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Correlation Between Interferon Alpha Receptor Protein Expression and Sensitivity to Interferon Alpha Subtypes in Human Renal Carcinoma Cell Lines

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Abstract. *Background: We have previously characterized the antitumor activities and immunological properties of interferon-alpha (IFN-α) subtypes on renal cell carcinoma (RCC). However, the mechanism responsible for the different biologic activities among the IFN-α subtypes is still unclear. To explain the different cellular sensitivities to IFN-α subtypes, detailed expression of the interferon-alpha receptor (IFNAR)-1 and IFNAR-2 subunits on different RCC cell lines was examined and compared with sensitivity of the cell lines to the IFN-α subtypes. Materials and Methods: We investigated the antiproliferative effects of natural IFN-α subtypes (IFN-α2 and IFN-α8) using eight RCC cell lines. IFNAR-1 and IFNAR-2 expression were determined by RT-PCR and Western blotting. To determine a possible relationship between IFN activity and IFNAR expression, the correlation between the 50% effective IFN dose (ED_{50}) for growth inhibition and the level of IFNAR expression was statistically examined. Results: We report here that IFN-α8 more potently induced growth inhibition than IFN-α2 in the majority of the RCC cell lines examined, this being in accordance with our previous results. The ED_{50} value of IFN-α8 was lower than 1000 (IU/ml) in six of the eight cell lines, whereas that of IFN-α2 was lower than 1000 (IU/ml) in three of the eight cell lines. The results of experiments using Western blotting analysis revealed that IFN-α subtype sensitivities were closely correlated with the expression level of IFNAR-2(c), a long form of the IFNAR-2 protein, in seven of the eight cell lines. Conclusion: These results suggest that the intensity of IFNAR-2(c) protein expression could be an important prognostic marker for clinical application of particular IFN-α subtypes in RCC.*

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Interferon-alpha (IFN-α) has diverse physiological activities that include enhancing immunological responses and expressing antiviral and antitumor effects (1-4). It is now well known that IFN-α is not a single molecule but that it is actually composed of many subtypes and that the expression level of each subtype depends upon the producing cells. All IFN-α subtypes compete for their common cell surface receptors and transmit signals intracellularly through these receptors, but exhibit variations in biologic potency (5-7). Because the IFN-α subtypes compete for the same cell surface receptor binding sites, and cell surface receptor binding is essential for all known IFN-α effects, it has been proposed that the response would be influenced by differences in the binding affinities exhibited by the different IFN-α subtypes.

Recently, we have reported that a number of IFN-α subtypes, in particular IFN-α8, show potent cytotoxic effects on renal cancer cell lines and chronic myelogenous leukemia (CML)-derived cell lines, suggesting that IFN-α8 is the most effective subtype as an antitumor agent and that it may be useful in the treatment of patients with RCC (3, 4).

IFN-α subtypes bind to the same cellular receptor, commonly designated as IFNAR (5-7). The high affinity IFNAR is composed of two chains, a 110 KDa α subunit (IFNAR-1) and a 102 KDa β subunit IFNAR-2 (7, 8). Additionally, three different forms of IFNAR-2 have been reported: a 40 KDa soluble form designated IFNAR-2(a), a 55 KDa short form known as IFNAR-2(b) and a 102 KDa

long form known as IFNAR-2(c). These different forms are derived from alternative splicing of the same gene. Since the IFNAR-2(b) and the IFNAR-2(c) mediate a biological response when associated with IFNAR-1, the expression of these subunits was the focus of this study.

Human RCC cell lines have reportedly shown resistance or sensitivity to the antiproliferative effects of IFN-α (4). However, the differences in sensitivities to the several IFN-α subtypes as well as the relationship between responsiveness to IFN-α subtype and IFNAR expression has not been clarified yet. To address the question of the sensitivity to IFN-α subtypes of human RCC cell lines, we examined the growth inhibition patterns and IFNAR expression at the mRNA and protein level.

Materials and Methods

Reagents. IFN-α2 and IFN-α8 were purified using NK-2 sepharose affinity column chromatography from a commercially available, clinically applied natural preparation of human IFN-α (9). The specific activities of the subtypes were determined in a cytopathic effect (CPE) reduction assay with target FL cells and Sindbis virus. Anti-human IFNAR-1 and anti-human IFNAR-2 rabbit polyclonal antibodies were kindly provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan).

Cell culture. Four human RCC cell lines (NKK-1, KPK-1, KPK-13 and SMKT-R3) were kindly provided by Dr. Kitamura of Tokyo University, Japan (10-12). A-498 (HTB-44) and ACHN (CRL-1611) were obtained from American Type Culture Collections (ATCC)(13,14) and Caki-1 (TKG 0436)(15) and VMRC-RCW (TKG 0447)(16) were provided by the Cell Resource Center for Biochemical Research, Institute of Development, Aging and Cancer, Tohoku University, Japan (IDAC), respectively. Cell lines were maintained for the duration of our experiments in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL; Grand Island Biological Co., Grand Island, NY, USA), 100 U/ml penicillin and 50 μg/ml streptomycin. Cells were cultured under standard conditions in a humidified 5% CO_2 and air mixture at 37˚C.

Growth inhibition assay. Cells (1-3 x 10^4/well) of the different lines were incubated in 96-well culture plates (Corning Glass Works, Corning, NY, USA) in 100 μl of RPMI-1640 medium containing 10% FBS with or without various concentrations of the IFN-α subtype preparations. After 96 hours, the supernatant was removed and the number of viable cells or determination of growth inhibition was performed using a cell counting kit-8 (Dojindo Molecular Technologies, Inc. Kumamoto, Japan) as described by the manufacturer. Triplicate wells were analyzed for each dose of IFN-α subtype. The ED_{50} value for growth inhibition was estimated by probit analysis for each cell line and subtype.

Human IFNAR-1 and IFNAR-2 mRNA expression by reverse transcription -polymerase chain reaction (RT-PCR). Total RNAs were extracted from the cell lines using RNeasy Mini kit (QIAGEN Inc., Hilden, Germany) as recommended by the manufacturer. One μg of RNA was subjected to reverse transcription with Omniscript reverse transcriptase (RT; QIAGEN Inc.) at 37˚C for 60 minutes. PCR primers used for the detection of IFNAR-1 consisted of the following sense and antisense primers, respectively; 5'-AGT GTT ATG TGG GCT TTG GAT GGT TTA AGC-3' and 5'-TCT GGC TTT CAC ACA ATA TAC AGT CAG TGG-3', resulting in a PCR product of 765 base pairs (bp). Thermocycle conditions were set at 20 cycles of 94˚C for 1 minute, 62˚C for 1 minute and 72˚C for 1 minute, and final extension at 72˚C for 10 minutes. For the detection of IFNAR-2, sense and antisense primers specific for the transcript were 5'-ATT TCC ATC TAT TGT TGA GG-3' and 5'-CAC TTT CTT CTT TCT GTT GA-3', respectively, which produced 350 <IFNAR-2(a)>, 481 <IFNAR-2(c)> and 713 <IFNAR-2(b)> bp products. The PCR conditions for IFNAR-2 were set at 20 cycles of 94˚C for 1 minute, 50˚C for 1 minute and 72˚C for 1 minute, and final extension at 72˚C for 10 minutes. For the RNA loading controls, sense and antisense primers specific for the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) transcript were 5'-TGT GAT GAA GGA GAT GGG AG-3' and 5'-TCA AGG GCA TAT CCT ACA AC-3', respectively, which produced a 427 bp product. The PCR conditions for HGPRT were set at 20 cycles of 94˚C for 1 minute, 55˚C for 1 minute and 72˚C for 1 minute, and final extension at 72˚C for 10 minutes. The PCR products were electrophoresed on a 1.2% agarose gel and visualized after ethidium bromide staining under UV light.

Hybridization with the respective probe. The PCR products were transferred to a nylon membrane (Hybond-N+: Amersham Pharmacia Biotech UK Limited, Amersham, UK), and hybridized with the respective ^{32}P-labeled primer. Labeling of the respective oligonucleotide primer was performed using the 5' prime oligonucleotide labeling kit (Amersham Pharmacia Biotech UK Limited) as described by the manufacturer. The sequences of the primers used are as follows:
IFNAR-1, 5'-TCT GGC TTT CAC ACA ATA TAC AGT CAG TGG-3';
IFNAR-2: 5'-GCA TTT TAA GGG AGA CTT-3';
HGPRT: 5'-GTG GGG TCC TTT TCA CCA GCA AGC-3'.
The hybridization was performed for 24 hours at 65˚C in 6 x SSC, 5 x Denhard's, 0.5 % sodium dodecyl sulfate (SDS), 20 mg/ml denatured salmon sperm DNA and 50 ng of the respective ^{32}P-labeled primers. The filter was rinsed with 2 x SSC at room temperature for 10 minutes, then washed at 65˚C with 2 x SSC/0.1% SDS solution for 10 minutes and exposed to X-ray film (Fuji Film Co. Ltd., Tokyo, Japan) with an intensifying screen at -80˚C for 1 to 3 days. The intensity of the specific bands was calibrated using ImageMaster (Amersham Pharmacia Biotech).

Western blotting. Cells were washed twice with Dulbecco's phosphate-buffered saline (D-PBS). The cells were dipped into 1 ml of 50 mM Tris-HCl (pH7.5) on ice for 20 minutes and homogenized by 50 strokes of a homogenizer. Then, the cell suspension was centrifuged at 3,000 rpm and 4˚C for 5 minutes. The supernatant was mixed with seven-fold homogenizing solution [0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 1mM ethylene diamine tetraacetic acid (EDTA)] and centrifuged at 30,000 rpm and 4˚C for 1 hour. The pellet was re-suspended into 2 ml of two-phase

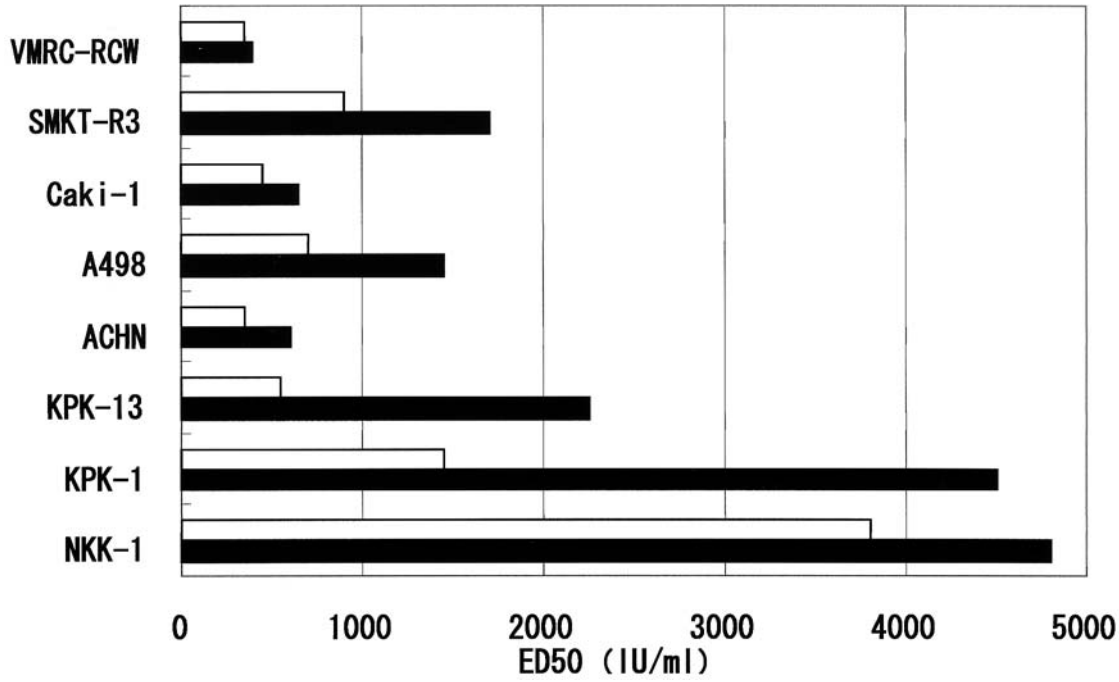

Figure 1. *Effects of the IFN-α subtypes on cell growth inhibition in eight RCC cell lines. Cells (1 x 10^4/well) of the different lines were incubated in 96-well culture plates in 100 μl of RPMI-1640 medium containing 10% FBS with or without various concentrations of the IFN-α subtypes. After 96 h, the supernatant was removed and viable cells were counted as a measure of growth inhibition using the cell counting kit-8 as described by the manufacturer. Triplicate wells were analyzed for each IFN-α dose. Results represent one typical experiment out of two similar experiments.* ■*, IFN-α2;* □*, IFN-α8.*

mixture solution [0.39% (W/V) dextran (Sigma, MW 500,000), 0.2% polyethylene glycol (Sigma, MW 6,000), 0.22M sodium phosphate (pH 6.5), 10 mM $ZnCl_2$] and centrifuged at 8,500 rpm and 4˚C for 10 minutes. The intermediate layer (membrane-rich protein fraction) of the solution was recovered and mixed with a five-fold volume of distilled water. Samples were centrifuged at 15,000 rpm and 4˚C for 10 minutes and the pellet was dissolved in D-PBS. Protein concentration was measured using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) (17) and the samples were frozen at -80˚C until assay by Western blotting.

Twenty μg of protein sample were boiled for 5 minutes in the presence of 20 mM Tris-HCl (pH 7.4) and 10% glycerol in a total volume of 20 μl and subjected to electrophoresis on a Multigel 10/20 (SDS-PAGE; Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). Routinely, the samples were then transferred onto nitrocellulose filter membranes (Millipore, Freehold, NJ, USA) and the membranes were blocked with TBS/BlockAce solution (Dainippon Pharmaceutical Co. Ltd., Tokyo, Japan). Next, the membranes were incubated with anti-human IFNAR-1 polyclonal antibody or anti-human IFNAR-2 polyclonal antibody at 4˚C overnight, washed with T-TBS and then incubated with anti-rabbit Igs-HRP antibody (1:1000; Sigma Chemical Inc., St. Louis, MO, USA) for 1 hour. The reaction was visualized using the ECL system (Amersham Pharmacia Biotech UK Limited). Hyperfilm ECL (Amersham Pharmacia Biotech) was exposed to the membrane, developed and the intensity of the specific bands was also calibrated by ImageMaster.

Results

Growth inhibitory effects of IFN-α subtypes on RCC cell lines. To determine the growth inhibitory effects of the IFN-α subtypes on the eight human RCC cell lines, a cell proliferation assay was performed. As shown in Figure 1, cell growth inhibition was observed in all of the cell lines tested and the ED_{50} values exhibited by the respective IFN-α subtypes varied widely from 350 to 4800 IU/ml. Among these cell lines, the effect on the VMRC-RCW cells was the most potent under our experimental conditions. In addition, the cell growth inhibition induced by IFN-α8 was generally confirmed and found to be remarkable in KPK-1, KPK-13, A-498 and SMKT-R3 cells when compared with IFN-α2. In VMRC-RCW, Caki-1 and ACHN cells, the cell growth inhibition induced by IFN-α8 was similar to that induced by IFN-α2. However, differences between the two subtypes of IFN-α as regards cell growth inhibition were not observed in NKK-1 cells.

Human IFNAR-1 and IFNAR-2 mRNA expression by RT-PCR. To assess the expression level of IFNAR-1 and IFNAR-2 mRNA, RT-PCR analysis was performed. IFNAR-1 mRNA expression in A-498 cells was relatively

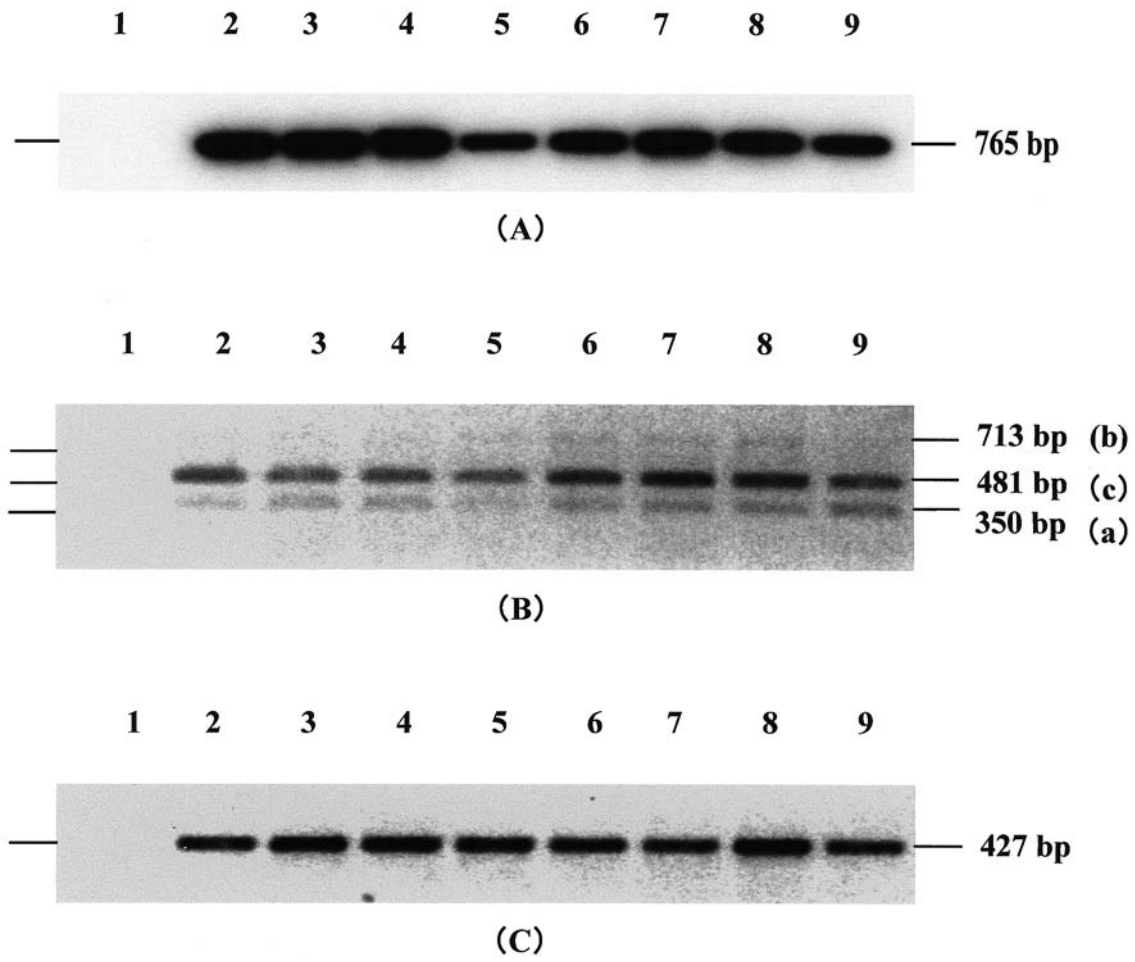

Figure 2. *IFNAR-1 and IFNAR-2 mRNA expression in the eight RCC cell lines as determined by RT-PCR. Total RNAs were extracted from the different cell lines as described in the text. One μg of RNA was subjected to reverse transcription with Omniscript RT at 37°C for 60 minutes. PCR was performed as described in the text. A, IFNAR-1 mRNA; B, IFNAR-2 mRNA [(a); soluble form: IFNAR-2(a), (b); short form: IFNAR-2(b), and (c); long form: IFNAR-2(c)]; C, HGPRT mRNA. Lane 1, no template; Lane 2, VMRC-RCW; Lane 3, SMKT-R3, Lane 4, Caki-1; Lane 5, A498; Lane 6, ACHN; Lane 7, KPK-13; Lane 8, KPK-1; Lane 9, NKK-1. Results represent one typical experiment out of two similar experiments.*

lower and that in the VMRC-RCW, SMKT-R3 and Caki-1 cells higher than that in the other cells as shown in Figure 2(A). Among the three types of IFNAR-2, IFNAR-2(c) mRNA expression was relatively stronger than those of IFNAR-2(a) and IFNAR-2(b). As far as could be tested, however, apparent differences in IFNAR-2 mRNA expression could not be identified in the cells as shown in Figure 2(B). HGPRT mRNA was detected at the same level in all cells tested as shown in Figure 2(C).

Human IFNAR-1 and IFNAR-2 protein expression by Western blotting. To analyze the actual expression of IFNAR-1 and IFNAR-2 proteins, Western blotting analysis was done and the results are shown in Figure 3. IFNAR-1 protein expression was detected in all the cell lines tested but no significant differences between the eight cell lines could be observed as shown in Figure 3(A). Interestingly, IFNAR-2(c) protein expression was significantly stronger when compared with IFNAR-2(b) protein expression (Figure 3B). IFNAR-2(c) protein expression in ACHN, Caki-1 and VMRC-RCW cells, cell lines established from metastatic lesions of RCC, was relatively stronger than that observed in the other cells that had been established from primary lesions. In addition, the IFNAR-2(c) protein expression in NKK-1, KPK-1 and SMKT-R3 cells was lower than that in KPK-13 and A498 cells (Figure 3B). IFNAR-2(b) protein expression did not show any significant differences among the cell lines.

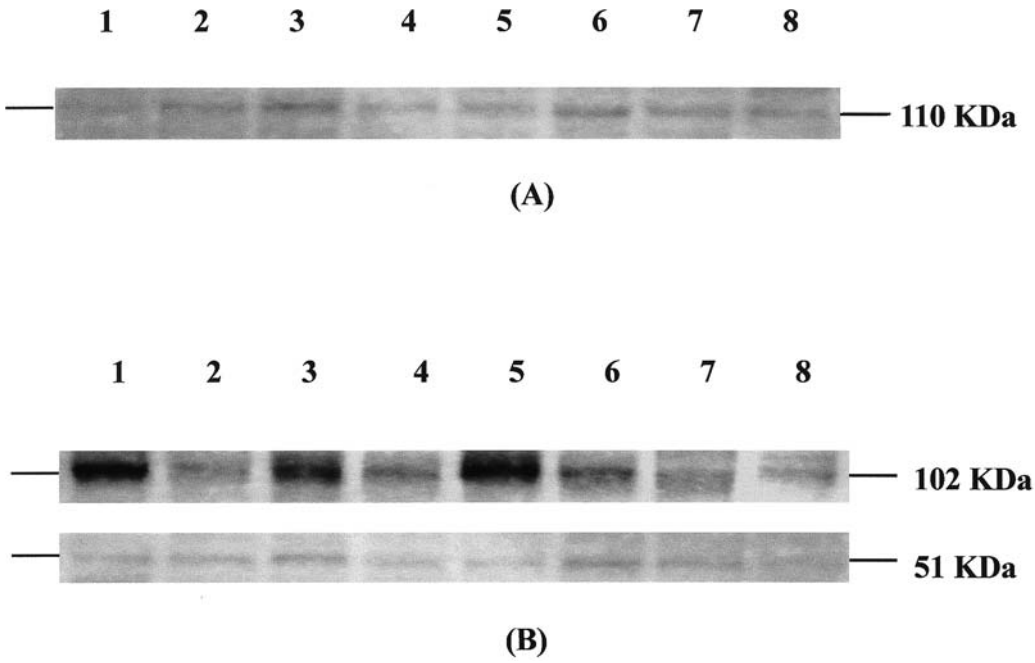

Figure 3. *Western blot analysis of IFNAR-1 and IFNAR-2 proteins in the eight RCC cell lines. Twenty μg of cell membrane proteins were subjected to SDS-PAGE and the proteins were transferred to a nitrocellulose filter membrane. The membranes were incubated with anti-human IFNAR-1 or anti-human IFNAR-2 polyclonal antibodies at 4˚C overnight, washed with T-TBS and then incubated with anti-rabbit Igs-HRP antibody (1:1000) for 1 h. The reaction was visualized using the ECL system. The blot was exposed to Hyperfilm ECL, developed and the strengths of the specific bands were determined by ImageMaster. A, IFNAR-1 protein; B, IFNAR-2 protein [(b); short form: IFNAR-2(b) and (c); long form: IFNAR-2(c)]. Lane 1, VMRC-RCW; 2, SMKT-R3, 3, Caki-1; 4, A498; 5, ACHN; 6, KPK-13; 7, KPK-1; 8, NKK-1. Results represent one typical experiment out of two similar experiments.*

Relationship between IFN-α subtype sensitivity and IFNAR expression. To analyze the relationship between IFN-α subtype sensitivity and the IFNAR mRNA expression in the eight RCC cells, the ED_{50} values and the intensities of IFNAR-1, IFNAR-2(a), IFNAR-2(b) or IFNAR-2(c) mRNA expression were compared. As far as could be tested, these parameters did not show any correlation (data not shown). IFNAR-2(b) protein was expressed at low levels relative to IFNAR-2(c); in addition, IFNAR-2(b) expression did not correlate with IFN-α subtype sensitivity (data not shown). Interestingly, IFN-α subtype sensitivity was significantly correlated with IFNAR-2(c) protein expression in all the eight RCC cell lines used in this study as shown in Figure 4. Concerning the correlations between IFN-α2, IFN-α8 and receptor expression, the correlation between IFN-α8 sensitivity and IFNAR-2(c) protein expression was slightly stronger than that of IFN-α2 sensitivity and IFNAR-2(c) protein expression.

Discussion

Estimates of annual newly diagnosed cases of RCC have been increasing steadily (18). Surgical resection of the primary tumor for patients with localized disease remains the mainstay of therapy. However, renal cell carcinoma is characterized by a lack of early warning signs, resulting in a high proportion of patients with metastases at diagnosis, or relapse following radical nephrectomy. The outlook for patients with distant metastases is poor, with a 5-year survival rate of less than 10% for those presenting with stage IV disease. Since IFN-α is an accepted therapeutical option for RCC therapy, the ability to predict the sensitivity to IFN-α subtypes of tumor cells in primary or metastatic lesions is an important consideration to improve RCC therapy.

In this study, we demonstrated that the cell growth inhibition activity of IFN-α subtypes (IFN-α2 and IFN-α8) correlated with IFNAR-2(c) expression at the protein level on eight RCC cells established from primary and metastatic lesions.

The effects of IFN-α are mediated through interaction with the IFN-specific cell-surface receptor, type I receptor, which consists of two chains, IFNAR-1 and IFNAR-2. Binding of IFN-α to its receptor induces IFN-sensitive gene expression through the activation of the JAK/signal transduction and activation of transcription signaling pathway (19). Since there are few data comparing the tumor cell growth inhibition induced by IFN-α subtypes and the

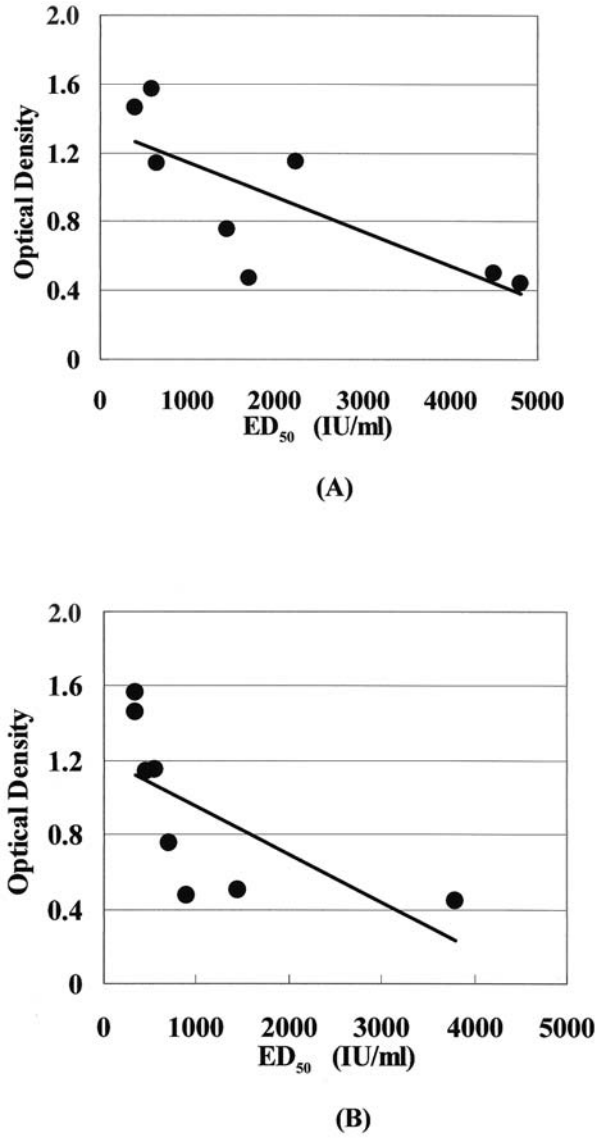

Figure 4. *Expression level of surface IFNAR-2(c) protein and IFN-α sensitivities (ED_{50}) in RCC cells. (A) IFN-α2; (B) IFN-α8.*

expression levels of IFNAR in RCC cells, we quantified mRNA and surface membrane protein levels of IFNAR in a panel of RCC cells with different IFN-α sensitivities. Only the protein levels of IFNAR-2(c) expressed on the RCC cells used in our present experiments correlated with their sensitivities to both IFN-α2 and IFN-α8, suggesting that IFN-α2 and IFN-α8 share a common signal pathway to some extent. On the other hand, there was no correlation between IFNAR mRNA expression and IFN-α sensitivities in the RCC cells, indicating that there is no correlation between IFNAR mRNA expression and protein expression in RCC cells. This discrepancy could also be observed in normal human peripheral blood mononuclear cells (PBMC). For example, in normal human PBMC IFN binding sites can be detected using radioisotope-labeled IFN (20) but IFNAR mRNA was undetectable (21). Therefore, quantification of IFNAR protein rather than mRNA may be crucial to assess IFN-α sensitivity of RCC cells.

In the present study, RCC cells showed similar or higher sensitivities to IFN-α8 in comparison with IFN-α2. These results indicate there is a possibility that IFN-α8 has a distinct signal transduction pathway while the mechanism is still obscure. Mouse cells expressing human IFNAR-1 did respond to human IFN-α8, but did not to the other human IFN-α subtypes. However, no apparent increased IFNAR-1 mRNA or IFNAR-1 protein was observed in RCC cells with higher sensitivity to IFN-α8 in our experiments. Although Pfeffer *et al.* (22) suggest that the IFNAR-2(b) acts in a dominant negative manner for the induction of the biological activities of several type I IFNs, IFNAR-2(b) protein was expressed at low levels relative to IFNAR-2(c) as Domanski *et al.* (23) reported. Additionally, there was no correlation between IFNAR-2(b) expression and IFN-α subtype biological activity. Taken together, our results suggest that IFNAR-2(b) may play a weak role in IFN-α subtype biological activity if its expression is associated with IFN-α sensitivity. Further studies must address the mechanism of growth inhibition activity induced by IFN-α8.

In summary, we propose that the intensity of IFNAR-2(c) protein expression could be an important parameter of prognostic value in partly selective clinical application of IFN-α subtypes. To clarify the clinical relevance of these experiments, we have been studying the relationship between the efficacy of IFN-α for RCC therapy and the IFNAR-2(c) protein expression. Furthermore, the panel of cell lines used in this study provides an interesting tool for the study of the mechanism of signal transduction pathways using different IFN-α subtype preparations, and for the study of the physiological significance of having the different IFN-α subtypes and their receptors.

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