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Insulin-like Growth Factor 1 Receptors Are Increased in Estrogen-induced Kidney Tumors[1]

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ABSTRACT

We have previously demonstrated that membrane receptor protein tyrosine kinase(s) activities are higher in estrogen-induced kidney tumors in comparison with such activities in the normal kidney. In the present work we have investigated the growth factor binding sites in estrogen-induced kidney tumor and in normal kidney membranes in an attempt to understand the mechanism of activation of membrane protein tyrosine kinase(s) and their possible relationship to the induction of estrogen-induced tumors. The characteristics of the normal hamster kidney membrane insulin-like growth factor 1 (IGF-1) receptor are similar to those reported for kidney and extrarenal tissues of other rodents. The binding of ^{125}I-IGF-1 to the normal kidney or tumor membranes was saturable and dependent on time, protein, pH, and temperature. The binding of ^{125}I-IGF-1 to the tumor membranes was significantly higher when compared to the binding activity of the membranes obtained from age-matched normal kidney. The Scatchard analysis of the binding data of both tumor and normal kidney revealed a single class binding site for IGF-1 with K_d of 1.7 and 1.8 nM and maximum binding capacities of 4150 and 2050 fmol/mg protein, respectively. Therefore, the difference observed in ^{125}I-IGF-1 binding between tumor and normal kidney membranes was due to an increase in the number of IGF-1 binding sites with no change in the affinity of receptors for IGF-1. An enhanced level of IGF-1 receptors in tumor membranes also was visualized by autoradiography following affinity labeling of membrane proteins subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Under reducing conditions of electrophoresis, two molecular bands of M_r 240,000 and M_r 130,000 were evident. The M_r 130,000 band represents the α subunit of IGF-1 receptors, and the M_r 240,000 band may represent the aggregates of the receptor subunits which were not reduced completely. IGF-1 stimulated normal kidney or tumor membrane protein tyrosine kinase(s) (wheat germ lectin agarose-purified membrane proteins) in a dose-dependent fashion. Therefore, the alteration of IGF-1 binding activity of the tumor membrane receptors and stimulation of IGF-1-mediated membrane protein tyrosine kinase activity in tumor tissues suggest that events coupled to this membrane receptor may play a role in estrogen stimulation of renal carcinoma.

INTRODUCTION

Estrogens are carcinogenic to both animals and humans. In addition to their carcinogenic actions, estrogens are known to control the growth of normal and neoplastic cells (1, 2). The mechanisms of estrogen-induced carcinogenesis or estrogen-regulated growth of normal and neoplastic cells are not clear. Studies using a variety of estrogen-dependent breast or pituitary normal and cancer cell lines have shown that estrogen controls the cell growth by regulating the expression of growth factors and/or their receptors (3). Among the growth factors known to elicit mitogenic responses in estrogen-dependent cell lines (IGF-1,[3] EGF, and basic fibroblast growth factor), the IGF-1 has been shown to elicit strongest growth stimulatory effects *in vitro* and *in vivo* in a variety of normal and neoplastic cells (4–8). The IGF-1 or somatomedin C is growth hormone-dependent polypeptide growth factor with insulin-like activity. The mitogenic or transforming activity of IGF-1 is mediated through binding to its specific receptor on the cell surface. The IGF-1 receptor is a glycoprotein that consists of α and β subunits. The extracellular α subunit contains the receptor binding domain. The transmembrane spanning β subunit contains tyrosine kinase activity which mediates the growth promoting actions of type I IGF receptor. An immediate response of ligand binding to IGF-1 receptors is the autophosphorylation of its own receptor and an increase in the property of tyrosine kinase activity. The phosphorylated site(s) and tyrosine kinase domain of the IGF-1 receptor reside in the same polypeptide chain of the receptors (6). Most studies related to the evaluation of mitogenic properties of IGF-1 in cancer cells have utilized an *in vitro* system (3–5). Less information is available about the possible role of IGF-1 and -2 or of IGF-1 and -2 receptors in transition from normal to malignant cells and in growth of neoplastic cells *in vivo*.

One of the organs widely used to study the relationship between the level of IGF or their receptors and growth responses after acute treatment with physiological doses of estrogen is uterine tissues (7, 8). However, no *in vivo* data are available relative to type 1 or 2 IGF receptors after chronic treatment with carcinogenic doses of estrogen or in estrogen-induced tumors in animals. In this study we have identified and characterized the IGF-1 receptors in normal and in neoplastic hamster kidney tissues. We show in this study that enhanced levels of IGF-1 receptors are present in estrogen-induced kidney tumors in comparison with levels found in age-matched normal hamster kidney.

MATERIALS AND METHODS

Chemicals. Diethylstilbestrol and PGT were purchased from Sigma Chemical Co., St. Louis, MO. Insulin, gastrin, EGF, TGF-β, and IGF were purchased from Bachem, Inc., Torrence, CA. VIP was purchased from Peninsula Laboratory, Belmont, CA. ^{125}I-IGF-1 (2000 Ci/mmol) was purchased from Amersham, Arlington Heights, IL, and WGA was purchased from Pharmacia. All other chemicals were the highest grade commercially available.

Tumor Induction by Estrogen Treatment to Hamsters. Male Syrian hamsters (6–8 weeks old) were purchased from Sasco. Laboratory chow and water were provided *ad libitum*. Hamsters were treated with one s.c. implant of diethylstilbestrol (25 mg containing 10% cholesterol) for 8 months (9). The hamsters which did not receive any treatment were considered controls and were kept for the same period of time under similar conditions. After 8 months of treatment the hamsters were killed, and the tumors in kidney were scored by gross visual examination. Kidney from treated hamsters were separated into tumor and tumor free tissue (surrounding to the tumor). Some of the kidney tumors were fixed in 10% buffered formalin and histologically evaluated using eosin-hematoxylin stain.

Preparation of Crude Plasma Membranes. Plasma membranes were prepared from age-matched normal kidney, tumor surrounding tissues, and tumors as described previously (10) with some modifications. The tissues were washed and homogenized in buffer containing 20 mM HEPES (pH 7.5), 2 mM PMSF, 0.1% soybean trypsin inhibitor, 100 units/ml trasylol, and 0.1% bacitrasin. The

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[3] The abbreviations used are: IGF, insulin-like growth factor(s); SDS, sodium dodecyl sulfate; TGF, transforming growth factor; VIP, vasoactive intestinal peptide; WGA, agarose wheat germ lectin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; EGF, epidermal growth factor; PGT, poly(Glu, Na-Tyr), 4:1; EC_{50}, the concentration of the unlabeled ligand (IGF-1) required to produce 50% competitive displacement of ^{125}I-IGF-1 binding to membrane.

homogenate was centrifuged at 40,000 × *g* for 45 min at 4°C. The pellet was washed once in the same buffer and finally suspended in homogenizing buffer which contained only 0.1 mM PMSF instead of 2 mM. The small aliquots of membrane suspensions were stored at −70°C for further analysis.

Assay of ^{125}I-IGF-1 Binding to Membrane Proteins. Aliquots of membranes were incubated with 0.01 nM ^{125}I-IGF-1, in the absence or presence of increasing concentrations of IGF-1 for 2 h (except time course binding, 0–4 h) at 22°C, in binding buffer (5 mM $MgCl_2$, 25 mM HEPES (pH 7.5), 0.1 mM PMSF, 0.1% soybean trypsin inhibitor, 100 units/ml trasylol, 0.1% bacitracin, 0.1% BSA). At the end of incubation, the membrane pellets were washed with ice-cold buffer and counted for ^{125}I radioactivity in a gamma counter. The binding affinity and the total binding capacity for IGF-1 to bind to membrane proteins were determined by Scatchard analysis. The specificity of the binding of IGF-1 was determined from inhibition of specific binding by various peptides IGF-1, IGF-2, insulin, EGF, TGF-β, and VIP as described previously (11).

Affinity Labeling of IGF-1 Receptors. For the affinity labeling experiments, we carried out binding of IGF-1 to membranes as described above, washed with ice-cold buffer devoid of BSA, and subjected to cross-linking in the presence of 1 mM disuccinimidyl suberate for 30 min at room temperature. The cross-linking agent, disuccinimidyl suberate, was dissolved in dimethylsulfoxide immediately prior to use and added to the membrane suspension at room temperature to give a final concentration of 1% dimethylsulfoxide in the binding buffer. The reaction was terminated by rapid centrifugation and washing of the membranes at 4°C with excess binding buffer. The washed membrane pellets were solubilized in 0.05 ml of electrophoresis sample buffer containing 6.25 mM Tris-HCl, 10% glycerol, 0.2% SDS, and 0.001% bromophenol blue, in the absence or presence of 0.5% mercaptoethanol. Samples were immediately heated at 100°C for 5 min and analyzed by one-dimensional gel electrophoresis on 5% acrylamide in the separating gel and 4% in the stacking gel with 0.1% SDS, per the method of Laemmli (12). At the end of the electrophoresis, the gels were stained, destained, dried, and exposed to x-ray films (Kodak X-Omat™AR).

Partial Purification of IGF-1 Receptors and Determination of IGF-1-stimulated Tyrosine Kinase Activities Using Exogenous Substrate PGT. Membrane proteins were solubilized in 1% Triton X-100 solution containing 50 mM HEPES (pH 7.5), 10 mM sodium-pyrophosphate, 10 mM NaF, 4 mM EDTA, 2 mM sodium-orthovanadate, 2 mM PMSF, and 100 units of trasylol (13). The solubilization was carried out at 4°C for 1 h. The supernatant was diluted with the solubilization buffer without Triton X-100 to get the final Triton X-100 concentration to 0.1%. The diluted samples were incubated with agarose WGA and shaken at 4°C for an additional hour. Agarose-WGA was then packed in a small column and washed 2–3 times with solubilization buffer containing 0.1% Triton X-100. Glycoproteins containing IGF-1 receptors were eluted by 0.3 M sodium-acetylglucosamine prepared in the washing buffer.

Protein tyrosine kinase activity in the WGA-purified proteins was assayed at room temperature using PGT, a random polymer (average M_r 43,000), as a phosphorylatable substrate (14). The phosphorylation reaction mixture consisted of 15 μg WGA-purified proteins, 20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 100 μM Na_3VO_4, 0.1 mM dithiothreitol, 2 mM PMSF, 1 mg/ml BSA, and 0.1% Nonidet P-40, in the presence or absence of various concentrations of IGF-1. The reaction mixture was preincubated for 6 min with 50 μM [^{32}P]ATP (0.5 μCi). The reaction was started by addition of PGT, incubated for an additional 20 min, and stopped by direct application on P-81 filters. The filters were washed three times with 0.5% phosphoric acid and finally with acetone to remove the unincorporated [^{32}P]ATP. Filters were dried for 5 min at 80°C and counted for radioactivity in a scintillation counter.

RESULTS

Characterization of ^{125}I-IGF-1 Binding to Normal Kidney Membranes. Prior to the detailed analysis of ^{125}I-IGF-1 binding to normal kidney and kidney tumor membranes, the optimal conditions for binding of ^{125}I-IGF-1 were established through use of normal kidney membranes. The data presented in Fig. 1 show that the binding of ^{125}I-IGF-1 to renal membranes was time and temperature dependent. Incubation at 22°C resulted in higher binding than that observed at 37°C. Maximum binding was observed at 22°C between 2 to 4 h. The binding was also pH dependent. The optimum pH for ^{125}I-IGF-1 binding to membranes was observed at 7.5 (data not shown). Therefore, all subsequent binding experiments were performed at 22°C for 2 h at pH 7.5. Membrane proteins equivalent to 250 μg/tube were used in each assay, which was found to give the maximum ^{125}I-IGF-1 bindings (data not shown).

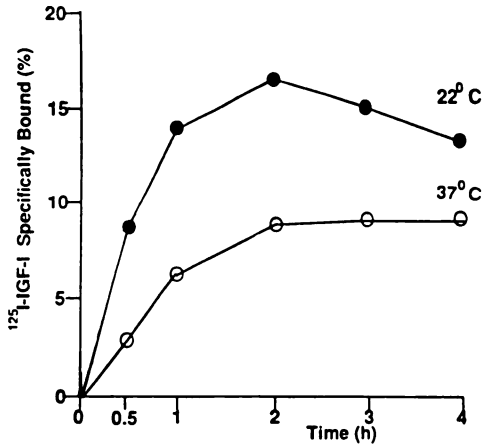

Fig. 1. Time- and temperature-dependent specific binding of ^{125}I-IGF-1 to normal kidney membranes. Values represent the percentage mean of two experiments.

Specific ^{125}I-IGF-1 Binding to Normal and Tumor Kidney Membranes. The binding specificity of ^{125}I-IGF-1 to normal and neoplastic kidney membranes is shown in Fig. 2. The binding of ^{125}I-IGF-1 to both normal kidney and tumor membranes was inhibited by unlabeled IGF-1 in a concentration-dependent fashion (1–100 ng/250 μl). The closely related peptides, IGF-2 and insulin, demonstrated a lower binding affinity for the IGF-1 binding sites. IGF-1 competed in low concentrations (0–5 ng/tube) with ^{125}I-IGF-1 binding by more than 70%, whereas displacement with IGF-2 and insulin was less effective at the same concentration. IGF-2 (1–250 ng/250 μl) displaced ^{125}I-IGF-1 binding, but it was less efficient than IGF-1 (Fig. 2). Insulin (1–1000 ng/250 μl) also competed with ^{125}I-IGF-1 but required about 100-fold-higher concentration to cause 40% inhibition, which is in agreement with previous reports (7, 11, 15). All other unrelated peptides (EGF as shown in Fig. 2; TGF-β, VIP, gastrin data not shown) did not compete for the normal kidney or tumor membrane ^{125}I-IGF-1 binding sites at 100 ng/250 μl. The relative affinity of IGF-1 receptor for IGF-1, IGF-2, and insulin exhibited typical type 1 binding (IGF-1 > IGF-2 > insulin). The specific displacement of ^{125}I-IGF-1 binding by IGF-1 peptide on kidney membranes indicates that the binding sites being measured are present on IGF-1 receptor-like proteins and not on the nonspecific binding proteins. Under optimal conditions, IGF-1 binding to the membranes from both normal kidney and tumor was analyzed. It was found that tumor membranes exhibited the highest binding activity for IGF-1, followed by normal kidney membranes.

Scatchard analysis was performed to further ensure that the observed higher binding activity for ^{125}I-IGF-1 in tumor membranes compared to that for the control kidney membranes represents the increase in number of binding sites *per se*. A log-dose dependent ^{125}I-IGF-1 binding displacement curve on normal kidney and tumor membranes is presented in Fig. 3. IGF-1 displaced the ^{125}I-IGF-1 binding efficiently in both normal kidney and tumor membranes; however, the saturation of the binding sites was reached earlier in normal kidney membranes than in the tumor membranes. The EC_{50} of ^{125}I-IGF-1 binding to normal kidney and tumor membranes was calculated to be 24 and 45 nM, respectively (Fig. 3 and Table 1). This gives the further indication that the population of IGF-1 receptors in tumor membranes is greater than in the normal kidney membranes. To determine K_d and binding capacity of the ^{125}I-IGF-1 binding on these membranes, the specific binding data from Fig. 3 were transformed into a Scatchard plot (Fig. 4). The result obtained showed a linear plot

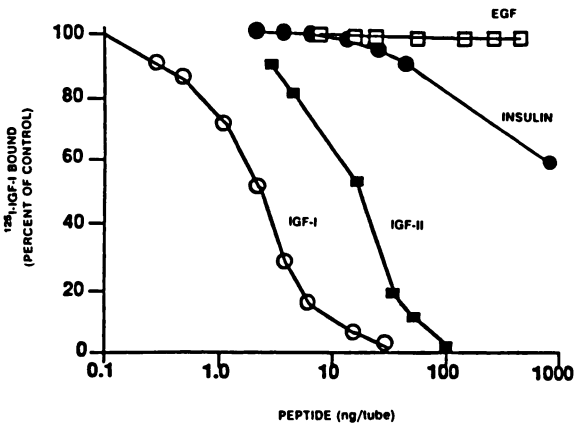

Fig. 2. Displacement of ^{125}I-IGF-1 binding to normal kidney membranes by IGF-1, IGF-2, insulin, and EGF. Membranes were incubated with ^{125}I-IGF-1 with or without the indicated concentrations of IGF-1 or related peptides at 22°C for 2 h. *Points*, means of triplicate measurements.

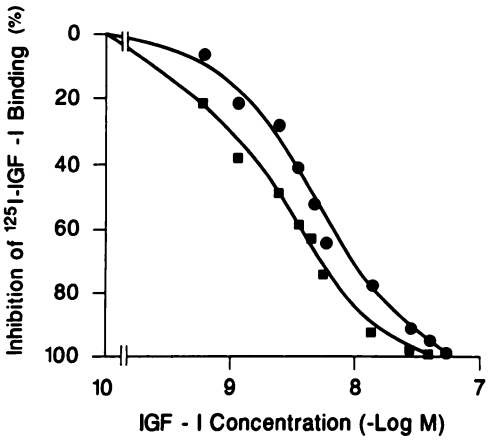

Fig. 3. Log-dose percentage inhibition of specific binding of ^{125}I-IGF-1 to membranes of normal age-matched kidney (■) and tumor (●) by increasing concentrations of IGF-1. *Points*, means of 2–3 experiments.

Table 1 *Comparison of ^{125}I-IGF-1 binding characteristics to normal and kidney tumor membrane*[a]

Parameters	Normal	Tumor
EC_{50} (nM)	24	45
K_d (nM)	1.8	1.7
Maximum binding capacity (fmol/mg protein)	2050	4150

[a] Each value is the mean of 3–4 experiments.

in both normal kidney and tumor membranes, suggesting a single class of high-affinity binding sites of IGF-1 on these membranes. The binding capacity of normal kidney and tumor membranes was 2050 and 4150 fmol/mg protein, respectively, and K_d was 1.8 and 1.7 nM, respectively (Table 1).

In order to further substantiate this increase in IGF-1 binding sites, we performed affinity-labeling studies. These results are shown in Fig. 5. For affinity labeling, membranes were incubated with ^{125}I-IGF-1, briefly washed by centrifugation to remove unbound ^{125}I-IGF-1, and cross-linked with 1 mM disuccinimidyl suberate. The cross-linked membranes were subjected to SDS-polyacrylamide gel electrophoresis; labeled material was detected by autoradiography. As seen in Fig. 5 major (M_r 240,000) and minor (M_r 130,000) species of cross-linked ^{125}I-IGF-1 are present, and tumor membranes exhibited higher levels of both species. The autoradiogram of the gels run under nonreducing conditions showed a single band of M_r 300,000, suggesting that protein bound to ^{125}I-IGF-1 is dimer-linked to a disulfide bond (data not shown), since under reducing conditions two bands of M_r 240,000 and M_r 130,000 were observed. It is important to note that both M_r 240,000 and M_r 130,000 ^{125}I-IGF-1-bound receptor proteins from normal kidney and tumors was displaced in a dose-dependent manner by the increase in concentrations of IGF-1 (Fig. 5). The concentrations of insulin equivalent to low concentrations of IGF-1 used for cross-linking experiment did not inhibit the labeling of ^{125}I-IGF-1 to M_r 240,000 protein band (data not shown). The M_r 130,000 band represents the α subunit of IGF-1 receptors (7, 11, 15). The M_r 240,000 band may represent aggregates of subunits of IGF-1 receptors that were not completely separated during reduction as suggested by some investigators (7, 11, 15), unlike the type 2 receptors.

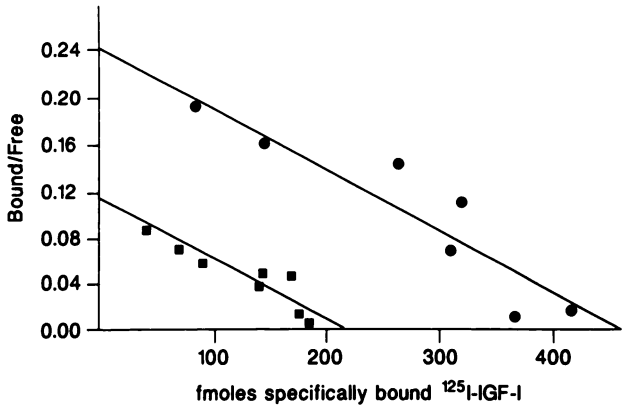

Fig. 4. Comparison of IGF-1 specific binding activity between normal kidney (■) and kidney tumor (●). Binding assays were performed with increasing concentrations of IGF-1 and the data were transformed into Scatchard plots.

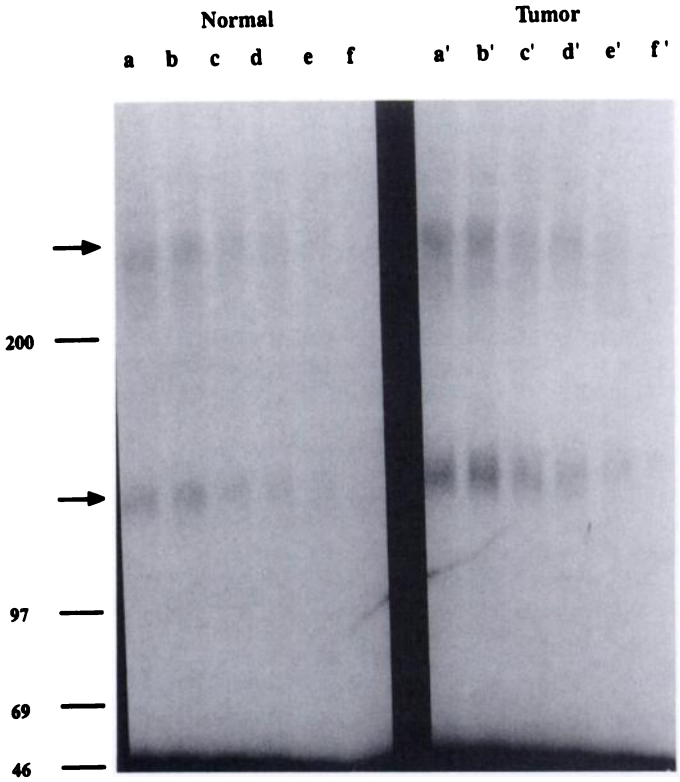

Fig. 5. Autoradiograph of affinity labeling of ^{125}I-IGF-1 to IGF-1 receptors from normal kidney (*a-f*) and tumor membranes (a'-f'). Membranes were incubated with ^{125}I-IGF-1 with or without increasing concentrations of unlabeled IGF-1 (*a, a'*, 0 nM; *b, b'*, 1 nM; *c, c'*, 10 nM; *d, d'*, 20 nM; *e, e'*, 30 nM; and *f, f'*, 50 nM) and cross-linked with disuccinimidyl suberate as described in "Materials and Methods." *Solid line*, position of standard molecular weight marker proteins. *Arrow*, molecular weight profiles of IGF-1 receptor proteins under reducing condition.

Table 2 *Stimulation of the activity of WGA-purified normal and estrogen-induced kidney tumor membrane protein tyrosine kinase(s) by IGF-I*

Protein tyrosine kinase activity was assayed using exogenous substrate as described in "Materials and Methods" (14). Each value is the mean of 3–4 experiments. The values in parentheses represent the specific activity of protein tyrosine kinase (pmol/mg protein/min).

Effector	% of control activity	
	Normal	Tumor
IGF (nM)		
0	100 (38 ± 5)	100 (96 ± 12)
1	121	148
10	142	220
100	158	280

Since tyrosine kinase mediated phosphorylation of cellular proteins by IGF-I receptor is a known phenomenon in signal transduction mechanisms, the membrane protein tyrosine kinase activity was measured. For these studies, membranes from normal kidney and tumors were prepared. The IGF-I receptor was solubilized by detergent treatment and partially purified by WGA column. Stimulation of membrane protein tyrosine kinase activity by IGF-I was then determined using PGT as an exogenous substrate. As shown in Table 2, tumor membranes had twice the capacity to phosphorylate PGT compared to age-matched control hamster kidney membranes. IGF-I stimulated membrane protein tyrosine kinase catalyzed phosphorylation of PGT was in a dose-dependent fashion.

DISCUSSION

In the present study we have demonstrated that hamster kidney tissues, both normal and neoplastic, have IGF-I receptors. The characteristics of the normal hamster kidney membrane IGF-I receptors are similar to those reported for kidney and extrarenal tissues of other rodents (7, 15). The binding of ^{125}I-IGF-I to the tumor membranes was significantly higher when compared to the binding activity of the membranes obtained from age-matched normal kidney. Both Scatchard analysis and visualization of receptors by autoradiography after affinity labeling of membrane proteins subjected to SDS-polyacrylamide gel electrophoresis revealed that an enhanced level of IGF-I receptors in tumor membranes was present, compared to the level observed in age-matched control kidney.

IGF-I and -II are known to produce mitogenic effects in both normal and neoplastic tissues (3–5, 7, 8, 16, 17). They are known to exert mitogenic effects through binding to their receptors. It is known that acute treatment of rats with estrogen up-regulates the IGF-I receptors in the uterus (7). Higher levels of IGF-I receptors in tumor tissues from estrogen-dependent target organs such as breast and uterus, compared to those of normal tissues, have been recently reported (11, 18). These studies have suggested that enhanced expression of IGF-I receptors may be involved in the growth of these human cancers. Thus, enhanced levels of IGF-I binding activity and stimulation of IGF-I- mediated membrane protein tyrosine kinase activity in tumor tissues observed in the present study suggest that events coupled to this membrane receptor may have played a role in estrogen stimulation of renal carcinoma.

Alternatively, the increased expression of IGF-I receptors of kidney tumor membranes may not play a causative role in tumorigenesis. It is known that hamster kidney requires estrogen for continued growth (1). IGF-I via its receptor also is known to play a role in the growth of estrogen-dependent tumor cells. Thus, the enhanced levels of IGF-I receptors in tumor membranes may simply be required for the stimulation of tumor growth by estrogen but may not be a primary carcinogenic event(s) produced by the estrogen. Studies in our laboratory are in progress to ascertain the role of IGF-I and IGF-I receptors in initiation and promotion of hormone-induced carcinogenesis.

Our findings are significant because the results demonstrate for the first time that the expression of IGF-I receptors is elevated in estrogen-induced kidney tumors, one of the major animal models of estrogen-induced carcinogenesis. Consequently, our results may provide specific markers for evaluating the similarities and differences of estrogen-induced hamster tumors with estrogen exposure-associated human and other animal cancers on the basis of expression of IGF-I receptors. However, further studies are needed to elucidate the relationship between the molecular basis of the activation of IGF-I receptor intrinsic protein tyrosine kinase, the expression of IGF-I receptors, and hormone-induced renal carcinogenesis.

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