Significant induction of apoptosis in renal cell carcinoma cells transfected with cationic multilamellar liposomes containing the human interferon-β gene through activation of the intracellular type 1 interferon signal pathway

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Abstract. We previously reported that cationic multilamellar liposome containing the human interferon-β *(huIFN-β)* gene (IAB-1) demonstrated significant cytotoxic effect in the NC65 human renal cell carcinoma (RCC) cell line. In this study, we investigated the molecular mechanisms of IAB-1-induced apoptosis and cytotoxicity in RCC cells. Remarkable *in vitro* cytotoxic and apoptosis-inducing effects of IAB-1 against NC65 cells were observed by a colorimetric method and TUNEL staining, respectively. In contrast, treatment of NC65 cells with exogenously added huIFN-β protein induced low-level cytotoxicity without apoptosis. Neutralizing antibodies against huIFN-β significantly suppressed the cytotoxic effect of huIFN-β protein, but they were unable to block the effect of IAB-1. Cytotoxicity assays using transwell plates revealed that NC65 cells treated with IAB-1 did not secrete cytotoxic soluble factors other than IFN-β. Substantial enhancement of interferon-stimulated response element (ISRE) activity of NC65 cells by IAB-1 was demonstrated by promoter reporter assays. In addition, immunofluorescence using confocal microscopy revealed the intracellular expression of IFN-β and its receptor induced by IAB-1. The induction of c-Myc by IAB-1 was suggested by a cDNA macroarray and was confirmed by western blot analysis. These findings indicate that IAB-1 induces significant cytotoxicity and apoptosis in NC65 cells, possibly through enhanced ISRE activity, that is associated with increased intracellular localization of huIFN-β and IFN-receptor. Our data support the potential clinical application of IAB-1 gene therapy for RCC resistant to IFN.

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Abbreviations: hu, human; IFN, interferon; PBS, phosphate-buffered saline; RCC, renal cell carcinoma; OD, optical density; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; STAT, signal transducer and activator of transcription; ISGF, interferon-stimulated gene factor; ISRE, interferon-stimulated response element; SD, standard deviation

Key words: apoptosis, gene therapy, IAB-1, IFN-β, renal cell carcinoma

Introduction

There are few effective therapeutic modalities for metastatic renal cell carcinoma (RCC). Among these treatment strategies, interferons (IFNs) have generally been included. Many combination treatments with other biological agents and chemotherapeutic agents have been developed to enhance the effectiveness of IFNs. However, response rates to these therapies have been reported to be ~15-20% (1-4), which is not satisfactory. Although molecular-targeted therapy has recently been introduced to treat metastatic renal cell carcinoma (mRCC) patients and has been shown to improve clinical outcome, a complete response has rarely been observed, and the therapy has various adverse effects (5-8). Therefore, the prognosis of mRCC remains poor, emphasizing the need to develop novel therapeutic modalities. One such promising treatment involves gene therapy.

We previously reported that cationic multilamellar liposomes containing the *IFN-β* gene (IAB-1) show significant antitumor activity against RCC both *in vitro* and *in vivo* (9).

Significant cytotoxic effects as well as apoptosis are induced by IAB-1, but not by the huIFN-β protein. Therefore, we speculated that one mechanism of enhanced cytotoxicity by IAB-1 may be via the induction of apoptosis. We even observed cytotoxicity and apoptosis against the relatively IFN-β protein-resistant NC65 cell line, which suggests that this method may be suitable for clinical application to treat IFN-resistant RCC. Elucidation of the molecular mechanisms of IAB-1-induced apoptosis and cytotoxicity in RCC cells would be useful for developing and optimizing effective gene therapies for RCC. In this study, we examined the molecular mechanisms responsible for the significant apoptosis and cytotoxicity of RCC cells induced by IAB-1.

Materials and methods

Cells. The human RCC cell line NC65 (10) was maintained in RPMI-1640 medium (Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies Inc.), and 10% heat-inactivated fetal bovine serum (Life Technologies Inc., Bio-cult, Glasgow, Scotland, UK), hereafter referred to as complete medium.

Reagents. IAB-1 is a plasmid DNA/lipid complex composed of a plasmid (pSV2IFNβ) that contains the SV40 early promoter and the *huIFN-β* gene coding sequence (11), and positively charged liposomes [N-(α-trimethylammonioacetyl)-didodecyl-D-glutamine chloride, dilauroyl phosphatidylcholine, and dioleoyl phosphatidylethanolamine in a molar ratio of 1:2:2], as described previously (11-13). The final concentration of IAB-1 was 50 nmol of lipid/μl with 1.0 μg plasmid DNA/μl. The empty liposome was composed of liposome alone (50 nmol of lipid/μl) without plasmid DNA (9). The IAB-1 and empty liposomes were dissolved in phosphate-buffered saline (PBS) and diluted to the indicated concentrations. Recombinant huIFN-β protein (IFN-β Mochida, 2.0×10^5 IU/mg) was provided from Mochida Pharmaceutical Inc., Tokyo, Japan (9). Neutralizing anti-huIFN-β monoclonal antibody (clone 76703.111) was purchased from R&D Systems Inc. (Minneapolis, MN, USA).

IFN-β gene transfer. Aliquots of 5.0×10^4 cultured cells were placed in each well of 6-well plates with 2 ml medium and were incubated for 24 h at 37˚C in a humidified atmosphere of 5% CO_2 (standard conditions). IAB-1 solution or empty liposome solution was then added, and incubation was continued for up to 3 days (9,14).

Cytotoxicity assay. Cytotoxicity was evaluated by a colorimetric method using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8; Nacalai Tesque, Kyoto, Japan) (15). Briefly, triplicate aliquots of cells were treated with IAB-1. After incubation under standard conditions, the culture supernatant was replaced with 1 ml fresh complete medium containing 0.5 μmol tetrazolium salt. After incubation for an additional 1 h, culture supernatants were harvested, and the optical density (OD) at 450 nm was measured. Cytotoxicity was calculated as follows: cytotoxicity (%) = [1 - (absorbance of experimental wells/average absorbance of control wells)] x100 (9).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay for detection of apoptotic cells. Apoptosis was examined by the TUNEL method, as previously reported (16). NC65 cells were incubated with 0.1 μl/ml IAB-1 (5.0 nmol lipid with 0.1 μg plasmid DNA/ml) or 10000 IU/ml recombinant huIFN-β protein for 24 h under standard conditions. After incubation, apoptotic cells were detected using an *in situ* Apoptosis Detection kit (Takara, Otsu, Japan), according to the manufacturer's protocol. Labeled cells were observed with a fluorescence microscope (16).

Western blot analysis. Sample proteins (20 μg) were separated on 10% polyacrylamide gels in Tris-glycine buffer and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked for 30 min with blocking buffer (5% skim milk in 0.1% Tween-PBS) and probed with anti-c-Myc monoclonal antibody (clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-signal transducer and activator of transcription 1 (STAT1) monoclonal antibody (clone 42; BD Biosciences, San Jose, CA, USA), anti-STAT2 monoclonal antibody (clone 22; BD Biosciences), or anti-interferon-stimulated gene factor 3γ (ISGF3γ, p48) monoclonal antibody (clone 6; BD Biosciences) for 1 h. The membrane was washed and then incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, USA). The signal was detected with the BCIP-NBT kit (Nacalai Tesque). The relative expression of c-Myc, STAT1, STAT2, and ISGF3 was determined with Gel Doc 2000 (Bio-Rad Laboratories, Osaka, Japan).

The measurement of interferon-stimulated response element (ISRE) activity. The activity of ISRE in NC65 cells treated with IAB-1 or recombinant huIFN-β protein was measured using Pathway Profiling Luciferase System 5 (Clontech Laboratories Inc., Mountain View, CA, USA), according to the manufacturer's protocol. pISRE-Luc is a signal transduction *cis*-reporter vector. It was designed to monitor the activation of IFN-triggered signal transduction pathways. pISRE-Luc contains five copies of the ISRE-binding sequence, located upstream of the TATA-like promoter (P_{TAL}) region of the herpes simplex virus thymidine kinase promoter. Located downstream from P_{TAL} is the firefly *luciferase* reporter gene. After activated transcription factors bind to the *cis*-acting enhancer element, ISRE, transcription is induced and the *luciferase* reporter gene is activated. The activity of luciferase is proportional to the level of induction of cellular gene transcription by type 1 IFN. pTA-Luc, the negative control plasmid vector, was used as a control.

NC65 cells were plated the day before transfection of the Pathway Profiling Luciferase vectors. Then, 1 μg pISRE-Luc or pTA-Luc was transfected with 9 μg SuperFect transfection reagent (Qiagen, Hilden, Germany), according to the manufacturer's protocol. One day after the transfection, IAB-1 or recombinant huIFN-β protein was added to the wells, as described above in '*IFN-β* gene transfer', and the cells were incubated for 24 h. Luciferase activity was measured using a Luciferase assay kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. Photoemission was measured for 10 sec using a luminometer.

Fluorescent confocal imaging of IFN-β protein and type 1 IFN-receptor. NC65 cells were treated with IAB-1 or huIFN-β

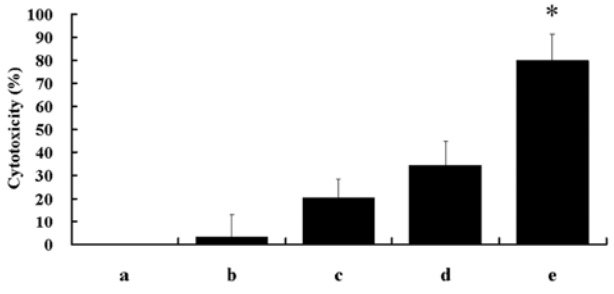

Figure 1. Cytotoxicity of IAB-1 against NC65 *in vitro*. NC65 cells ($1.25x10^4$) were placed in each well of a 24-well plate with 0.5 ml medium and incubated for 24 h. PBS (control), recombinant huIFN-β protein, empty liposome, or IAB-1 was added to the medium, and the cells were further incubated for 3 days. Cytotoxicity was evaluated by a colorimetric method using tetrazolium salt as described in Materials and methods. a, control (PBS); b, 0.1 μl/ml of empty liposome (5.0 nmol lipid/ml); c, 1000 IU/ml recombinant huIFN-β; d, 0.01 μl/ml of IAB-1 (0.5 nmol lipid with 0.01 μg plasmid DNA/ml); e, 0.1 μl/ml of IAB-1 (5.0 nmol lipid with 0.1 μg plasmid DNA/ml). Results were derived from three independent experiments and are expressed as mean ± SD. *p<0.05, compared to other treatment groups.

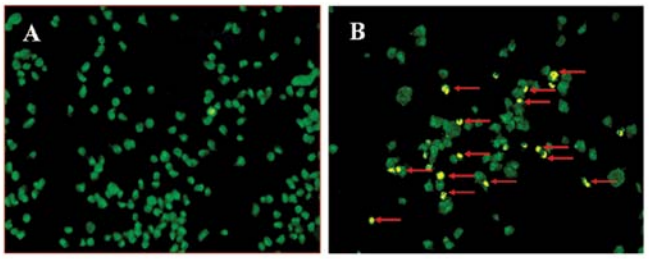

Figure 2. TUNEL assay for detection of apoptosis. NC65 cells were treated with (A) 10000 IU/ml recombinant huIFN-β protein or (B) 0.1 μl/ml of IAB-1 (5.0 nmol lipid with 0.1 μg plasmid DNA/ml). Apoptotic cells (yellow) were determined by TUNEL staining 24 h after treatment and visualized by fluorescence microscopy at x200 magnification. Red arrows indicate representative apoptotic cells.

protein in single-well chamber slides. After 24 h of incubation, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS, and then incubated with 1:25 mouse monoclonal anti-huIFN-β antibody (clone 76703.111; R&D Systems Inc.) and 1:10 rabbit polyclonal anti-IFNα/βRβ antibody (the beta chain of type 1 IFN-receptor, C-18; Santa Cruz Biotechnology) for 1 h at room temperature. After washing with PBS twice, the slides were incubated with AlexaFlour 488 goat anti-mouse IgG antibody (Invitrogen Corp., Carlsbad, CA, USA) and AlexaFlour 568 goat anti-rabbit IgG antibody (Invitrogen Corp.) at a dilution of 1:200 for 45 min. Slides were washed in PBS and coverslipped. Cells were viewed at x600 magnification. Images were collected with an Olympus Fluoview laser scanning confocal microscope (Olympus).

cDNA macroarray. Gene expression profiles using mRNA from NC65 cells treated with medium, 10000 IU/ml recombinant huIFN-β protein, 0.1 μl/ml empty liposome solution, or 0.1 μl/ml IAB-1 were compared by Atlas Human Cancer 1.2 Array (Clontech Laboratories Inc.). This array includes 1185 human genes. Cells were harvested 48 h after each treatment.

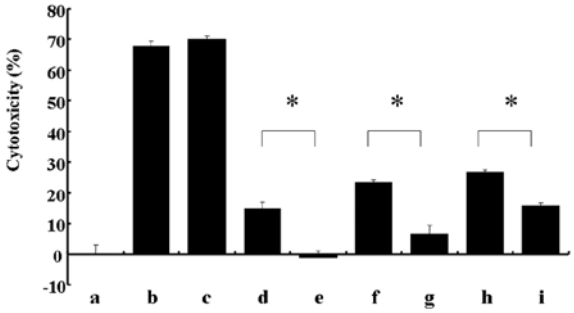

Figure 3. Neutralization effect of anti-IFNβ antibody on cytotoxicity of IAB-1 against NC65 *in vitro*. NC65 cells ($2.5x10^4$) were placed in each well of a 12-well plate with 1.0 ml medium and incubated for 24 h. PBS (control), recombinant huIFN-β protein, or IAB-1 was added to the medium with or without 20 μg/ml anti-huIFN-β monoclonal antibody (Ab). After 2 days of incubation, cytotoxicity was evaluated by a colorimetric method using tetrazolium salt as described in Materials and methods. a, control (PBS); b, 0.1 μl/ml of IAB-1 (5.0 nmol lipid with 0.1 μg plasmid DNA/ml); c, 0.1 μl/ml of IAB-1 + Ab; d, 100 IU/ml IFN-β; e, 100 IU/ml IFN-β + Ab; f, 1000 IU/ml IFN-β; g, 1000 IU/ml IFN-β + Ab; h, 10000 IU/ml IFN-β; i, 10000 IU/ml IFN-β + Ab. Results are expressed as mean ± SD (n=4 per treatment group). *p<0.05, comparing cells with antibody versus those without.

Statistical analysis. Multiple independent experiments were performed for each data set, and the results are presented as the mean ± standard deviation (SD). For statistical analysis, unpaired t-tests were used. Differences were considered statistically significant at $p<0.05$.

Results

Transfection of IAB-1 induces significant cytotoxicity and apoptosis in the human RCC line NC65. The cytotoxicity against NC65 cells induced after 3 days of incubation with 0.1 μl/ml IAB-1 was substantially higher than that after exogenously adding 1000 IU/ml recombinant huIFN-β protein (Fig. 1), consistent with our previous report (9). However, we previously found that NC65 cells treated with IAB-1 secreted IFN-β protein, and the concentration of IFN-β protein in the culture medium of NC65 cells treated with 0.1 μl/ml IAB-1 was consistently lower than that of cells treated with 1000 IU/ml recombinant huIFN-β protein (9). In this study, TUNEL staining demonstrated that transfection of 0.1 μl/ml IAB-1 significantly induced apoptosis, while treatment of NC65 cells with 10000 IU/ml recombinant huIFN-β protein did not (Fig. 2), which is also consistent with our previous study (9). These findings suggest that the ability of IAB-1 to induce cytotoxicity and apoptosis is not simply the result of IFN-β protein secretion.

Secreted huIFN-β protein is not the sole cause of cytotoxicity in IAB-1-transfected NC65 cells. Utilizing neutralization antibodies against huIFN-β, we evaluated the contribution of secreted huIFN-β protein to the cytotoxic and apoptotic effects of IAB-1. Neutralizing antibodies to huIFN-β protein significantly suppressed the cytotoxic effect of exogenously supplied huIFN-β protein, but did not block the lysis of IAB-1-transfected NC65 cells (Fig. 3). These results suggest that secreted huIFN-β protein was not the sole factor that induced cytotoxicity or apoptosis of IAB-1-transfected NC65 cells.

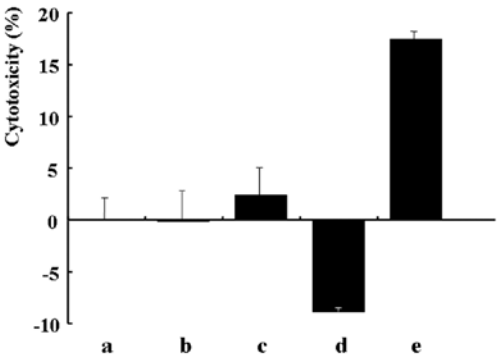

Figure 4. Effects of soluble factors secreted by IAB-1-transfected NC65 cells on untransfected NC65 cells using transwell plates. NC65 cells ($5.0x10^4$) were seeded in each compartment of a transwell plate (6-well plate). Two days after the addition of PBS (control), empty liposomes, IAB-1, or huIFN-β protein to the upper wells, the growth of NC65 cells in the lower wells was determined and cytotoxicity was evaluated by a colorimetric method using tetrazolium salt as described in Materials and methods. a, control (PBS); b, 0.1 μl/ml of empty liposome (5.0 nmol lipid/ml); c, 0.01 μl/ml of IAB-1 (0.5 nmol lipid with 0.01 μg plasmid DNA/ml); d, 0.1 μl/ml of IAB-1 (5.0 nmol lipid with 0.1 μg plasmid DNA/ml); e, 1000 IU/ml recombinant huIFN-β.

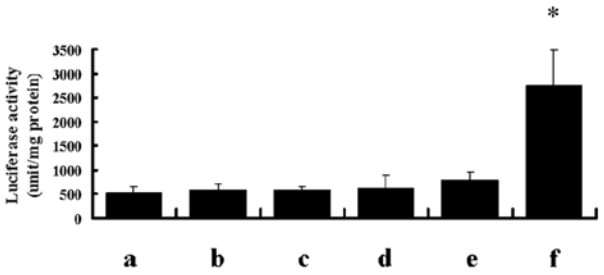

Figure 5. Activation of ISRE by IAB-1. NC65 cells were plated 1 day prior to transfection with control or ISRE promoter luciferase vectors. One day post-transfection, IAB-1 or recombinant huIFN-β protein was added to the wells, and the cells were incubated for an additional 24 h before measuring luciferase activity. a, control (PBS); b, 100 IU/ml recombinant huIFN-β; c, 1000 IU/ml recombinant huIFN-β; d, 10000 IU/ml recombinant huIFN-β; e, 0.01 μl/ml of IAB-1 (0.5 nmol lipid with 0.01 μg plasmid DNA/ml); f, 0.1 μl/ml of IAB-1 (5.0 nmol lipid with 0.1 μg plasmid DNA/ml). Results are expressed as mean ± SD (a-d, n=3; e and f, n=6). *$p<0.05$, compared to other treatment groups.

Lack of evidence for other cytotoxic or pro-apoptotic soluble factors secreted by IAB-1-transfected NC65 cells. To examine if IAB-1-transfected NC65 cells secreted other molecules that contributed to autocrine-mediated induction of cell lysis or apoptosis, we employed a transwell system, which is composed of two compartments (upper and lower) separated by a 1-mm space containing a polycarbonate membrane with 8.0 μm pores. NC65 cells were seeded into both compartments at $5.0x10^4$ cells/well (6-well plate), and IAB-1 or huIFN-β protein was added to the upper wells. Two days after the addition of IAB-1 or huIFN-β protein, the growth of NC65 cells in the lower well was determined and cytotoxicity was evaluated. Using this system, molecules secreted from the IAB-1-transfected NC65 cells in the upper wells transfer through the porous membrane and affect NC65 cells in the lower wells. IAB-1-transfected NC65 cells in the upper wells resulted in lower cytotoxicity of NC65 cells in the lower wells compared to huIFN-β protein-treated cells (Fig. 4). This suggests that IAB-1 does not induce the secretion of soluble factors, other than IFN-β, that significantly impact cell growth or cytotoxicity.

No difference in STAT1, STAT2, or p48 expression between IAB-1-transfected NC65 cells and huIFN-β protein-treated NC65 cells. We next examined the expression of ISGF3, which transduces signals triggered by IFN/IFN-receptor interactions through the cytoplasm and into the nucleus, activating the ISRE. ISGF3 consists of STAT1, STAT2, and p48, and their expression in NC65 cells treated with PBS or empty liposome solution was detected at very low levels by western blot analysis. In contrast, the expression of STAT1, STAT2, and p48 in NC65 cells was upregulated by the addition of 1000 IU/ml huIFN-β protein or 0.1 μl/ml IAB-1, but their levels of expression did not significantly differ between the two treatment groups (data not shown).

The activity of ISRE is enhanced by IAB-1. ISRE is a signal transduction *cis*-acting response element that is activated through IFN-triggered signal transduction pathways to induce the transcription of type 1 IFN-responsive genes. The addition of 0.1 μl/ml IAB-1 induced significant activation of ISRE, whereas 0.01 μl/ml IAB-1 or various concentrations of huIFN-β protein did not activate ISRE (Fig. 5). Luciferase activity was not demonstrated in control transfected with pTA-Luc (data not shown). These results suggest that the IFN-triggered signal transduction pathway is significantly activated in IAB-1-transfected NC65 cells.

IAB-1-induced intracellular expression of IFN-β and IFN-receptor. To further investigate the molecular mechanisms of IAB-1-induced apoptosis and cytotoxicity, compared to those of exogenously added huIFN-β protein, we examined the expression of huIFN-β protein and type 1 IFN-receptor by immunofluorescence. Confocal microscopy enabled us to assess the subcellular expression patterns of huIFN-β protein and type 1 IFN-receptor. In PBS-treated NC65 cells, type 1 IFN-receptor was expressed only on the cell membrane (Fig. 6A). When NC65 cells were treated with huIFN-β protein, huIFN-β protein and type 1 IFN-receptor were also detected on the cell membrane (Fig. 6A). In contrast, in IAB-1-treated NC65 cells, huIFN-β protein and type 1 IFN-receptor were observed predominantly in the cytoplasm and were co-localized (Fig. 6A and B).

c-Myc expression is enhanced by IAB-1. To better understand the mechanism of enhanced cytotoxicity and apoptosis in IAB-1-treated versus huIFN-β protein-treated NC65 cells, we performed a cDNA macroarray. We identified eight genes among the 1185 human genes represented on the array, for which expression differed by ≥3-fold in NC65 cells treated by IAB-1 compared to NC65 cells treated with huIFN-β protein. Among these eight genes, *c-Myc* was of particular interest, because it is reported to be associated with proliferation and apoptosis (17-20). cDNA expression of c-Myc increased by 3.4-fold in NC65 cells treated with IAB-1 compared to huIFN-β protein. The level of c-Myc protein in whole cell lysates extracted 24 h after each treatment was examined by western blot analysis and

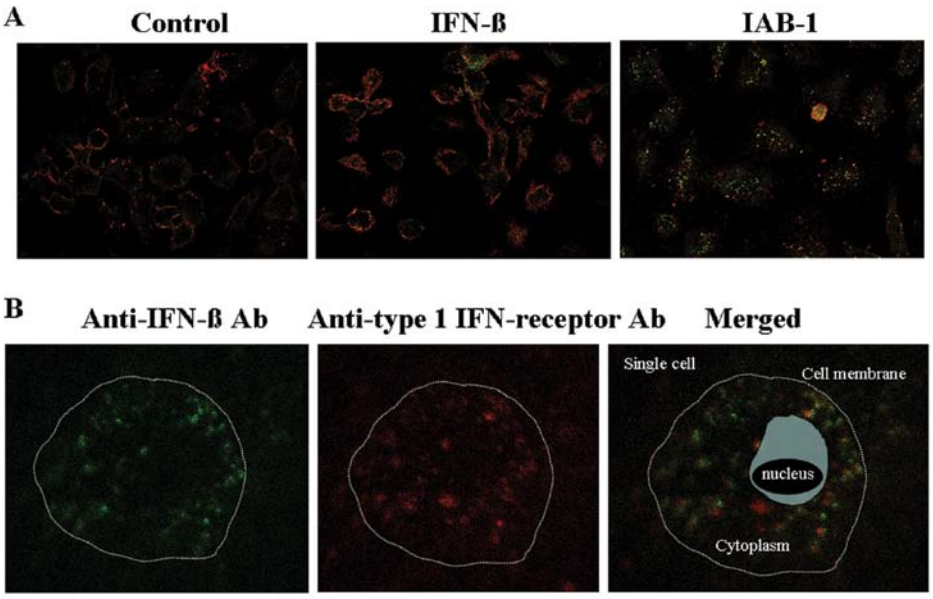

Figure 6. Subcellular localization of IFN-β protein and type 1 IFN-receptor. (A) Merged confocal microscopic images show the subcellular localization of IFN-β protein (green) and type 1 IFN-receptor (red) in NC65 cells by immunofluorescence. Cells were treated with PBS (control), 10000 IU/ml recombinant huIFN-β (IFN-β), or 0.1 μl/ml IAB-1 (IAB-1) . (B) In NC65 cells treated with IAB-1, co-localization of huIFN-β protein (green) and type 1 IFN-receptor (red) was observed in the cytoplasm. In the merged image, superimposed white dotted lines delineate the cell membrane, and the gray area demarcates the nucleus.

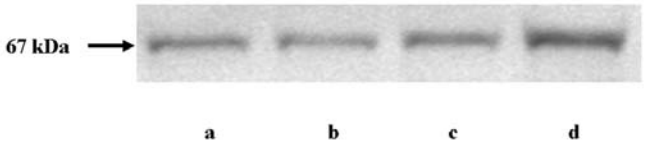

Figure 7. Western blot analysis of c-Myc expression. The relative expression levels of c-Myc protein were determined by western blot analysis using 20 μg whole lysate extracted from NC65 cells after treatment with (a) PBS (control), (b) 0.1 μl/ml of empty liposome (5.0 nmol lipid/ml), (c) 10000 IU/ml recombinant huIFN-β, or (d) 0.1 μl/ml of IAB-1 (5.0 nmol lipid with 0.1 μg plasmid DNA/ml). Cell lysates were separated on 10% polyacrylamide gels, and c-Myc protein (67 kDa) was detected with an anti-c-Myc antibody. Relative expression levels of c-Myc protein were quantified as follows: (a) 1.00, (b) 0.91, (c) 0.93, and (d) 1.91.

was found to be 2.1-fold higher in NC65 cells treated with IAB-1 compared to those treated with huIFN-β protein (Fig. 7).

Discussion

We previously reported that IAB-1 causes significant cytotoxicity against human RCC cells and that apoptosis was induced by IAB-1, but not by recombinant huIFN-β protein (9). However, the molecular mechanisms by which IAB-1, but not recombinant huIFN-β protein, induced apoptosis in the human RCC cell line NC65 were unclear. In this study, we examined the type 1 IFN signal transduction pathway in NC65 cells treated with IAB-1 and compared it to that in NC65 cells treated with recombinant huIFN-β protein. We first examined the status of extracellular signals induced by IAB-1 and huIFN-β protein. Neutralizing anti-huIFN-β antibodies decreased cytotoxicity in NC65 cells exogenously applied with huIFN-β protein, but not in NC65 cells transfected with IAB-1. This suggests that the IFN-β secreted from IAB-1-treated NC65 cells did not contribute to the enhanced cytotoxic and apoptotic effects. Furthermore, transwell experiments suggested that IAB-1 did not induce secretion of other molecules that may have contributed to increased cytotoxicity. Assessing downstream effects of the type 1 IFN signal transduction pathway, luciferase reporter assays indicated that ISRE activity was substantially enhanced by IAB-1.

To elucidate the molecular changes responsible for this increased activity, we examined the expression of ISGF3, which propagates signals triggered by IFN-IFN-receptor associations at the cell membrane through the cytoplasm and into nucleus, activating the ISRE. We found increased expression of the components of ISGF3 (STAT1, STAT2, and p48) in NC65 cells treated with either huIFN-β protein or IAB-1, although their levels did not significantly differ between the two treatments. We speculate that the activation of ISGF3, including phosphorylation, translocation into the nucleus, and expression of co-activators, is more highly induced by IAB-1 compared to huIFN-β protein. However, the molecular mechanism by which only IAB-1, but not huIFN-β protein, activates ISGF3 to enhance ISRE activity is unclear.

It has been reported that transfection of Fas ligand into prostate cancer cells resistant to monoclonal antibody-induced apoptosis induces Fas-mediated apoptosis associated with intracellular Fas ligand expression (21). In addition, it was recently reported that adenovirus encoding *IFN-α* caused marked cell growth inhibition and apoptosis in bladder, prostate, and ovarian cancer cell lines, all of which were resistant to IFN-α protein, in association with the perinuclear expression of IFN-α protein (22). Therefore, we examined the subcellular localization of huIFN-β protein and type 1 IFN-receptor after the transduction of IAB-1 into NC65 cells. Immunofluorescence revealed the co-localization of huIFN-β protein and type 1 IFN-receptor. We postulate that intracellular expression of huIFN-β protein

by IAB-1 transduction into RCC cells leads to the association of huIFN-β protein with type 1 IFN-receptor, enhancing ISRE activity. Our findings, together with those of previous studies (22), suggest that the intracellular expression of IFN protein by way of gene transfer may induce cytotoxicity of cancer cells that are otherwise resistant to IFN protein. Our results highlight the potential clinical application of IAB-1 for gene therapy in RCC cases resistant to IFN.

To elucidate the molecular mechanism of IAB-1-induced cytotoxicity and apoptosis, we compared gene expression profiles of IAB-1- versus huIFN-β protein-treated NC65 cells by cDNA macroarray. The cDNA macroarray revealed that c-Myc mRNA expression increased by 3.4-fold in NC65 cells treated with IAB-1 compared to huIFN-β protein. Western blot analysis confirmed a 2-fold increase in c-Myc protein in the same cells. The *c-Myc* promoter contains four regulatory sequences that are similar to ISRE (23). IRLB has been shown to bind this sequence, and its molecular cloning has been reported (23). The expression of c-Myc is generally suppressed by IFNs (23-26), but its transcriptional regulation is not well understood (23).

Furthermore, c-Myc has been reported to induce apoptosis under certain conditions (17-20). Enhanced expression of c-Myc protein in NC65 cells might be related to IAB-1-induced apoptosis. Additional studies are needed to confirm whether this is the case. It is also possible that other molecules play a role in IAB-1-induced apoptosis. Detailed understanding of the molecular mechanism of *IFN* gene transduction-induced apoptosis in IFN-resistant RCC will help develop more powerful therapeutic modalities for RCC patients that are resistant to conventional immunotherapies.

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