Impaired α-Interferon Signaling in Transitional Cell Carcinoma: Lack of p48 Expression in 5637 Cells

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ABSTRACT

The limited success of IFN-α therapy for clinical treatment of transitional cell carcinoma (TCC) has prompted us to investigate the responsiveness of TCC lines to IFN-α. The response to IFN-α in terms of 561 gene induction, an IFN-stimulated response element-containing IFN-α/β-inducible gene, and IFN-stimulated gene factor 3 (ISGF3) formation was normal in primary human urothelial cells. We tested the antiproliferative effects of IFN-α in three TCC lines as a measure of IFN-α responsiveness, and variable patterns of growth inhibition were observed in three TCC lines. More than 90% growth inhibition was noted in TCCSUP cells, whereas only 40% and 10% inhibition by IFN-α was observed in 5637 and HT1197 cells, respectively. IFN-α treatment formed extremely low levels of ISGF3 in electrophoretic mobility shift assays in these later two relatively insensitive cells. In addition, expression of the 561 gene was significantly reduced in these two TCC lines by Northern blots. We have further identified a low expression level of Tyk2 in HT1197 cells compared with two other TCCs. This suggests that an extremely low ISGF3 level after IFN-α treatment may be due to low Tyk2 expression or other unidentified defects. In 5637 cells, p48 protein expression was undetectable. This undetectable p48 expression is not due to a deletion in the coding region because the correct size protein is detected following IFN-γ treatment. Consequently, the ISGF3 complex formation and 561 gene induction were restored by IFN-γ pretreatment plus IFN-α treatment. Introduction of p48 expressing plasmid into 5637 cells was sufficient to form the ISGF3 complex by IFN-α treatment, suggesting the defect lies in the expression of p48 protein in 5637 cells. Detailed mechanistic understanding of the action of IFNs in bladder cancer cell lines may explain the abrogated therapeutic response of IFN-α in the clinical treatment of TCCs.

INTRODUCTION

IFNs are known to have a variety of biological activities including antitumor, antiviral, and immunomodulatory effects. The biological actions of type 1 (IFN-α and -β) and type II (IFN-γ) IFNs are mediated by IFN-inducible gene expression (1). The Jak-signal transducer and activator of transcription (STAT) pathway (2, 3) mediates IFN signaling. Binding of IFN-α/β to its specific receptor at the cell surface stimulates tyrosine phosphorylation of many proteins, including the receptor and receptor-associated Janus kinases Tyk-2 and Jak-1 (2–10). These activated tyrosine kinases then phosphorylate specific amino acids of the latent cytoplasmic protein called STAT (2). Tyrosine-phosphorylated STAT1 and STAT2 (IFN-stimulated gene factor 3 [ISGF3α]) then translocate to the nucleus and associate with a DNA-binding protein, p48 (ISGF3-γ), to form a multiprotein complex, ISGF3. This complex binds with the IFN-stimulated response element (ISRE) present in most of the IFN-α/β-inducible genes to induce transcription of several IFN-inducible genes (2, 3, 8, 9, 11). STAT2 is a 113-kDa protein involved in the response to IFN-α or -β but not to IFN-γ, whereas STAT1, a 91-kDa protein and its spliced 84-kDa product, is activated by both IFNs (12, 13).

Clinically, IFN-α is currently used for the treatment of several diseases such as Kaposi’s sarcoma, hairy cell leukemia, multiple sclerosis, papillomavirus infection, and bladder cancer (14–16). In the United States, about 53,000 patients are diagnosed with bladder cancer and approximately 25% of bladder cancer patients die each year from their disease (17). Bacillus Calmette-Guérin instillation in the bladder is a highly successful treatment for superficial transitional cell carcinoma (TCC), although occasional severe side effects can lead to discontinuation of therapy (18). In vitro studies have indicated that IFN-α in combination with Bacillus Calmette-Guérin at very low concentrations inhibit the proliferation of human bladder cancer cells (19). However, IFN-α as therapy has shown limited efficacy in the clinical treatment of bladder cancer (15, 16). It has been reported that the expression or affinity of IFN-α receptors in malignant urothelial cells is not less than in normal urothelial cells (20). Recent evidence indicates that IFN-resistant chronic lymphocytic leukemia (CALL) cells are defective in ISGF3 formation by IFN-α treatment (21). This defect in the formation of ISGF3 in CALL cells was correlated with poor induction of the IFN-inducible enzyme 2′,5′-oligoadenylate synthetase (2′,5′-A-synthetase) (22). IFN-resistant human melanoma and breast cancer cells have also been reported to have defective ISGF3 formation in response to IFN-α due to the deficiency of STAT1 expression in these cells (23, 24). Previous studies have evaluated the in vitro antiproliferative effects of IFN-α in TCCs, showing variable effects in different cells (25).

In this report, the antiproliferative effects of IFN-α and formation of the ISRE-binding complex, ISGF3, and its constituent components were measured in TCCSUP, 5637, and HT1197 cell lines. Activation of ISGF3 was severely reduced, and consequently very poor induction of the 561 mRNA was observed in response to IFN-α in relatively insensitive TCCs. Markedly low level activation of ISGF3 was correlated with undetectable levels of p48 expression in 5637 cells. The p48 protein expression level in 5637 cells was highly inducible by IFN-γ treatment. Moreover, restoration of ISGF3 formation and 561 gene induction was achieved by pretreatment of cells with IFN-γ followed by IFN-α treatment. ISGF3 complex formation by IFN-α treatment was demonstrated by transfecting the p48 expression plasmid into 5637 cells.

MATERIALS AND METHODS

Cell Culture. The culture of normal human urothelial cells was performed according to our previously published procedure (26). The 5637 cells were cultured in RPMI 1640 and the other two cell lines (TCCSUP and HT1197) were cultured in modified Eagle’s medium. All TCCs were supplemented with 10% fetal bovine serum containing penicillin and streptomycin according to the American Type Culture Collection (Manassas, VA). All cell culture materials were obtained from Life Technologies, Inc. (Rockville, MD). The
2TGH and mutant cells defective in IFN signaling (negative controls) were provided by Dr. George Stark (The Cleveland Clinic Foundation). These cells were cultured in DMEM with supplementation described above. Antibodies such as Jak-1, p48, and IFN-α/β receptor were purchased from Transduction Laboratories (Lexington, KY), STAT2 from Santa Cruz Biotechnologies (Santa Cruz, CA), and actin from Boehringer Mannheim (Indianapolis, IN). LipofectAMINE plus reagent were purchased from Life Technologies.

**Proliferation Assays.** Cell proliferation was measured using the Cell Titer 96 AQueous cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer’s protocol. Two thousand cells were plated in 96-well plates with 200 μl of appropriate culture medium. The next day fresh medium was added and cells were treated with 0, 500, 5000, and 10,000 units/ml IFN-α. Fresh medium and IFN-α were added every 36 h. Assay reagents were added in each well at specific time points. AQueous assay is composed of solutions of a tetraczenium compound 3,4,5-dimethylthiazole-2-yl-5-(3-carboxymethoxy-phenoxy)-2-(4-sulfophenyl)-2H-tetrazolium and an electron coupling reagent phenazine methosulfate. 3,4,5-Dimethylthiazole-2-yl-5-(3-carboxymethoxy-phenoxy)-2-(4-sulfophenyl)-2H-tetrazolium was reduced into the aqueous soluble formazone by dehydrogenase enzymes present in metabolically active cells. Absorbance readings were taken at 490 nm in a ThermoMax Microplate Reader (Molecular Devices, Menlo Park, CA). Calculation of growth inhibition was determined by the formula: percent inhibition = [(1 – (A/B)) × 100], where A represents absorbance of treated wells and B the absorbance of control wells (27). All results are represented by the average value from triplicate samples and are corrected for background absorbance with culture medium.

**Oligonucleotide Probe and Electrophoretic Mobility Shift Assay.** Double-stranded synthetic oligonucleotides containing the ISRE sequences from −125 to −93 of the 561 gene were labeled by kinase reaction with [γ-32P]ATP and were used as a probe in the electrophoretic mobility shift assay (EMSA). The sequence of the probe is 5′-TCTAGCCTTTAGTCTACCTCTCCCCCTTTCTGCTTT-3′ (11). IRF-1 γ-activating sequence (GAS) probe was used as described previously (28). Extracts were prepared for EMSA as previously published (11). Briefly, 10 μg of extract were incubated in 20 μl of reaction volume in binding buffer [20 mM HEPES (pH 7.9), 5% glycerol, 60 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 0.5 mM EDTA] containing 2 μg of poly(dIdC), 2 μg of salmon sperm DNA, and 0.2 ng of labeled probe. After 20 min at 22°C incubation, the reaction mixture was resolved in a 5% nondenaturing polyacrylamide gel in TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA) at 180 V for 2 h. The gel was dried, then visualized and analyzed by autoradiography.

**Determination of Protein Concentration.** Protein concentration was determined by Bio-Rad reagent (Bio-Rad, Richmond, CA) using BSA as standard (29).

**RNA Isolation and Northern Blot Analysis.** All of these methods were carried out according to our previously published procedures (11, 30). Total RNA was prepared from 80–90% confluent cells after treatment with 500 units/ml IFN-α for the indicated time. The dose of IFN-γ was 100 units/ml for 18 h whenever cells were pretreated with IFN-γ prior to treatment with IFN-α. Equal amount of mRNA was electrophoresed in a 1% agarose-formaldehyde gel and then transferred onto a GeneScreen nylon membrane. cDNA probes were prepared by a random priming kit (Boehringer Mannheim) using [α-32P]dCTP in the reaction mixture. Hybridization and washing were done by standard procedure. Blots were reprobed with actin cDNA for normalization. Results were visualized by autoradiography and normalized with actin by quantitation with Phospholmage (Molecular Dynamics) analysis.

**Immunoprecipitation and Western Blot Analysis.** Cell extracts were prepared with or without treatment according to published procedure (5). Immunoprecipitations were performed with 100 μg of protein for STAT1 (Transduction Laboratories) and 200 μg of protein for Tyk-2 (Upstate Biotechnology, Lake Placid, NY) using 40 μl of protein G-Sepharose beads (1:1 suspension of beads/lysis buffer; Pharmacia, Uppsala, Sweden) with appropriate antibodies. After washing, bound proteins were eluted in reducing SDS loading buffer (5, 13). For Western blots, equal amounts of protein (20 μg) for each lane were electrophoresed on SDS-10%–polyacrylamide gel and transferred to a polyvinylidene difluoride-type transfer membrane at 4°C (Immobilon-P; Millipore, Bedford, MA) by standard procedure. Blocking was performed in PBS-Tween 20 (PBST) containing 5% nonfat dry milk or Tris-buffered saline-Tween 20 (TBST) in 5% BSA containing 0.1 mM sodium vanadate, in case of phosphotyrosine blots, for 2 h at room temperature. Diluted primary antibodies were incubated with membranes in 5% milk-PBST or horseradish peroxidase-conjugated phosphotyrosine antibody (Transduction Laboratories) in 1% BSA-TBST in the presence of 0.1 mM sodium vanadate for 16 h at 4°C. After five washes with PBST or TBST, horseradish peroxidase-conjugated secondary antibody (1:5000) was incubated for 1 h at room temperature. Blots were washed four times with PBST and twice with PBS and analyzed using an enhanced chemiluminescence kit from Amersham according to their protocol.

**Transfection.** Approximately 50% confluent 5637 cells were transiently transfected with pDNA3, a p84-expressing plasmid (31) (obtained from Dr. Dhananjaya Kalvakolanu, University of Maryland Cancer Center, Baltimore, Maryland via Dr. Andrew Lerner, Cleveland Clinic Foundation), using LipofectAMINE plus reagent (Life Technologies) according to their protocol. Briefly, 5 μg of DNA in 750 μl of serum-free medium plus 20 μl of plus reagent was incubated for 15 min at room temperature. DNA solution was then added to 30 μl of LipofectAMINE in 750 μl of medium and mixed, incubated for another 15 min, and the mixture was added into 10-cm plates containing cells in 5 ml of serum-free medium. Cells were incubated for 3 h at 37°C in a cell culture incubator. Then 6.5 ml of 20% serum-containing medium were added and next-day cells were treated with IFN-α for 30 min, and cell extracts were prepared and used for EMSA, p48, and actin expression.

**RESULTS**

**Growth Inhibition by IFN-α Is Variable in Three TCCs.** The growth pattern of three TCC lines, TCCSUP, 5637, and HT1197, were characterized to determine the in vitro responsiveness to IFN-α in the presence and absence of different doses of IFN-α (Fig. 1). More than 90% growth inhibition was observed at 72 h of treatment with either 5,000 or 10,000 units/ml IFN-α in TCCSUP cells. In contrast, 5637 and HT1197 cells were less inhibited by IFN-α treatment at the same doses. Only 40% inhibition for 5637 cells and about 10% inhibition for HT1197 cells was observed when cells were challenged with the same doses of IFN-α for 72 h under the same conditions. Fifty and 30% growth inhibition at 96 h was observed with 10,000 units/ml IFN-α for 5637 and HT1197 cells, respectively (data not shown). Thus, using growth inhibition as an assay for IFN-α responsiveness, the TCCSUP cells were the most sensitive among these three cell lines tested and the order of IFN-α sensitiveness was TCCSUP > 5637 > HT1197.

![Fig. 1. IFN-α-induced growth inhibition in TCCs. TCCSUP, 5637, and HT1197 cells were untreated or treated with 500, 5,000, and 10,000 units/ml IFN-α for 24, 48, and 72 h. Results represent percent growth inhibition from the average of triplicate samples.](cancerres.aacrjournals.org)
IFN-α Treatment Markedly Reduced ISGF3 Levels in 5637 and HT1197 Cells. Most IFN-α/β-inducible genes are induced transcriptionally by IFN-α/β through binding of the ISGF3 complex to promoter elements called ISREs. The IFN-α/β-inducible, p56 or 561, gene is one such gene whose induction is mediated by the ISRE sequence present in the −125 to −93 position of the transcription start site of the gene. Since formation of the ISGF3 complex after treatment with IFN-α/β correlates strongly with IFN-α/β-inducible gene expression (11), we determined the formation of the transcription factor complex ISGF3 in three TCC lines by EMSA. EMSA was performed to check the formation of ISGF3 using synthetic 33-bp ISRE oligonucleotides from IFN-inducible 561 gene (11). All three TCCs and normal urothelial cells were untreated or treated with IFN-α for 30 min and extracts were prepared to determine the formation of ISGF3 by the same EMSA. A strong ISGF3 complex was seen in IFN-α-treated normal urothelial cells (Fig. 2). The ISGF3 complex was formed after 30 min of IFN-α treatment in TCCSUP cells, whereas no ISGF3 band was seen at 30 min of IFN-α treatment in both 5637 and HT1197 cells (Fig. 2). However, in some experiments, a faint ISGF3 band was observed at very long exposure in these two cells. The ISGF3 band in IFN-α-treated normal urothelial cells was abolished when a 100-fold excess of the same cold oligonucleotide was added in the reaction mixture (Fig. 2). These experiments indicate that the relatively insensitive cells (in terms of growth in the presence of IFN-α) form markedly low levels of ISGF3 complex after IFN-α treatment.

Induction of 561 Gene Induction by IFN-α Is Impaired in 5637 and HT1197 Cells. All biological actions of IFN-α/β are mediated by IFN-inducible gene products such as 561. Since ISGF3 formation was drastically reduced after IFN-α treatments in 5637 and HT1197 cells, we investigated the steady-state level of 561 mRNA, a representative of IFN-α/β-inducible genes, after IFN-α treatment in three TCC cell lines. We first determined whether the 561 gene is inducible by IFN-α treatment in normal urothelial cells. Total RNA was isolated from cells either untreated or treated with IFN-α and subjected to Northern blot analysis using a 561-cDNA fragment as a probe. In normal urothelial cells, 561 mRNA expression was detected initially at 2 h (12.6-fold), reached maximum at 4 h (16.8-fold), and slightly declined at the 8 h-time point (13.2-fold; Fig. 3A). Like other cell types, 561mRNA was not expressed in untreated normal urothelial cells. Since 561 mRNA induction in normal urothelial cells was maximum at 4 h after IFN-α treatment, we treated all three TCCs (TCCSUP, HT1197, and 5637) with IFN-α for 4 h for Northern blot analyses using 561 cDNA as probe. All blots were deprobed and rehybridized with labeled actin as a probe for verifying equal loading and normalization.

After normalization with actin, the induction was maximal for TCCSUP (10.7-fold) and very poor induction was observed for both HT1197 (2-fold) and 5637 (2.9-fold) cells (Fig. 3B). These observations indicate that the induction of the 561 gene was markedly reduced in relatively less IFN-α-sensitive cells (measured by growth inhibition studies) compared to the more IFN-α-sensitive cell line, TCCSUP.

Low Level of IFN-signaling Protein Expression in HT1197 and 5637 Cells. ISGF3 complex formation by IFN-α/β treatment is dependent on intact IFN-α/β-signaling components. Since ISGF3 complex formation was drastically reduced in the 5637 and HT1197 cell lines, we first investigated Jak-1 and Tyk-2 levels in these cell lines. To measure these two kinase levels, Western blot analysis was performed on three TCCs using antibodies against Jak-1 and Tyk-2. The results show that all three TCCs are expressing Jak-1 protein at similar levels (Fig. 4A). In HT1197 cells, the expression of Tyk-2 is low compared to the other two TCCs (5637 and TCCSUP, Fig. 4A). We included a positive control lane (2TGH cell extracts, Fig. 4B) and also extracts from mutant cells lacking individual components of IFN-signaling proteins (Fig. 4). U1A, U3A, U4A, and U6A mutant cells are lacking Tyk-2, STAT1, Jak-1, and STAT2, respectively. These mutant extracts serve as negative controls in these experiments (Fig. 4). The next components of the IFN-α/β-signaling pathway include STAT1 and STAT2 proteins. We measured these two levels by Western blot. Both STAT1 and STAT2 levels are low in 5637 cells compared to the other two TCCs (Fig. 4B). These blots showed equal loading by GAPDH expression (data not shown). In a separate experiment, equal amount of all of these same extracts from Fig. 4, A and B, were also tested for actin expression, showing similar expression in all extracts (Fig. 4C).

Lack of p48 Expression in 5637 Cells. Since the level of p48 (also known as ISGF3γ) is a rate-limiting factor for the assembly and DNA-binding component of ISGF3, we measured the levels of p48 in all three cell lines. Western blot analyses using p48 antibody show that the expression of p48 was undetectable in 5637 cells, whereas p48 protein is strongly expressed in HT1197 and TCCSUP cells (Fig. 5A).
that both Tyk-2 and STAT1 are functional with respect to tyrosine phosphorylation by IFN-γ for Tyk-2, STAT1, Jak-1, and STAT2, respectively, in these experiments. Results show that Tyk-2 is tyrosine phosphorylated in all three IFN-γ-treated cells, we investigated the DNA-binding ability of STAT1 in IFN-γ-treated TCC extracts using the IRF-1 GAS element. Fig. 6A shows a comparable level of DNA-binding activity in all three TCCs after IFN-γ treatment. The STAT1 molecules that are tyrosine phosphorylated by IFN-γ treatment from the total STAT1 pool are similar, although constitutive STAT1 expression is low in 5637 cells. These results suggest that functions of STAT1 mediated through tyrosine phosphorylation are not altered compared to the other two cell lines. The markedly low level of ISGF3 formation is possibly not attributable to low STAT1 protein expression in 5637 cells. We also measured the levels of tyrosine phosphorylated Tyk-2 and STAT1 expression by immunoprecipitation followed by Western blot. Cells were treated as indicated and immunoprecipitated with either Tyk-2- or STAT1-specific antibody and then blots were probed with phosphotyrosine. Results show that Tyk-2 is tyrosine phosphorylated in all three IFN-α-treated cells (Fig. 6B). STAT1 is also tyrosine phosphorylated after IFN-γ treatment in all three cells. These results implicate that both Tyk-2 and STAT1 are functional with respect to tyrosine phosphorylation after IFN-α or IFN-γ treatment in these three cells.

**Overcoming the Defect in 5637 Cells by Pretreatment with IFN-α and also by p48 Expression.** Since the p48 protein expression level in 5637 cells is undetectable under this assay condition and p48 protein expression is inducible by IFN-γ treatment, we attempted to induce the p48 expression by treatment with IFN-γ for 18 h. Results show that expression of p48 and STAT1 are highly inducible by IFN-γ treatment in 5637 cells and the induced protein is immunoreactive and possibly functionally active (Fig. 7A). In the next experiment, we tested whether ISGF3 formation could be restored by pretreating the 5637 cells with IFN-γ prior to IFN-α treatment. Indeed, ISGF3 complex formation was restored by pretreatment with IFN-γ followed by IFN-α treatment for 30 min (Fig. 7B). Since 561 gene induction was poor in IFN-α-treated 5637 cells, we next examined whether we could induce 561 mRNA expression by pretreatment with IFN-γ followed by IFN-α treatment, as we demonstrated previously that p48 is involved in ISGF3-mediated 561 gene induction (11). Northern blot analysis in Fig. 7C shows that the expression of 561 mRNA is highly inducible by IFN-α if the cells were pretreated with IFN-γ. We transiently transfected p48 expression plasmid into 5637 cells to determine whether ISGF3 could be formed after IFN-α treatment. Cells were treated with IFN-α for 30 min after 24 h of transfection and extracts were used for similar EMSA. Fig. 7D shows that ISGF3 was formed in p48-expressing 5637 cells by IFN-α treatment. Transfection of p48 expressing plasmid into 5637 cells without any treatment did not form ISGF3 (data not shown). We also used the same extracts to determine the level of p48 expression and results show that p48 is expressed at a high level in transfected cells (Fig. 7D). Actin levels were also monitored for equal loading in the same samples.

**DNA-binding Activity of STAT1 Is Intact in All TCCs.** STAT1 is rapidly phosphorylated, dimerized, and binds to the GAS after IFN-γ treatments. Since STAT1 protein expression was low in 5637 cells, we investigated the DNA-binding ability of STAT1 in IFN-γ-treated TCCs using the IRF-1 GAS element. Fig. 5 shows that expression of STAT1 and STAT2 antibodies (A), STAT1 and STAT2 antibodies (B), and antiantibody (C). 2TGH (2T) cells serve as positive control, while U1A, U3A, U4A, and U6A cellular extracts were used as negative controls for Tyk-2, STAT1, Jak-1, and STAT2, respectively, in these experiments.

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IFNs have shown limited responses to IFN-α in the IFN system have delineated these critical pathways. Although signal transduction of IFNs. In the past decade, discoveries of signaling molecules have led to the identification of the p48 protein, which is involved in the regulation of IFN-α signaling.

**DISCUSSION**

IFNs have a wide range of biological functions including antitumor, antiproliferative, antiviral, immunomodulatory, and growth regulatory effects. Products of IFN-inducible genes mediate the biological functions of IFNs. In the past decade, discoveries of signaling molecules in the IFN system have delineated these critical pathways. Although IFN therapy is successful for many diseases, transitional cell carcinoma has shown limited responses to IFN-α as intravesical therapy. Cellular mechanisms responsible for the unresponsiveness of many TCCs to the antitumor effects of IFN-α remain unknown. In this study, we used three bladder cancer cell lines, TCCSUP, 5637, and HT1197, to investigate the molecular mechanisms responsible for these variable sensitivities of TCCs to IFN-α in vitro.

IFNs are known to affect cell viability in different ways. IFNs induce cell death by direct cytotoxic effects in some malignant cells, whereas in some others malignancies the antitumor effect is due to the induction of apoptosis (32–35). It has been suggested that IFN-α-mediated growth inhibition and apoptosis are two distinct and separable pathways and in some cells IFN-α is in fact an inducer of apoptosis (36). We have examined whether IFN-α treatment (up to 96 h) could induce apoptosis in these three TCCs to determine whether the observed variable IFN-α sensitivities were attributable to apoptosis. Our observations indicate that IFN-α treatment did not cause any apoptosis in these three cells as measured by terminal deoxynucleotidyl transferase-mediated nick end labeling, DNA fragmentation, and trypan blue exclusion assays (data not shown). A previous study has evaluated the in vitro antiproliferative effects of IFN-α for TCCs (25). Like many other malignancies, TCCs displayed a varied spectrum of responses to the antiproliferative effects of IFN-α (25, 37), and this report also demonstrates that one cell line, TCCSUP, is highly sensitive to the antiproliferative effects of IFN-α. Two other TCCs are less sensitive to IFN-α. The 5637 and HT1197 cell lines show approximately 50% and 10% growth inhibition, respectively, in the presence of IFN-α. The relative insensitivity toward the antiproliferative effects of IFN-α is possibly attributable to defects in the IFN-α-signaling components or the expression of proteins interfering with the IFN-α-signaling components (38).

ISGF3 formation is critical for most of the biological actions of IFN-α/β, and reports have demonstrated defective ISGF3 formation after IFN-α/β treatment in CLL, melanoma, and breast cancer cells (21–24). We present evidence that the ISGF3 complex formation in 5637 and HT1197 cells is extremely low after IFN-α treatment. The antiproliferative action of IFN-α may be correlated with the levels of ISGF3 formation, since the relatively insensitive TCC cells are forming significantly low levels of ISGF3 after IFN-α treatment. Neither receptor expression nor the binding affinity was altered in TCCs in other studies (20). In this study, a similar level of receptor expression is demonstrated in all three cell lines (Fig. 5B). The finding of impaired ISGF3 formation in TCC has not been previously reported.

Results from Western blot analyses of individual signaling components indicate that Jak-1 levels are relatively equal in all three TCCs, whereas both STAT1 and STAT2 levels are low in 5637 cells. STAT1-DNA-binding activity and tyrosine-phosphorylated STAT1 levels in IFN-γ-treated cells are comparable in all three TCCs, implicating that STAT1 is not defective in 5637 cells. Our experiments demonstrate the lack of p48 protein expression in 5637 cells. If the defect lies in the regulatory region within this gene or mutations are present in the coding region so that the functional protein is not expressed, then treatment with IFN-γ should not induce the functional protein, since p48 protein is known to be up-regulated by IFN-γ treatment. However, treatment of 5637 cells with IFN-γ resulted in an increase and easily detectable level of p48 expression. Therefore, IFN-γ pretreatment followed by IFN-α treatment should not only form ISGF3 but also increase the level of expression of p48 mRNA in 5637 cells. Indeed, not only is formation of ISGF3 restored by pretreatment with IFN-γ followed by IFN-α in 5637 cells, but 561 mRNA expression is also restored. Detection of the ISGF3 band after IFN-α treatment in p48-expressing cells indicates that relative insensitivity to IFN-α is most likely due to the undetectable level of p48 in 5637 cells. The molecular mechanisms involved in this undetectable expression of p48 are under investigation.

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Fig. 7. Restoration of impaired conditions in 5637 cells by pretreatment with IFN-γ and ISGF3 formation by p48 expression. A, using equal amount of proteins from 5637 cells either untreated (Unt) or treated with 500 units/ml IFN-γ for 18 h, Western blot analyses were performed with p48 and STAT1 antibodies. B, ISGF3 complex formation was measured by EMSAs in 5637 cells either untreated (Unt), treated for 30 min with 500 units/ml IFN-α, or pretreatment with 100 units/ml IFN-γ for 18 h followed by a 30-min treatment with 500 units/ml IFN-α (IFN-γ, IFN-α). C, 5637 cells were treated as indicated in B and 10 μg of total RNA were subjected to Northern blot analysis for the steady-state level of 561 mRNA expression. D, 5637 cells were transiently transfected with 5 μg of p48-expressing plasmid DNA. After 24 h of transfection, cells were treated with 500 units/ml IFN-α for 30 min and 10 μg of protein were used for ISGF3 assay by EMSA. Equal amounts of extracts were used for Western blots with p48 antibody. The same blot was also used for actin expression verifying equal loading.
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