Urol Res (1993) 21:27–32

Urological Research

Characterization of insulin-like growth factor I binding sites in human bladder cancer cell lines

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Received: 24 May 1992 / Accepted: 30 July 1992

Summary. The role of insulin-like growth factor I (IGF-I) in the growth and development of bladder cancer cells was investigated using cultured human cell lines representing differentiated (RT-4, 5637) or undifferentiated (T-24, J-82, TCC-SUP) transitional cell carcinoma (TCC). In the presence of 2% serum, IGF-I significantly stimulated the growth of all cell lines. The proliferation of T-24, 5637, and RT-4 cells was more sensitive to IGF-I than that of J-82 and TCC-SUP cells. [^{125}I]IGF-I binding to 5637 and J-82 cells was significantly higher than that to T-24 and TCC-SUP cells ($P < 0.001$). RT-4 cells possessed the lowest binding capacity among the cell lines tested. Scatchard analysis of [^{125}I]IGF-I binding to four of the five cell lines indicated a single binding site for IGF-I, with apparent dissociation constants (K_d) of 1.27, 1.18, 1.34, and 1.39 nmol/l for TCC-SUP, J-82, 5637, and T-24, respectively. Therefore, the difference observed in [^{125}I]IGF-I binding among the bladder cancer cell lines was attributed to the difference of IGF-I binding sites and not to a change in receptor binding affinity. Cross-linking studies supported the suggestion that [^{125}I]IGF-I was bound to a receptor on these cells. The results indicate that cultured human bladder cancer cells contain functional IGF-I receptors. A differentiated cell line, RT-4, possesses significantly fewer IGF-I receptors than other cell lines. This suggests that the overexpression of IGF-I receptor may reflect the malignant potential of bladder cancer cells.

Key words: Human bladder cancer – IGF-I receptor

The importance of polypeptide growth factors in the molecular mechanism of cell transformation and tumor cell proliferation has been demonstrated [11, 12]. Since epidermal growth factor (EGF) is excreted in urine in very high concentrations in biologically active form, the role of EGF in the growth of normal and malignant urothelium has been well studied [1, 19, 23, 24]. EGF stimulates the proliferation of bladder cancer cells in vitro [19]. The invasive bladder cancers have significantly higher EGF receptor levels than the noninvasive cancers [23]. Moreover, death of bladder cancer is associated with EGF receptor expression [24].

IGF-I, also termed somatomedin C, is a polypeptide growth promoter which has amino acid sequence and functional homology to insulin [10]. IGF-I is synthesized in many tissues and stimulates the growth of diverse cell types [7, 10, 18, 21]. It has been suggested that besides mediating the effects of growth hormone in an endocrine fashion, this factor can act via autocrine and/or paracrine pathways [6, 7, 8]. The mitogenic activity of IGF-I is known to be mediated by its receptor, which is a tetrameric membrane glycoprotein consisting of 2 α-chains of molecular weight 130 kDa and two β-chains of molecular weight 98 kDa each [26].

Evidence has accumulated that IGF-I appears to play an important role in the progression of malignant tumors. Overexpression of IGF-I receptors, and a positive correlation between IGF-I receptor concentration and the malignant potential of a tumor, has been reported in various malignant tumors and cell lines [6, 27, 30, 34]. Furthermore, Reiss et al. [28] demonstrated recently that the expression of the protooncogene c-*myb* causes an increase in both IGF-I and IGF-I receptor mRNA levels in Balb/c3T3 cells.

To our knowledge, no reports demonstrating the influence of IGF-I on urothelial tumors have been published. We therefore investigated whether IGF-I may play a role in the development of bladder cancer by the measurement of IGF-I binding sites using five different human bladder cancer cell lines.

Materials and methods

Growth factors

Recombinant IGF-I and IGF-II were purchased from Bachem (Torrance, Calif.). EGF was obtained from Amgen Biologicals

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Table 1. Effect of IGF-I on TCC Growth

EGF (ng/ml)	Doubling Time (h)				
	J-82	T-24	TCC-SUP	5637	RT-4
0	27.8 ± 2.5[a]	66.9 ± 9.9	28.9 ± 1.0	55.2 ± 4.5	80.9 ± 5.1
5	*24.8 ± 1.9*[b]	*48.4 ± 6.3*	*25.1 ± 0.7*	*36.9 ± 2.1*	*71.9 ± 3.2*
10	24.1 ± 1.2	50.6 ± 5.8	26.4 ± 0.9	36.7 ± 2.0	*60.1 ± 4.2*
50	24.5 ± 1.4	*43.6 ± 4.1*	25.4 ± 0.7	42.7 ± 2.7	57.6 ± 9.0

[a] Mean ± SD
[b] Numbers in *italics*, $P < 0.05$ between italic number and value above

(Thousand Oaks, Calif.). Bovine insulin was obtained from Sigma (St. Louis, Mo.).

Cell culture

Five different human bladder cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). TCC-SUP [22], J-82 [25], and T-24 [4] are undifferentiated transitional cell carcinoma (TCC); 5637 [16] and RT-4 [29] are differentiated TCC. All cancer cell lines were grown on plastic culture dishes containing Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% heat-inactivated fetal calf serum (DMEM/10% HI-FCS). Cells from subconfluent cultures were used for all assays. Routine assays of all cell lines for *Mycoplasma* were negative (Mycotrim-T.C., Hana Media Inc., Berkeley, Calif.).

Cell growth assay

The in vitro growth-stimulating effect of IGF-I on bladder cancer cells was measured calorimetrically using sulforhodamine B (SRB; Sigma). Cells ($0.8–1.0 \times 10^4$) suspended in 600 µl DMEM/10% HI-FCS per well were plated in triplicate in 24-well plates (Corning, NY) and allowed to adhere overnight. The following day culture medium was replaced with 600 µl/well of DMEM/2% HI-FCS supplemented with penicillin, streptomycin, and gentamicin (Gibco, Grand Island, NY), and cells were incubated for an additional 48 h. Culture supernatant was replaced with fresh medium with various concentrations (0–50 ng/ml) of IGF-I (day 0) and cells were cultured for 0 or 72 h. At the end of IGF-I exposure, culture medium was removed and cells were fixed with ice-cold 10% trichloroacetic acid (TCA). To minimize the intra-assay variations, culture plates fixed at 0 h were preserved at 4°C and the following procedure was done with 72 h plates at the same time. Wells were washed five times with distilled water, then 0.5 ml 4% SRB (in 1% acetic acid) was added and incubated for 10 min at room temperature. Wells were rinsed five times with 1% acetic acid and air dried. Dye was solubilized into unbuffered tris base (10 mmol/l) at an appropriate concentration to create an optical density of less than 1.8 and read at a wavelength of 564 nm. Cell doubling time was calculated using the absorbance of dye at 0 and 72 h and the formula

$$\text{Cell doubling time (h)} = \frac{\text{Log } 2}{\text{Log } (A_0/A_{72})} \times 72$$

where A_0 and A_{72} equal the value of absorbance of dye at 0 and 72 h, respectively [17].

Binding assays

[^{125}I]IGF-I (specific activity 2000 Ci/mmol) was obtained from Amersham (Arlington Heights, Il.). Cells were harvested from culture dishes by short-term trypsinization. Between 4×10^4 and 6×10^4 cells in 600 µl/well of DMEM/10% HI-FCS were plated in 24-well plates and incubated for 2 days. Wells were then rinsed with ice-cold phosphate-buffered saline (PBS) and incubated at 4°C for 30 min with serum-free binding buffer (SF-BB) consisting of DMEM, 25 mmol/l HEPES and 0.1% fatty acid free bovine serum albumin (Sigma). Medium was replaced with 0.3 ml SF-BB containing various concentrations of radioligand (0.037–2.4 nmol/l) in quadruplicate. Following incubation for 5 h at 4°C, SF-BB was removed and wells were gently washed three times with ice-cold PBS. Finally, cells were extracted in 0.3 N sodium hydroxide and the amount of cell-bound label was measured in a gamma counter (Gamma 5500, Beckman). Specific binding (SB) was determined by subtracting nonspecific binding (excess unlabeled IGF-I, NSB) from total binding (TB). For the competition assay, cells were incubated with radioligand (1 nmol/l, 50000 cpm) and a graded amount of unlabeled hormones. Because some cell lines (TCC-SUP and J-82) easily detach from culture wells during a 5-h incubation at 4°C, cells for counting were plated into additional wells and cell counts were performed by the trypan blue exclusion method following the same treatments with the exception of addition of hot or cold ligand. The binding activity was expressed as the amount of [^{125}I]IGF-I IGF-I (fmol) bound to a million cells.

Affinity cross-linking studies of [^{125}I]IGF-I to bladder cancer cells

Bladder cancer cells (J-82) were seeded onto 6-well plates (Corning, NY) with DMEM/10% HI-FCS and incubated until subconfluent. Culture supernatant was replaced with SF-BB containing [^{125}I]IGF-I (1 nmol/l, 200000 cpm) with or without unlabeled peptides and incubated for 5 h at 4°C. After rinsing with cold PBS, cells were incubated for 30 min at 4°C with 1 mmol/l disuccinimidyl suberate (DSS; Pierce, Rockford, Il.) dissolved in SF-BB. Once the supernatant was aspirated, the cells were scraped with a rubber policeman in the presence of EDTA buffer (10 mmol/l in PBS) and transferred to microfuge tubes. Cells were washed with EDTA buffer and the resultant pellets were boiled for 5 min in 100 µl SDS sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromphenol blue). Electrophoresis was performed by SDS-PAGE with a 7.5% running gel and a 4% stacking gel [14]. Autoradiograms were made by exposing Kodak XRP-5 film (Rochester, NY) to the dried gel for 14 days at −80°C.

Statistical analysis

Data were compared using Student's *t*-test. Competitive binding data were fitted to a linear regression equation analysis. The difference was reported to be significant when $P < 0.05$.

Results

The mitogenic effect of IGF-I on human bladder cancer cell lines is summarized in Table 1. IGF-I stimulated the proliferation of all cell lines tested. Growth of J-82, TCC-SUP, and 5637 cells was fully stimulated by the lowest concentration of IGF-I (5 ng/ml). The response of T-24, 5637, and RT-4 cells to IGF-I appeared to be more sensitive than that of J-82 and TCC-SUP cells.

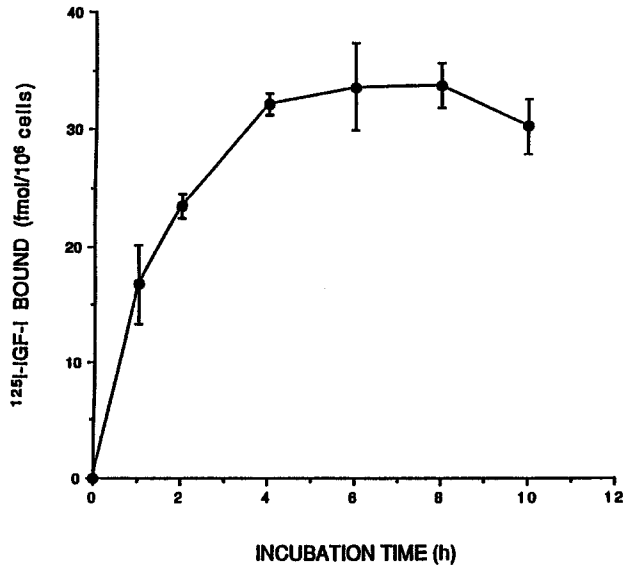

Fig. 1. Time course of [^{125}I]IGF-I specific binding to human bladder cancer cells (5637) at 4°C. For each time point, cells were incubated in quadruplicate with [^{125}I]IGF-I (0.5 nmol/l) in the absence or presence of unlabeled peptide (100 nmol/l). Nonspecific binding in the presence of unlabeled IGF-I was subtracted from total binding to give specific binding. *Points,* mean; *bars,* SD

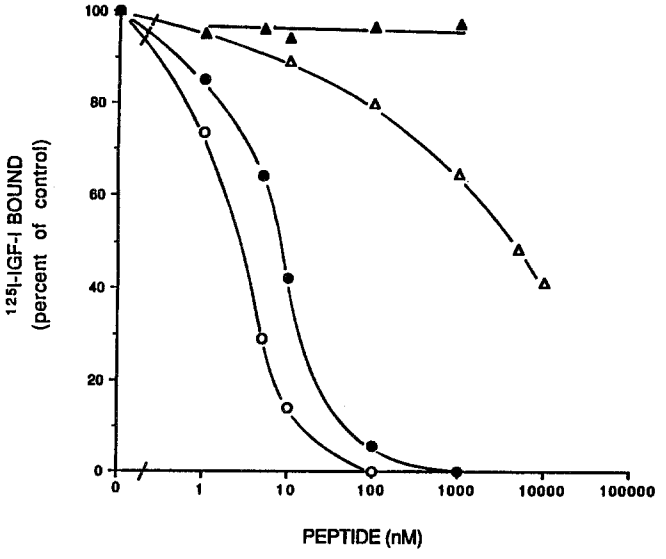

Fig. 2. Displacement of [^{125}I]IGF-I binding to human bladder cancer cells (5637) by IGF-I (○), IGF-II (●), insulin (△), and EGF (▲). Cells were incubated with [^{125}I]IGF-I (1 nmol/l, 50000 cpm) with or without the indicated concentration of peptides at 4°C for 5 h. *Points,* mean of quadruplicate measurements

The data presented in Fig. 1 show that the binding of IGF-I to human bladder cancer cells (5637) was time dependent. Steady-state binding conditions at 4°C were observed between 4 and 8 h. Similar findings were obtained in all of the other cell lines (data not shown). Therefore, a 5 h incubation was employed in subsequent studies to characterize the binding site.

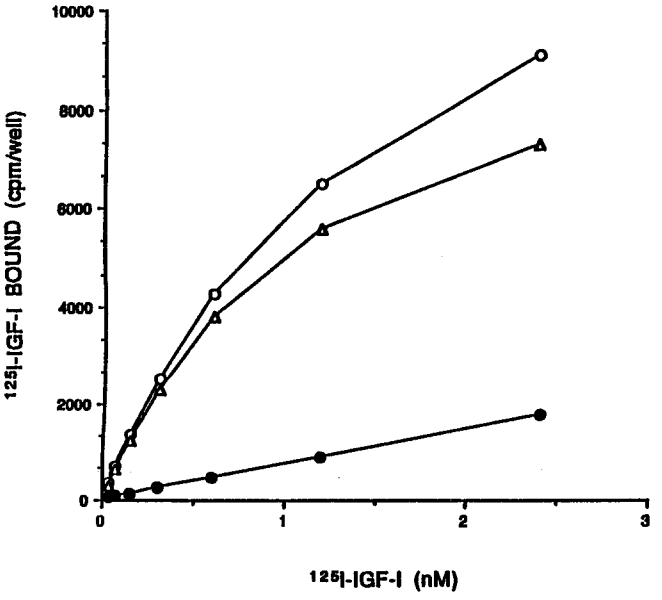

Fig. 3. IGF-I binding to human bladder cancer cells (5637). Cells were incubated with various concentrations of [^{125}I]IGF-I (0.037–2.4 nmol/l) for 5 h at 4°C. To determine nonspecific binding, incubations were performed in the presence or absence of unlabeled IGF-I (100 nmol/l). Specific binding (△) was calculated by subtracting nonspecific binding (●) from total binding (○). *Points,* mean of quadruplicate measurements

Specificity of [^{125}I]IGF-I binding to the cell surface is shown in Fig. 2. Radioligands could be inhibited by unlabeled IGF-I and, at higher concentrations, IGF-II. Insulin (10–10000 nmol/l) competed with [^{125}I]IGF-I, but required much higher concentrations than either IGF-I or IGF-II. EGF failed to compete at any concentrations tested (up to 1000 nmol/l). These observations are consistent with IGF-I receptor specificity for ligands as has been reported for other types of cells.

Figure 3 shows binding of [^{125}I]IGF-I to cells (5637) was dose dependent. Specific binding (SB) approached saturation at approximately 2.5 nmol/l radioligand. Nonspecific binding (NSB) was linear over the range of [^{125}I]IGF-I concentration studied and represented 10% or less of total binding (TB).

IGF-I binding activities of various human bladder cancer cell lines were compared using a single concentration (0.3 nmol/l) of [^{125}I]IGF-I under steady-state conditions (Fig. 4). The highest binding activity (20.2 ± 0.45 fmol/10^6 cells) was seen with 5637 cells, whereas RT-4 showed the lowest activity (0.61 ± 0.23 fmol/10^6 cells) among the cell lines tested. These observations demonstrate significant differences in the IGF-I binding capacity (SB) among various human bladder cancer cells. Such differences could be due to differences in IGF-I receptor number or binding affinity (K_d).

In order to determine whether the difference in IGF-I binding among these cell lines was due to a difference in binding affinity or to a difference in receptor number, binding data were analyzed by the method of Scatchard [31] (Fig. 5). SB to RT-4 cells was too low to allow

Table 2. Relationship between IGF-I receptor status and histological grade of original tumors

Cell lines	Grade[a]	IGF-I binding sites	
		Affinity (nM)	Receptor number (site/cell)
RT-4	I	N.D.[b]	N.D.[b]
5637	II	1.34	6.92×10^4
T-24	III	1.39	3.34×10^4
J-82	III	1.18	6.02×10^4
TCC-SUP	IV	1.27	2.83×10^4

[a] Histological grade of original tumor from which cell lines tested were derived
[b] Not detected

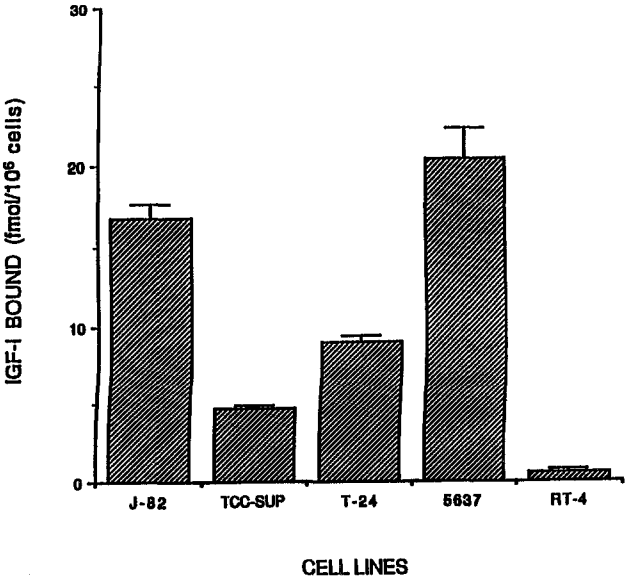

Fig. 4. IGF-I binding to different human bladder cancer cell lines. Cells weere incubated with [^{125}I]IGF-I (0.3 nmol/l) at 4°C for 5 h in the presence or absence of unlabeled peptide (60 nmol/l). The specific binding was determined as described in "Materials and methods". Each *column* represents mean ± SD of quadruplicate measurements. Each of the five cell lines differs significantly from the other cell lines

Scatchard analysis. Analysis of binding data from the other cell lines showed linear plots with similar apparent affinity constants (slope, $1/K_d$), which are summarized in Table 2. Thus, bladder cancer cells express a single class of IGF-I binding sites. The number of receptors on 5637 and J-82 cells was similar and higher than that on TCC-SUP and T-24 cells (Table 2).

To confirm that the IGF-binding, which illustrated the kinetics and specificity typical of the IGF-I receptor, was due to the interaction with membrane receptors, bound [^{125}I]IGF-I was cross-linked with DSS and analyzed by SDS-PAGE under reducing conditions. Affinity-labeled J-82 cells displayed radiolabeled bands corresponding to molecular weights of 130 and 260 when analyzed by

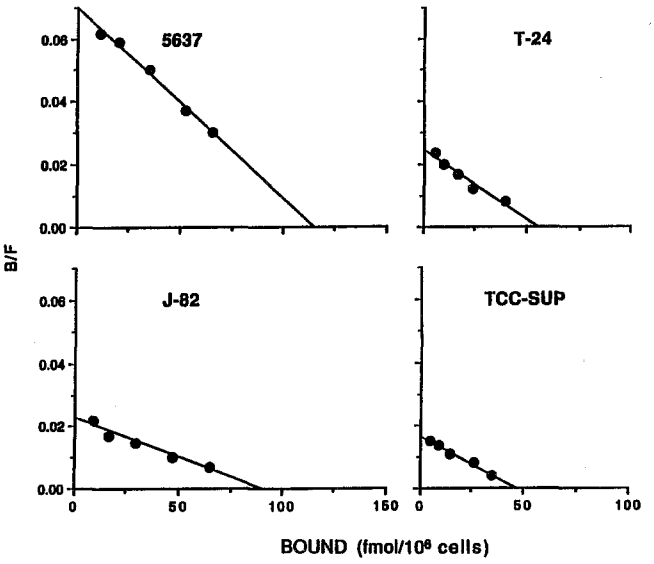

Fig. 5. Comparison of IGF-I binding activity among different human bladder cancer cell lines. Binding assays were performed as described in Fig. 3 using various concentrations of [^{125}I]IGF-I (0.037–2.4 nmol/l) and data were transformed into Scatchard plots. *Points,* mean from three independent experiments

autoradiography (Fig. 6). Formation of these bands was completely blocked by the presence of unlabeled IGF-I (100 ng/ml) and partially displaced by IGF-II (100 ng/ml), but not affected by EGF (100 ng/ml). Insulin (10 µg/ml) and an anti-IGF-I receptor monoclonal antibody, αIR-3 (Oncogene Science, Uniondale, NY), also displaced the bands, but less effectively than IGFs. These findings regarding receptor specificity were consistent with the results obtained from a competitive binding assay using 5637 cells.

Discussion

The action of IGF-I on normal or neoplastic urothelial cells has not been reported, even though this peptide is synthesized in diverse tissues and stimulates the growth of numerous cell types [7, 10, 18, 21]. Our results indicate that human bladder cancer cells possess functional binding sites for IGF-I. IGF-I binding to bladder cancer cells was characterized by competitive binding studies (Fig. 2). [^{125}I]IGF-I binding to cells was inhibited by IGF-I, IGF-II, and insulin, but not by EGF. IGF-II (ED_{50} = 7.92 nmol/l and insulin (ED = 4.16 µmol/l) showed relatively weak competition compared with IGF-I (ED_{50} = 2.35 nmol/l). This rank order of potency (IGF-I > IGF-II >> insulin) is consistent with IGF-I receptor selectivity reported previously for other tissues [27, 30].

Affinity cross-lined [^{125}I]IGF-I displayed, under reducing conditions, radiolabeled bands corresponding to molecular weights of 130 and 260 (Fig. 6). Appearance of this radiolabeled protein was totally blocked by unlabeled IGF-I. Since the 260 kDa band was displaced similarly to the 130 kDa band by αIR-3 (100 ng/ml) we speculate that

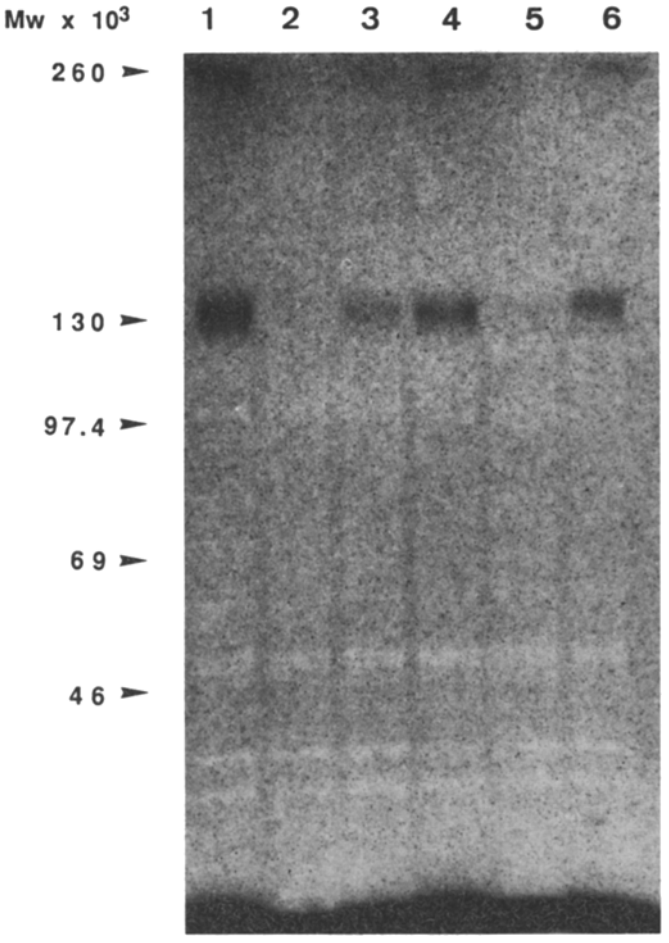

Fig. 6. Autoradiogram of affinity-labeled [^{125}I]IGF-I to bladder cancer cells. Cells (J-82) were incubated with [^{125}I]IGF-I (200000 cpm) in the absence of unlabeled peptide (*lane 1*) and in the presence of IGF-I (100 ng/ml in *lane 2*); anti-IGF-I receptor monoclonal antibody, αIR-3 (100 ng/ml in *lane 3*); EGF (100 ng/ml in *lane 4*); IGF-II (100 ng/ml in *lane 5*); or insulin (1 μg/ml in *lane 6*). [^{125}I]IGF-I was cross-linked to receptor and analyzed by SDS-PAGE under reducing conditions. Autoradiography was performed as described in "Materials and methods"

the 260 kDa band represents a dimer of the α-subunit of IGF-I receptor rather than a 260 kDa monomer of IGF-II receptor [34]. Additionally, present data clearly show binding to a large molecular membrane protein. There was no evidence that [^{125}I]IGF-I bound to small (24–32 kDa) IGF-I binding proteins [5].

Scatchard analysis of the binding data for four cell lines (5637, J-82, T-24, and TCC-SUP) indicated that bladder cancer cells have a single, high-affinity (K_d 1.18–1.39 nmol/l) binding site for IGF-I (Fig. 5). Since the binding affinity of the IGF-I receptor was similar for the various cell lines tested, the observed differences in specific binding reflect variation in receptor numbers.

Levels of IGF-I receptor appear not to correlate directly with the mitogenic effect of IGF-I (Tables 1, 2). For example, despite high IGF-I receptor numbers, the growth of 5637 or J-82 cells was stimulated less by exogenous IGF-I than the growth of TCC-SUP, T-24, or RT-4 cells. However, it may be dangerous to compare the data from the receptor assay and the cell growth assay directly because cells were grown in different concentrations of serum in the two assays (10% in binding assay and 2% in growth assay). It is known that the expression of IGF-I receptors is modulated by various factors in the serum [9, 13]. Therefore, serum concentrations in the culture medium may have a significant impact on the status of IGF-I receptors. Nevertheless, a similar discrepancy between the receptor concentration and response to growth factors was reported in several studies [2, 20]. One likely explanation regarding this discrepancy is that the proliferation of 5637 and J-82 cells may have been activated by certain autocrine growth factor(s) [2]. Thus, exogenous factors might show little additional stimulation in these cell lines.

Overexpression of IGF-I receptors is found in several malignant tumors [6]. In addition, a positive correlation between IGF-I receptor number and malignant potential of the tumor, such as metastatic or invasive potential, has been reported [34]. Using established cell lines, we determined the relationship between IGF-I receptor numbers and histological differentiation of original tumors from which cell lines were derived (Table 2). The results show that the IGF-I receptor numbers appeared not to correlate with histological differentiation of original bladder cancers. The cell line 5637 (grade II) possessed higher numbers of IGF-I receptor than did TCC-SUP (grade IV) and T-24 (grade III). It is important to consider that cultured cells might not completely reflect the malignant characteristics of original tumors, since cell differentiation is easily altered by the in vitro environment [3]. However, it should be noted that RT-4 cells expressed significantly fewer receptors than the other cell lines. RT-4 cells were derived from superficial, low-grade papillary TCC and are known to reflect well the biological characteristics of the original tumor when they are implanted in nude mice [35]. Therefore, it may be possible to speculate that invasive TCC might overexpress IGF-I receptors as compared with normal epithelial cells or low-grade non-invasive TCC.

For malignant cells to be invasive, they must penetrate the basement membrane components and migrate into surrounding tissues. Cellular proteolysis appears to play a central role in the processes of tumor invasion and metastasis. Some growth factors have the ability to stimulate not only cellular proliferation but also the secretion of proteolytic enzymes that can degrade the basement membrane and the stromal components [15]. Simultaneously, malignant cells express increased numbers of cell surface receptors for these growth factors [32]. This may underlie the positive correlation between invasive potential and number of growth factor receptors. However, effects of IGF-I or insulin have not been found in the regulation of these enzymes [15]. Nevertheless, several reports indicate that the number of IGF-I receptors is a useful marker for the malignant potential of tumors [34]. Alternatively, it is possible to speculate that in some tumors IGF-I receptors increase concomitantly with increasing receptor numbers of other growth factors, such as EGF, which can affect both cell proliferation and secretion of proteolytic enzymes [15]. A more likely

explanation is that IGF-I may stimulate the motility of malignant cells, since cell motility has been regarded as the other important factor for tumor cell invasiveness [17]. In fact, Stracke et al. [33] demonstrated that IGF-I enhances the motility of human melanoma cells.

In conclusion, established human bladder cancer cell lines have functional receptors for IGF-I. The data suggest that IGF-I may be an important growth factor for the development of human bladder cancer.

Acknowledgements. This study was supported in part by NIH Grant #CA 33148. The authors wish to thank Susan Schoen, Patricia Smith and Ellen M. Kane for excellent technical help, and Lora Tiede for her assistance in preparing this manuscript.

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