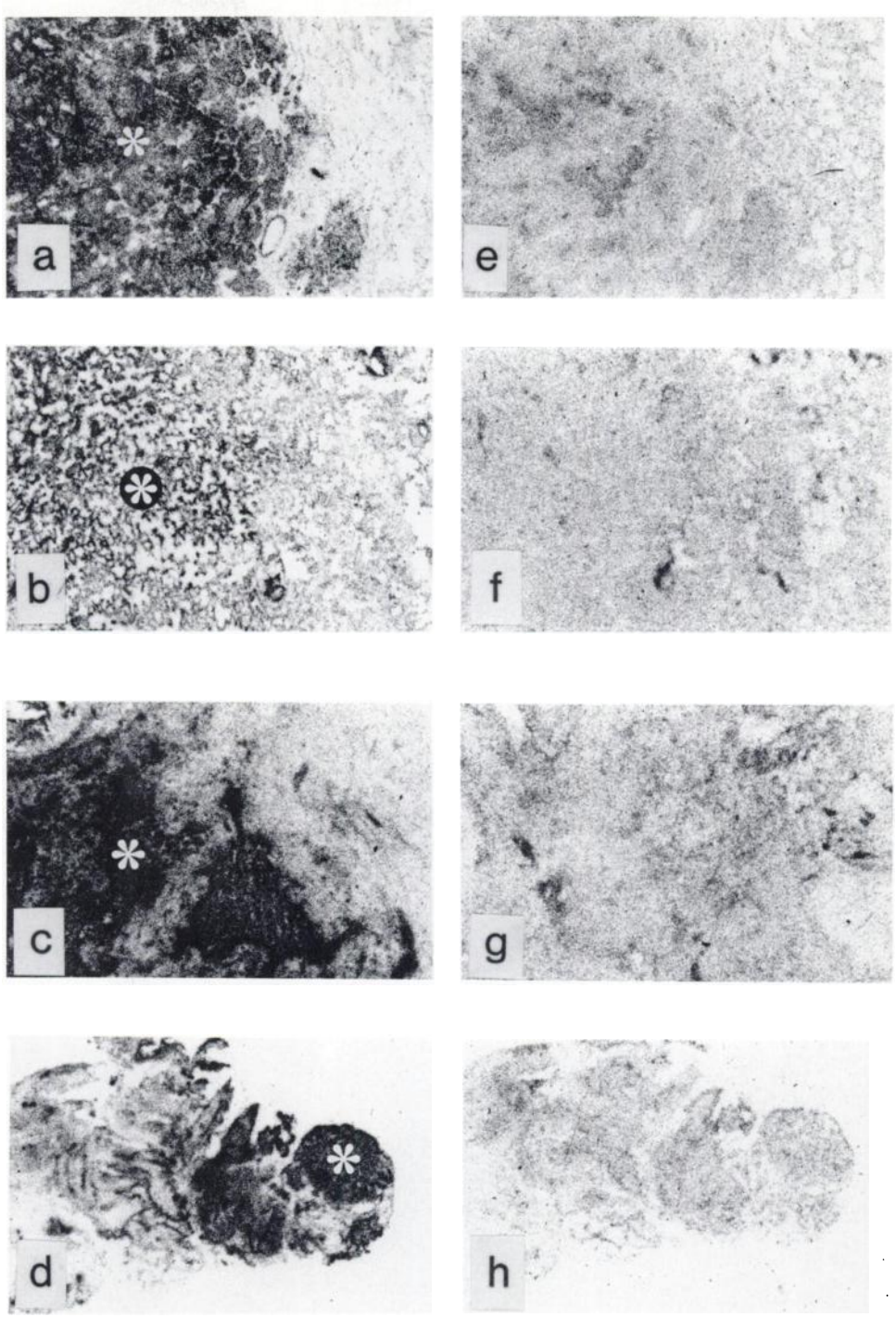

Fig. 2. Receptor autoradiographic localization of ^{125}I-IGF-I binding sites in SQC (*a*, *e*), ADC (*b*, *f*), LCC (*c*, *g*), and SCC (*d*, *h*). *a* to *d* show total binding incubated with 80 pM ^{125}I-IGF-I and exposed to ^{3}H-hyperfilm for 7 days. Nonspecific binding (*e* to *h*) was determined in adjacent sections of *a* to *d* that were incubated with the same amounts of the radioligands in the presence of 10^{-7} M unlabeled IGF-I. *, areas of carcinomas.

vitro. Whether the immunostaining shown in this study is due to a primary *in situ* production of IGF-I or to uptake of circulating IGF-I remains the subject of ongoing study.

In lung cancers, specific binding sites for IGF-I have been noted on SCC cell lines (4, 12, 13). We found that specific ^{125}I-IGF-I binding sites were present on SQC, ADC, and LCC as well as SCC, determined using quantitative receptor autoradiography. However, there was no relationship between the intensities of immunostaining and the densities of ^{125}I-IGF-I binding sites. Although cases of SQC showed both a strong immunoreactivity and high densities of ^{125}I-IGF-I binding sites, high concentrations of IGF-I binding sites, without immunoreactivity, were observed in cases of SCC. The densities of the binding sites in cases of LCC ranged from high to low. LCC is a microscopically pleomorphic epithelial tumor with no squamous or glandular differentiation (10). It may not represent a

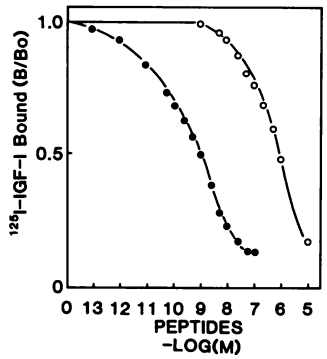

Fig. 3. Displacement of ^{125}I-IGF-I binding from human lung cancers by different concentrations of unlabeled IGF-I (●) and human insulin (○).

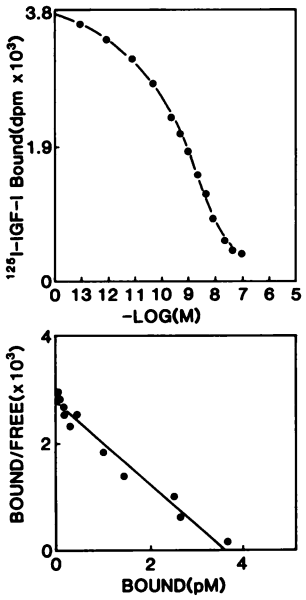

Fig. 4. Binding of ^{125}I-IGF-I to SQC (Case 3) and Scatchard plot of IGF-I dose-response data.

Table 2 *Dissociation constant (K_d) and maximum binding capacity (B_{max}) of IGF-I binding sites in human lung cancers*

The K_d and B_{max} were calculated from the competition curve using the LIGAND computer program (18).

Case	K_d (nmol)	B_{max} (fmol/mg of protein)
1	1.28	316.66
2	1.29	311.26
3	1.84	461.49
4	1.25	191.62
5	1.04	199.71
14	1.39	178.35
16	0.82	298.50

specific entity; rather it may be undifferentiated variations of SQC and ADC (19). Hence, our cases of LCC may possess a potential SQC or ADC.

We characterized IGF-I binding sites in cases of SQC (Cases 1 to 5), LCC (Case 14), and SCC (Case 15). Receptors for IGF-I have been characterized using competitive binding experiments (12, 20, 21). At least two types of receptors, IGF-I and IGF-II, have been proposed (9). To determine which type of receptor was present in lung cancer tissue, we performed competitive experiments with IGF-I and insulin. ^{125}I-IGF-I bindings were competitively inhibited by unlabeled IGF-I, with an IC_{50} of $1.84 \pm 0.31 \times 10^{-10}$ mol. Insulin also displaced ^{125}I-IGF-I binding sites, albeit less potently. Our results from the competitive binding study indicate that ^{125}I-IGF-I binding to human lung cancers is consistent with characteristics of IGF-I receptors (12, 20, 21). Further study, especially cross-linking experiments, is required to characterize the bindings.

Scatchard analysis of the data obtained from a LIGAND computer program (18) showed a single class of high affinity IGF-I binding sites, with a K_d of about 1 nmol, the values of which are similar to those noted in SCC cell lines by Jaques *et al.* (12). However, Nakanishi *et al.* (13) noted two classes of IGF-I binding sites in the cell line NCI-H345. This discrepancy probably relates to differences between tissue sections and cell lines and to different techniques.

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REFERENCES

1. Reeve, A. E., Eccles, M. R., Wilkins, R. J., Bell, G. I., and Millow, L. J. Expression of insulin-like growth factor-II transcripts in Wilms' tumour. Nature (Lond.), *317:* 258–260, 1985.
2. Sporn, M. B., and Roberts, A. B. Autocrine growth factors and cancer. Nature (Lond.), *313:* 745–747, 1985.
3. Tricoli, J. V., Rall, L. B., Karakousis, C. P., Herrera, L., Petrelli, N. J., Bell, G. I., and Shows, T. B. Enhanced levels of insulin-like growth factor messenger RNA in human colon carcinoma and liposarcoma. Cancer Res., *46:* 6169–6173, 1986.
4. Minuto, F., Monte, P. D., Barreca, A., Alama, A., Cariola, G., and Giordano, G. Evidence for autocrine mitogenic stimulation by somatomedin/insulin-like growth factor 1 on an established human lung cancer cell line. Cancer Res., *48:* 3716–3719, 1988.
5. Söderdahl, G., Betsholtz, C., Johansson, A., Nilsson, K., and Bergh, J. Differential expression of platelet-derived growth factor and transforming growth factor genes in small- and non-small-cell human lung carcinoma lines. Int. J. Cancer, *41:* 636–641, 1988.
6. D'Ercole, A. J., Stiles, A. D., and Underwood, L. E. Tissue concentration of somatomedin C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. Proc. Natl. Acad. Sci. USA, *81:* 935–939, 1984.
7. Svoboda, M. E., Van Wyk, J. J., Klapper, D. G., Fellows, R. E., Grissom, F. E., and Schlueter, R. J. Purification of somatomedin-C from human plasma: chemical and biological properties, partial sequence analysis, and relationship to other somatomedins. Biochemistry, *19:* 790–797, 1980.
8. Rechler, M. M., and Nissley, S. P. The nature and regulation of the receptors for insulin-like growth factors. Annu. Rev. Physiol., *47:* 425–442, 1985.
9. Massague, J., and Czech, M. P. The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. J. Biol. Chem., *257:* 5038–5045, 1981.
10. The Japan Lung Cancer Society. General rule for clinical and pathological record of lung cancer. Tokyo: Kanehara Publishing Co., 1987.
11. Minuto, F., Monte, P. D., Barreca, A., Fortini, P., Cariola, G., Catrambone, G., and Giordano, G. Evidence for an increased somatomedin/insulin-like growth factor 1 content in primary human lung tumors. Cancer Res., *46:* 985–988, 1986.
12. Jaques, G., Rotsch, M., Wegmann, C., Worsch, U., Maasberg, M., and Havemann, K. Production of immunoreactive insulin-like growth factor 1 and response to exogenous IGF-1 in small cell lung cancer cell lines. Exp. Cell Res., *176:* 336–343, 1988.
13. Nakanishi, Y., Mulshine, J. L., Kasprzyk, P. G., Natale, R. B., Maneckjee, R., Avis, I., Treston, A. M., Gazclar, A. F., Minna, J. D., and Cuttita, F. Insulin-like growth factor 1 can mediate autocrine proliferation of human small cell lung cancer cell lines *in vitro*. J. Clin. Invest., *82:* 354–359, 1988.
14. Sporn, M. B., and Todaro, G. J. Autocrine secretion and malignant transformation of cells. N. Engl. J. Med., *303:* 878–880, 1980.
15. Israel, A., Correa, F. M. A., Niwa, M., and Saavedra, J. M. Quantitative determination of angiotensin II binding sites in rat brain and pituitary gland by autoradiography. Brain Res., *322:* 341–345, 1984.
16. Shigematsu, K., Niwa, M., Shimomura, C., Ohta, H., Tsuchiyama, H., and Ozaki, M. Autoradiographic evidence of angiotensin II binding sites in the human adrenal gland. Biomed. Res., *9:* 27–31, 1988.
17. Goodyer, C. G., De Stephano, L., Lai, W. H., Guyda, H. J., and Posner, B. I. Characterization of insulin-like growth factor receptors in rat anterior pituitary, hypothalamus, and brain. Endocrinology, *114:* 1187–1195, 1984.
18. Munson, P. J., and Rodbard, D. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem., *107:* 220–239, 1980.
19. Byrd, R. B., Miller, W. E., Carr, D. T., Payne, W. S., and Woolner, L. B. The roentgenographic appearance of large cell carcinoma of the bronchus. Mayo Clin. Proc., *43:* 333–336, 1968.
20. Davoren, J. B., Kasson, B. G., Li, C. H., and Hsueh, A. J. W. Specific insulin-like growth factor (IGF) I- and II binding sites on rat granulosa cells: relation to IGF action. Endocrinology, *119:* 2155–2162, 1986.
21. Lin, T., Haskell, J., Vinson, N., and Terracio, L. Characterization of insulin and insulin-like growth factor I receptor of purified Leydig cells and their role in steroidogenesis in primary culture: a comparative study. Endocrinology, *119:* 1641–1647, 1986.