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Characterization and mutual ligand-induced modulation of epidermal growth factor and interferon-α receptors on renal carcinoma cells *in vitro*

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We have determined the binding of epidermal growth factor (EGF) and interferon (IFN)-α to their specific receptors on four renal carcinoma cell lines. CaKi-2, A-498 and ACHN cell lines express high numbers, and CaKi-1 expresses low number of EGF receptors (EGFRs). On all four renal carcinoma cell lines, we have also detected specific IFN-α binding sites. EGF and IFN-α binding to their receptors caused modulation of the other ligand's receptor binding. Scatchard analyses of binding data showed that IFN-α treatment leads to an increase of EGFR number in three out of four cell lines and to a decrease of EGFR number in one out of four (CaKi-1). No significant changes in EGF binding affinities were detected. EGF induced a reduction in IFN-α receptor number in all four cell lines without significant changes in IFN-α binding affinities. We hypothesize that presence of EGF in the microenvironment of renal cancer cells may modulate the biological effects of IFN and consequently decrease its antiproliferative activity.

Key words: Epidermal growth factor, interferon, modulation, renal carcinoma.

Introduction

Interferon (IFN)-α, an inducible protein produced by leukocytes in response to viral infections, plays a key role in the regulation of cell growth and differentiation. It induces inhibition of proliferation of normal and transformed cells, and modulates cell structure and function.[1,2] Interaction with specific high-affinity receptors on the surface of target cells is the initial step in IFN-α action.[3] It results in an activation of multiple pathways of signal transduction leading to the stimulation of expression of immediate-early genes termed the IFN stimulated genes (ISG) which are central in mediating biological effects.[4–10] Similarly, polypeptide tumor growth factors like epidermal growth factor (EGF) bind to membrane-spanning receptors. Ligand binding by the EGF receptor (EGFR) activates its intrinsic tyrosine kinase activity leading to autophosphorylation of EGFRs and multiple physiological changes in target cells, including alterations in gene expression and regulation of differentiation and proliferation of normal and tumor cells.[11–13] There is evidence that IFN-α may modulate the biological effects of EGF.[13–17] However, both up-regulation and down-regulation of EGFRs have been reported after incubation with IFN-α. In earlier studies using a capillary cloning system we have found that EGF significantly decreased the antiproliferative activity of IFN-α in freshly obtained renal cell cancer specimens. Hence it is possible that one ligand binding to its receptor may cause modulation of a second ligand–receptor interaction and permit unrelated receptors to regulate both common and unique sets of genes.[18,19] In particular, modulation of a cytokine receptor by agents like EGF is of potential clinical interest in tumors amenable to biologic therapies. The purpose of our present study was to examine the binding ability of IFN-α and EGF to established renal cancer cell lines, particularly the effects of IFN-α on EGF binding and the effects of EGF on IFN-α receptor binding.

Materials and methods

Materials

Receptor grade EGF was purchased from Flow Laboratories (Meckenheim, Germany). [^{125}I]EGF was obtained from Amersham Buchler (Braunschweig, Germany). Human recombinant IFN-α-2a (2.5×10^8 IU/mg) was a generous gift of Dr Behrens (Hoffmann-La Roche, Grenzach-Whylen, Germa-

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ny). Tissue culture plastic material was from Costar (Cambridge, MA). McCoy's 5a and Eagle's minimal essential medium (MEM), fetal calf serum (FCS), L-glutamine, penicillin–streptomycin, sodium pyruvate and non-essential amino acids were purchased from Gibco-BRL (Eggenstein, Germany), trypsin (0.5%)/EDTA (0.2%) from Seromed (Berlin, Germany), sodium hydrogen carbonate from Merck (Darmstadt, Germany), and bovine serum albumin (BSA) from Serva (Heidelberg, Germany).

Cell culture

Four human renal carcinoma cell lines from the ATCC (Rockville, MD) were used. CaKi-1 and CaKi-2 cell lines, clear cell carcinomas of primary renal cancer, were maintained in McCoy's 5a medium supplemented with 10% (v/v) heat-inactivated FCS, sodium hydrogen carbonate, L-glutamine and penicillin–streptomycin. The ACHN line, a human metastatic renal adenocarcinoma cell line, was grown in Eagle's MEM with Earle's salts containing 10% (v/v) heat-inactivated FCS, non-essential amino acids, L-glutamine and penicillin–streptomycin. The A-498 line, a hypertriploid kidney carcinoma cell line, was cultured in Eagle's MEM with Earle's salts, 10% (v/v) heat-inactivated FCS, non-essential amino acids, sodium pyruvate, L-glutamine and penicillin–streptomycin. The cells were grown in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

[^{125}I]IFN-α

Human recombinant IFN-α was labeled with ^{125}I using Iodogen for 5 min in 0.5 M phosphate buffer. After labeling, was added to a final concentration of 1 mg/ml. The specific activity of the labeled IFN-α was 150–300 Ci/mmol IFN-α. [^{125}]IFN-α was stored at 4°C prior to use.

EGF binding assays

EGF binding to cell surface receptors was studied in a competitive assay.[20] Renal carcinoma cells were seeded in 24-multiwell plates at a density of 5–10 $\times 10^4$ cells/well. After 24 h, the culture medium was exchanged. Subconfluent monolayers were washed twice with 1 ml ice-cold PBS supplemented with 1 mg/ml of BSA and incubated for 4 h at 4°C with 0.5 ml/well of binding buffer (Eagle's MEM or McCoy's each with 25 mM HEPES and 1 mg/ml BSA) and [^{125}I]EFG (1250–1500 Ci/mmol) in a concentration of 1.5×10^{-11} M and increasing concentrations of unlabeled EGF (1.5×10^{-11} to 1×10^{-7} M) to compete for [^{125}I]EGF binding. Cells were then rapidly washed twice with a 2 ml ice-cold PBS/BSA (1 mg/ml) and lysed in 1 ml 0.5% sodium dodecyl sulfate (SDS) for 10 min. Total radioactivity was counted in a Beckmann γ-counter. The EGFR binding affinities and receptor numbers were determined by Scatchard analyses of the specific EGF binding data.[21] To study the influence of IFN-α on the EGF binding of renal carcinoma cells, the medium was replaced with fresh medium containing IFN-α at various concentrations. Controls received no IFN-α. At 48 h after IFN-α addition, [^{125}I]EGF binding assays were carried out as described above.

IFN-α binding assays

For analysis of IFN-α binding to cell surface receptors, cells were seeded in 24-multiwell plates at a density of 5–10 $\times 10^4$ cells/well. After 1 day, the cells were refed with fresh medium. Subconfluent monolayers were washed twice with 1 ml PBS supplemented with 1 mg/ml BSA, 0.5 ml of binding buffer (Eagle's MEM or McCoy's with 25 mM HEPES and 5% FCS) was added together with [^{125}I]IFN-α (60–500 fmol). The cells were incubated for 3 h at 4°C, then rapidly washed twice with 2 ml PBS/BSA (1 mg/ml) and solubilized with 1 ml 0.5% SDS. Specific binding was determined as the difference between the binding of IFN-α in the absence and presence of a 100-fold excess of unlabeled IFN-α. Cell-associated radioactivity was counted in a Beckmann γ-counter. The value of the IFN-α binding affinities and the receptor numbers were determined by Scatchard analyses.[21] To study the effect of EGF on IFN-α binding, cells were refed with fresh medium containing EGF at specified concentrations. Controls received no EGF. At 48 h after EGF addition, [^{125}I]IFN-α binding assays were carried out as described above.

In parallel experiments, cell proliferation was determined by cell counts and Trypan blue staining.

Results

Expression of EGFRs on renal carcinoma cells

EGF receptor EGFRs binding on renal carcinoma cells was studied in a competitive assay[20] at 4°C to avoid receptor internalization. Specific binding of

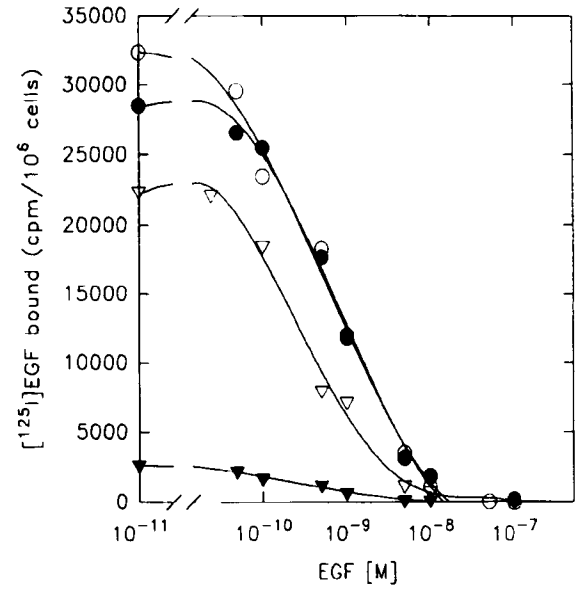

Figure 1. Binding data of competitive [^{125}I]EGF binding assays (4 h at 4°C) to renal carcinoma cells: ACHN (○), A-498 (●), CaKi-2 (▽) and CaKi-1 (▼).

EGF occurred in all four cell lines tested (Figure 1). Cell lines CaKi-2, A-498 and ACHN exhibited high level of specific EGF binding, while CaKi-1 cells bound considerably less of the ligand. Scatchard analyses of specific EGF binding data are summarized in Figure 2(a and b) and indicate a single class of EGF receptor sites. Between 142 000 and 411 000 EGFRs/cell were observed in CaKi-2, A-498 and ACHN, while CaKi-1 cells expressed 28 000 EGFR/cell. K_d values of binding affinities ranged from 1.4 to 4.6 × 10^{-10} M (Table 1).

Expression of IFN-α receptors on renal cancer cells

All cell lines specifically bound IFN-α (Figure 3). Specific binding of IFN-α was markedly lower than specific binding of EGF. Scatchard analyses indicate a density of IFN-α receptors between 1000 (ACHN) and 3300 (A-498) per cell with binding affinities of 0.5 to 1.6 × 10^{-9} M (Table 2).

Modulation of EGF binding by IFN-α

Because of co-expression of EGF and IFN-α receptors on renal carcinoma cells, we have studied whether preincubation with IFN-α modulates sub-

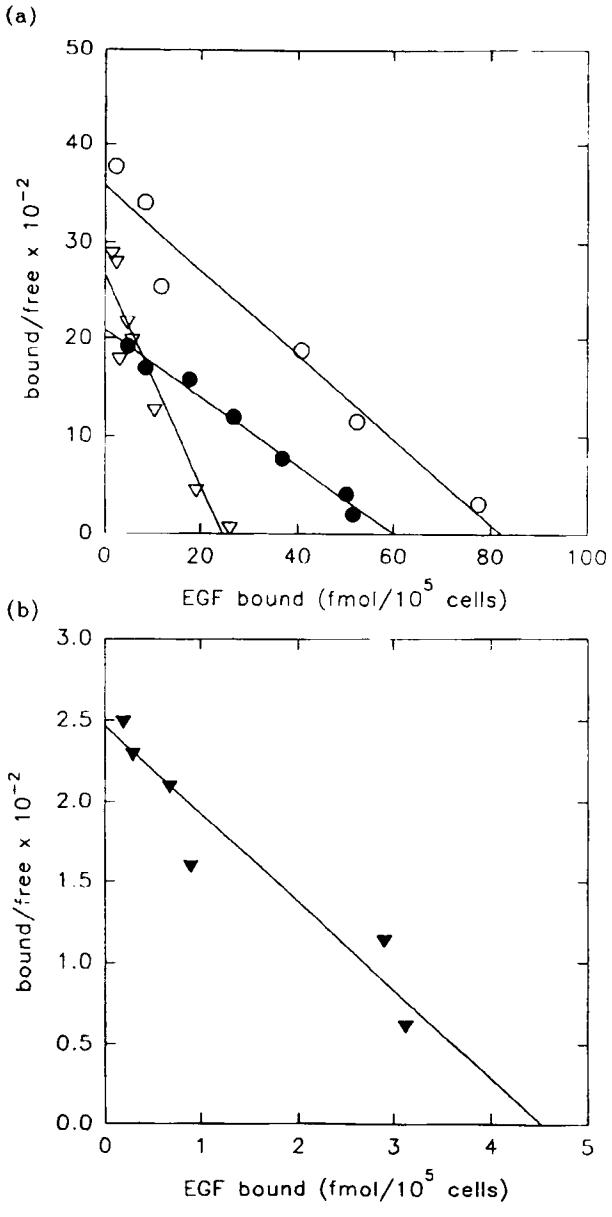

Figure 2. Scatchard plots of binding data from competitive [^{125}I]EGF binding assays. (a) ACHN (○), A-498 (●) and CaKi-2 (▽). (b) CaKi-1 (▼).

Table 1. Characterization of EGF receptors in four renal carcinoma cell lines

Cell line	EGFRs/cell	K_d (mol/l)
ACHN	411 000	3.6 × 10^{-10}
A-498	300 000	1.4 × 10^{-10}
CaKi-2	142 000	1.6 × 10^{-10}
CaKi-1	28 000	4.6 × 10^{-10}

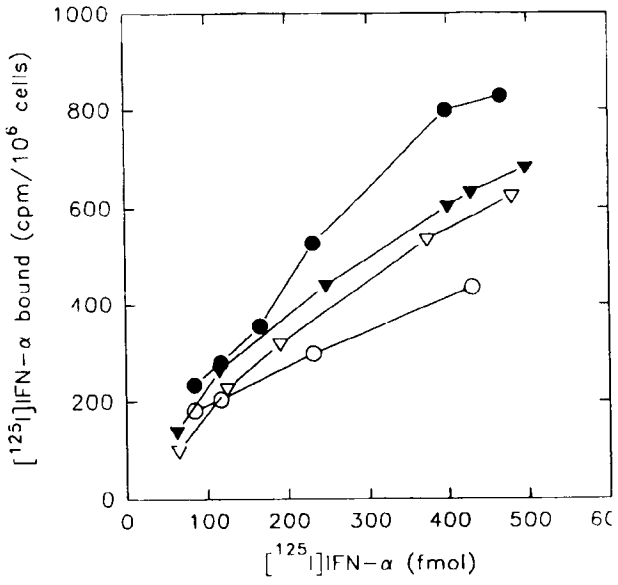

Figure 3. Binding of [^{125}I]IFN-α to renal cancer cells: A-498 (●), CaKi-1 (▼), CaKi-2 (▽) and ACHN (○) (3 h at 4°C). Data are corrected for specific receptor binding.

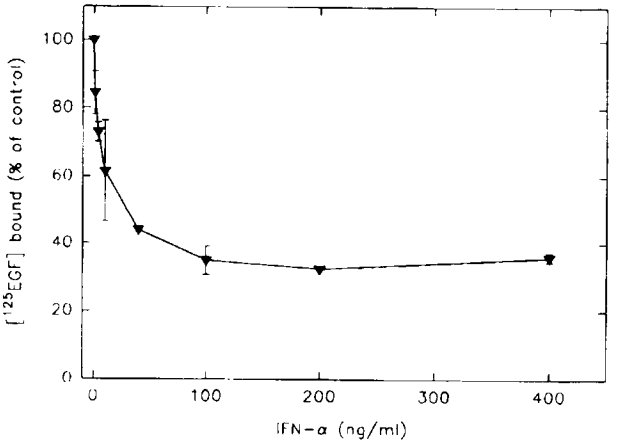

Figure 4. Inhibition of [^{125}I]EGF binding to CaKi-1 cells after exposure to IFN-α for 48 h.

sequent binding of EGF. In pilot experiments, time-dependency of the modulation of [^{125}I]EGF binding after IFN-α (100 ng/ml) pretreatment was studied using CaKi-1 cells. Maximal inhibition of EGF binding occurred after pretreatment with IFN-α for 48 h. To determine the concentration of IFN-α that causes a maximal effect on EGF binding, CaKi-1 and ACHN cells were subsequently exposed to IFN-α for 48 h at increasing concentrations (0–400 ng/ml). IFN-α was removed after 48 h and 1.5×10^{-11} M [^{125}I]EGF was added. EGF binding was determined after 1 h at 37°C. In CaKi-1 cells, EGF binding was significantly decreased to 35–40% of untreated controls with a maximal inhibition at 100 ng/ml IFN-α (Figure 4). In ACHN cells, IFN-α preincubation up to 400 ng/ml IFN-α slightly increased EGF binding to 130% of controls (SD < 10%) data not shown). Subsequent experiments were performed with all four renal carcinoma cells to determine whether the effects of IFN-α on EGF binding were due to a change in receptor number or binding affinity. Cells were exposed to IFN-α (100 ng/ml) for 48 h. IFN-α was then removed and EGF binding was determined in a competitive binding assay at 4°C. Scatchard analyses of specific EGF binding data indicated a moderate increase in the EGFR number in A-498, CaKi-2 and ACHN cells, while CaKi-1 cells showed a decrease in receptor density (Figure 5). In ACHN cells, the receptor number increased from 330 000 to 360 000 receptors/cell (109% of control); on A-498 from 236 000 to 296 000 receptors/cell (125% of control); and on CaKi-2 from 143 000 to 180 000 receptors/cell (126% of control). In CaKi-1 cells, receptor density decreased from 36 000 to 26 000 receptors/cell (72% of control). Standard deviations of repetitive experiments were below 10%. No changes in binding affinities were observed.

In parallel experiments, the growth-inhibitory effect of IFN-α was determined. After 48 h, 100 ng/ml of IFN-α caused 30–50% growth inhibition of all cell lines.

Table 2. Characterization of IFN-α receptors in four renal carcinoma cell lines

Cell line	IFN receptors/cell	K_d (mol/l)
A-498	3300	1.6×10^{-9}
CaKi-2	2400	1.6×10^{-9}
CaKi-1	2200	1.1×10^{-9}
ACHN	1000	0.5×10^{-9}

Modulation of IFN-α binding by EGF

In pilot experiments, CaKi-1 cells were exposed to EGF for 48 h at increasing concentrations (5–100 ng/ml). Maximal inhibition of IFN-α binding occurred at 25 ng/ml EGF (data not shown). In subsequent experiments, all cell lines were exposed to 25 ng/ml EGF for 48 h. After removal of EGF, IFN-α binding was determined at 4°C using increasing concentrations of [^{125}I]IFN-α. EGF decreased the number of available IFN-α receptors in all cell lines

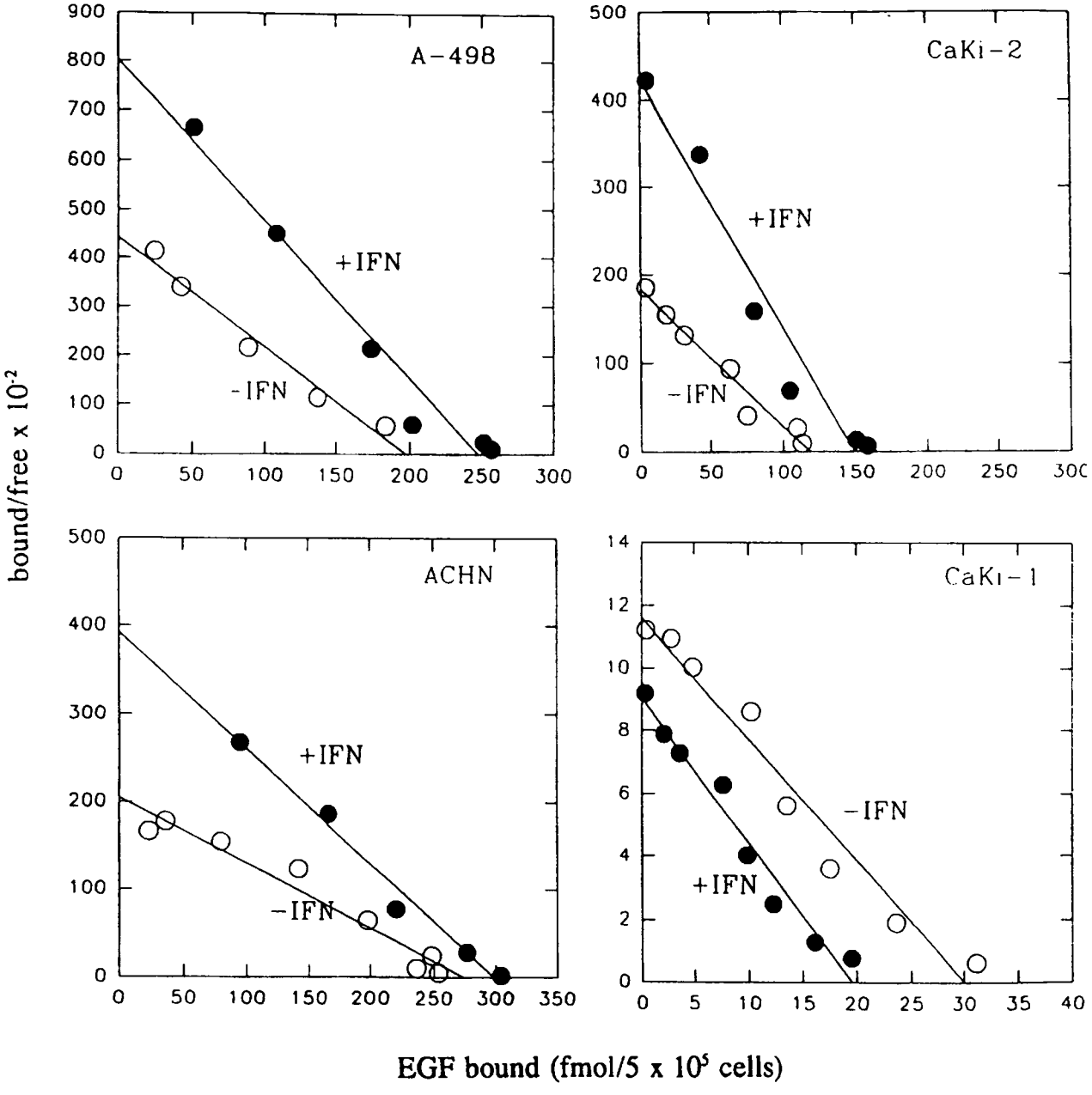

Figure 5. Modulation of [^{125}I]EGF binding by IFN-α (100 ng/ml). Binding data were obtained from competitive binding assays (4 h at 4°C) and were corrected for specific binding.

without affecting binding affinities. In A-498 cells, the number of IFN-α receptors decreased from 3360 to 2220 receptors/cell (66% of control), in CaKi-2 from 2480 to 1650 receptors/cell (67% of control), in CaKi-1 from 2200 to 1620 receptors/cell (74% of control) and in ACHN from 980 to 750 receptors/cell (77% of control).

Discussion

We have studied the inter-relationship between EGF and IFN-α binding to human renal carcinoma cells *in vitro*.

First, we have observed that three out of four renal carcinoma cell lines express high levels of cell surface EGFRs (CaKi-2, A-498 and ACHN). Over-ex-

pression of EGFR has been reported for numerous human cancers of epithelial origin, including renal and breast carcinoma, suggesting that magnification of EGFR-mediated cellular events may be an important component of the transformed phenotype of epitheloid cells and therefore may be considered a marker of cell transformation.[15,16]

Second, we have examined the binding of IFN-α to renal carcinoma cell lines. All cell lines bound IFN-α, but expressed relatively few high-affinity IFN-α binding sites. IFN-α inhibited the proliferation of all cell lines examined.

Third, we have found that after pre-exposure cells to IFN-α, binding of EGF is modulated. In three cell lines EGFR up-regulation occurred while in CaKi-1 cells EGFR down-regulation was noted.

Radioligand-binding experiments reported by Budillon *et al.* using human epidermoid cancer cells have shown that the growth inhibitory response to IFN-α is accompanied by a time- and dose-dependent increase in the number of EGF receptors due to a specific increase in receptor protein synthesis.[16] On the other hand, IFN-α has also been reported to inhibit EGF binding. Zoon *et al.* showed that IFN-α decreases the apparent number of EGFRs in Mardin–Darby bovine kidney cells (MDBK).[14] This was interpreted to be due to down-regulation of EGFRs through inhibition of EGFR protein synthesis.[15] Down-regulation of EGFR was reported to be specific for renal carcinoma cells sensitive to IFN-α and may thus contribute to the antiproliferative activity of IFN-α.[15] Therefore, the hypothesis was developed that IFN-α may attenuate the cellular response to mitogens by down-regulating the expression of growth factor receptors.[15] Our data are in agreement with Eisenkraft *et al.*[15] who reported an EGFR down-regulation in CaKi-1 cells. However, we have found evidence that other renal carcinoma cells (CaKi-2, A-498 and ACHN) up-regulate EGFR after exposure to IFN-α. Since IFN-α inhibited growth of all cell lines, up-regulation of EGFR is not restricted to IFN-resistant cells.

In concordance with Boudillon *et al.*[16] it may be possible that up-regulation of EGFR on renal carcinoma cells depends on high EGFR expression. In our experiments, CaKi-1 cells showed low EGFR expression and IFN-α-induced down-regulation of EGFR. CaKI-2, A-498 and ACHN cells express few IFN receptor compared with EGFR, the ratio of IFN receptors to EGFR being between 1:100 (CaKi-2 and A-498) and 1:340 (ACHN). However, the receptor ratio in CaKi-1 cells is 1:14. It is unclear whether this difference is relevant for the mutual modulation of receptor systems.

Presence of EGF also modulates IFN-α binding through down-regulation of IFN-α receptors. This was consistently observed in all cell lines and is in accordance with the results of an earlier study using a capillary cloning system, where we have found that EGF antagonizes the antiproliferative effect of IFN-α on freshly obtained renal cancer specimens (A-R Hanauske *et al.*, submitted).

Based on our data, we hypothesize that the EGF and IFN-α signal transduction systems constitute mutually controlling elements for the proliferation of renal cancer cells. The study of intermediates of the EGF and IFN-α signal transduction pathways will be of further interest and may help clarify underlying molecular mechanisms.[18,19]

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