Antitumor effects of a combination of interferon-alpha and sorafenib on human renal carcinoma cell lines

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ABSTRACT

To support the role of interferon (IFN)- α and sorafenib combination therapy against renal cell carcinoma (RCC), the effects of IFN- α and sorafenib on tumor growth, vascular endothelial growth factor (VEGF) production, and phosphorylation levels of extracellular signal-regulated kinase (ERK) and mitogen-activated protein/ERK kinase (MEK) were examined using several cultured RCC cell lines (ACHN, Caki-1, Caki-2, SMKT-R1, SMKT-R2, SMKT-R3 and SMKT-R4). IFN- α or sorafenib alone inhibited the proliferation of all the cell lines except Caki-2, while combined treatment with the two agents showed enhanced inhibitory effects compared to treatment with each agent alone. VEGF production was inhibited by IFN- α alone in ACHN and SMKT-R2 cells and by sorafenib alone in ACHN, Caki-1, SMKT-R1 and SMKT-R2 cells. However, sorafenib increased VEGF production by Caki-2 cells. Interestingly, combined treatment with the two agents suppressed VEGF production by SMKT-R1 and SMKT-R2 cells more strongly than IFN- α or sorafenib alone. Although phosphorylated ERK (p-ERK) was increased after 30 min of treatment with IFN- α alone, no difference was observed between control and IFN- α -treated cells after 2 h. Sorafenib decreased p-ERK in ACHN, Caki-1, SMKT-R1 and SMKT-R2 cells, but increased p-ERK in Caki-2, SMKT-R3 and SMKT-R4 cells, after 2 h. Combined treatment with IFN- α and sorafenib decreased p-ERK compared to treatment with each agent alone in all cell lines except Caki-2. However, IFN- α did not inhibit the p-ERK increase induced by sorafenib in Caki-2 cells. Phosphorylated MEK showed similar patterns to p-ERK after the various treatments. In conclusion, combined treatment with IFN- α and sorafenib suppressed cell proliferation and VEGF production more strongly than treatment with each agent alone in several RCC cell lines.

Since renal cell carcinoma (RCC) is resistant to conventional therapies such as chemotherapy and radiotherapy, immunotherapy regimens using recombinant or natural human interferon- α (IFN- α) and recombinant human interleukin-2 (IL-2) either alone

or in combination have been the standards for treating metastatic RCC due to its known immunogenicity. However, the efficacies of these therapy regimens tend to be insufficient due to their toxicity and generally poor overall response rates.

Recently, the clinical knowledge that RCC is a highly vascular cancer has made antiangiogenic strategies an attractive approach. The treatments for advanced RCC have undergone a major change with the development of potent angiogenesis inhibitors and targeted agents. Sorafenib and sunitinib have already been approved for the treatment of advanced

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RCC and their benefits have been recognized by significant increases in the progression-free survival rates (3).

Sorafenib is a small molecule tyrosine kinase inhibitor selected in screening experiments against c-Raf, and results in diminished phosphorylation of extracellular signal-related kinase (ERK) and mitogen-activated protein/ERK kinase (MEK). ERK links growth factor signaling via receptor tyrosine kinases (RTKs), such as vascular endothelial growth factor (VEGF) receptor, to many downstream effectors, including transcriptional activation, that broadly promote cancer growth and survival. Sorafenib inhibits tumor growth in many xenograft and autograft models, and its antitumor efficacy is primarily attributable to angiogenesis inhibition as a consequence of its inhibition of Raf-MEK-ERK signaling in endothelial cells (2, 10, 13, 26).

Sorafenib can directly inhibit tumor cell growth via Raf-MEK-ERK signaling (10, 26). IFN- α is also well-known to directly inhibit tumor proliferation and induce apoptosis, in addition to its immunomodulatory effects. It has been reported that the actions of IFN- α , including its inhibition of VEGF and basic fibroblast growth factor production, enhance the antitumor and antiangiogenic effects of sorafenib (4). As a consequence, clinical trials have been conducted to assess the efficacy and safety of combination therapies of sorafenib and IFN- α 2b for RCC (5, 19). The combination of sorafenib and IFN- α 2b was found to exhibit substantial activity in treatment-naïve and IL-2-treated patients with RCC. However, despite starting these clinical studies, the basic data required to support the efficacy of combined therapy of IFN- α and sorafenib remain insufficient. A previous study only indicated that MEK and ERK inhibitors enhance the antiproliferative effects of IFN- α (18).

In the present study, we examined the effects of IFN- α and/or sorafenib on tumor growth and VEGF production using several cultured RCC cell lines. We demonstrate that the combined effects of IFN- α and sorafenib differ among the various cell lines. In addition, our data indicate that MEK/ERK signaling may be partially related to the combined effects of IFN- α and sorafenib.

MATERIALS AND METHODS

IFN- α and sorafenib. Human natural IFN- α , which is clinically available in Japan, was purchased from Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan). It was derived from Sendai virus-induced BALL-1

cells and mainly composed of IFN- α 2 and IFN- α 8 (28). Sorafenib [N-(3-trifluoromethyl-4-chlorophenyl)-N'-(4-(2-methylcarbamoyl pyridin-4-yl) oxyphenyl) urea, molecular weight 464.7] was synthesized at Otsuka Pharmaceutical Corporation Co. Ltd. It was dissolved in 100% dimethyl sulfoxide (DMSO) and stored at -80°C until use. After thawing, it was dissolved in culture medium and added to the cells. The final concentration of DMSO was 0.1%.

Cell lines and cell culture. ACHN, Caki-1 and Caki-2 were obtained from the American Type Culture Collection (Rockville, MD). ACHN cells were cultured in Earle's minimal essential medium (MEM) (Invitrogen Inc., Carlsbad, CA) containing 10% fetal bovine serum (FBS; Biological Industries, Kibbutz, Israel) under 5% CO_2 at 37°C , while Caki-1 and Caki-2 cells were cultured in McCoy's 5A medium (Invitrogen Inc.) containing 10% FBS. SMKT-R1, SMKT-R2, SMKT-R3 and SMKT-R4 cells, which were established in our laboratory, were maintained in MEM with D-valine modification medium (Cell Science & Technology Institute Inc., Sendai, Japan) containing 10% FBS. The SMKT-R1, SMKT-R2, SMKT-R3 and SMKT-R4 cell lines were derived from the primary lesion of a patient with RCC, and histopathologically proven to be of conventional RCC origin (12).

Proliferation assay. ACHN, Caki-1 and Caki-2 cells were seeded at 2×10^3 , 1×10^3 and 1×10^3 cells/well, respectively, in 96-well culture plates in 100 μL of medium. SMKT-R1, SMKT-R2, SMKT-R3 and SMKT-R4 cells were seeded at 1×10^3 cells/well. After 1 day of culture, 100 μL of medium containing IFN- α and/or sorafenib at varying concentrations was added. After 5 days, 100 μL of each supernatant was discarded and the numbers of viable cells were determined by the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt] assay using a cell counting kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Four wells were analyzed for each dose of IFN- α and/or sorafenib.

Detection of VEGF produced by cultured cells. ACHN, Caki-1 and Caki-2 cells were seeded at 4×10^4 , 2×10^4 and 2×10^4 cells/well, respectively, in 96-well culture plates in 200 μL of medium. SMKT-R1, SMKT-R2, SMKT-R3 and SMKT-R4 cells were seeded at 1×10^4 cells/well. After 1 day of culture, the supernatants were discarded and the

cells were washed three times with fresh medium to eliminate VEGF. Aliquots (200 μ L) of culture medium containing final concentrations of 1000 IU/mL IFN- α and/or 7 μ M sorafenib were added. After 12 and 24 h, 100 μ L of each supernatant was collected and the amounts of VEGF were determined using an enzyme immunoassay (EIA). The EIA for human VEGF detection was performed essentially as described previously (27). Briefly, 96-well microplates were coated with a mouse anti-VEGF monoclonal antibody in 0.1 M NaHCO₃ (pH 8.0) overnight at 4°C. The wells were then blocked with 0.1% bovine serum albumin in phosphate-buffered saline (PBS) and washed three times. After the addition of each sample to duplicate wells, the plates were incubated at 37°C for 24 h. After washing, a rabbit anti-VEGF antibody was added to each well. The plates were then incubated for 2 h at 37°C, washed three times, and incubated with peroxidase-conjugated goat anti-rabbit immunoglobulins (heavy and light chains; Zymed Laboratories, San Francisco, CA) at room temperature for 2 h. Finally, the plates were washed five times and the enzyme substrate (1 mg/mL o-phenylenediamine in 0.1 M sodium citrate buffer, pH 5.0) was added to each well. After 5 min, the absorbances at 492 nm were measured using a Lab-systems iEMS Reader MF (MTX Lab Systems Inc., Vienna, VA).

Detection of p-MEK, MEK, p-ERK and ERK in cultured cells treated with IFN- α and/or sorafenib. ACHN, Caki-1 and Caki-2 cells were seeded at 4×10^5 , 1.5×10^5 and 2×10^5 cells/well, respectively, in 6-well culture plates in 1.8 mL of culture medium. SMKT-R1, SMKT-R2, SMKT-R3 and SMKT-R4 cells were seeded at 1×10^5 cells/well. After 4 days of culture, 200 μ L of medium containing IFN- α and/or sorafenib was added to each well. The final concentrations of IFN- α and sorafenib were 1000 IU/mL and 7 μ M, respectively. After 0.5 or 2 h, the supernatants were discarded and the wells were washed three times with PBS. Next, 150 μ L of lysis buffer (50 mM Tris-HCl pH 8.0, 280 mM NaCl, 0.2 mM EDTA, 2 mM EGTA, 100 μ M NaVO₄, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40TM and 10% glycerol) was added to each well. After dissolution of the cells, the lysates were centrifuged for 30 min at $30,000 \times g$ at 4°C. The supernatants were used for detection of phosphorylated ERK (p-ERK), ERK, phosphorylated MEK (p-MEK) and MEK. The protein concentrations of the supernatants were determined using the Lowry assay (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis. The samples for MEK/ERK determination were mixed with a loading buffer, boiled for 5 min and subjected to SDS-PAGE. After the electrophoresis, the separated proteins were transferred onto nitrocellulose membranes and incubated with primary antibodies for 16 h at 4°C. The primary antibodies against p-ERK, ERK, p-MEK and MEK (Cell Signaling, Beverly, MA) were each used at a dilution of 1 : 1000. Next, the membranes were washed with 0.9% NaCl containing 0.05% Tween 20 and incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling) at a dilution of 1 : 2000 for 1 h. Antibody-bound protein bands were visualized by enhanced chemiluminescence (Cell Signaling) and images of the bands were scanned. The protein expression levels and phosphorylation statuses were analyzed by measuring the signal intensities using Scion Image (Scion Corporation, Frederick, MD).

Statistical analysis. Values were expressed as the mean $\pm$ standard error (SE). To evaluate the differences between sorafenib and IFN- α doses in proliferation assays, data were analyzed by two-way analysis of variance (ANOVA) followed by the two-tailed Dunnett's test. In other assays, the effects of single agents were analyzed by comparing control data with data for IFN- α or sorafenib alone. Combined effects were analyzed by comparing data for the combined treatment with data for IFN- α or sorafenib alone. The significance of differences in each test was assessed by one-way ANOVA, followed by the two-tailed Dunnett's test. The analyses were performed with SAS software version R8.1 (SAS Institute Japan Ltd., Tokyo, Japan). Values of $P < 0.05$ were considered significant.

RESULTS

Effects of IFN- α and sorafenib on the growth of several RCC cell lines

IFN- α dose-dependently inhibited the proliferation of all RCC cell lines except Caki-2, but the inhibition rates were only 40-50% even at a dose of 1000 IU/mL (Fig. 1A). Sorafenib also dose-dependently inhibited the proliferation of all RCC cell lines except Caki-2 (Fig. 1B). The degrees of inhibition by increasing doses of sorafenib were more prominent than those by increasing doses of IFN- α . Each RCC cell line showed various degrees of inhibition by sorafenib. SMKT-R1 and SMKT-R2 were the most sensitive cell lines. Combinations of IFN- α and sorafenib suppressed the proliferation rates of

Fig. 1 Effects of IFN- α and sorafenib on the proliferation rates of several RCC cell lines. Seven RCC cell lines were seeded into 96-well culture plates. After 1 day of culture, the cells were treated with IFN- α and/or sorafenib at varying concentrations. After 5 days, the numbers of viable cells were determined using a cell counting kit. Values are expressed as the mean $\pm$ SE. (A, B) Effects of IFN- α alone (A) and sorafenib alone (B) on ACHN (-○-), Caki-1 (-△-), Caki-2 (-□-), SMKT-R1 (-◇-), SMKT-R2 (-●-), SMKT-R3 (-▲-) and SMKT-R4 (-■-) cell lines. (C) Combined effects of IFN- α and sorafenib. IFN- α was added at doses of 0, 1, 3, 10, 30, 100, 300 and 1000 IU/mL, while sorafenib was added at doses of 0 (-●-), 1 (-×-), 3 (-□-), 5 (-◇-), 7 (-△-) and 10 (-○-) μ M. The data were analyzed by two-way ANOVA followed by the two-tailed Dunnett's test. In all cell lines except Caki-2, the overall mean values for all doses of IFN- α and sorafenib are significant.

the 6 affected RCC cell lines more strongly than the single agents (Fig. 1C). However, the growth of Caki-2 cells was not inhibited, even by combinations of IFN- α and sorafenib.

Effects of IFN- α and sorafenib on VEGF production by several RCC cell lines

As shown in Table 1, IFN- α alone inhibited VEGF production by ACHN and SMKT-R2 cells at 12 and 24 h. Sorafenib alone inhibited VEGF production by ACHN, Caki-1, SMKT-R1 and SMKT-R2 cells, but increased the VEGF production by Caki-2 cells. The combination of IFN- α and sorafenib suppressed VEGF production by SMKT-R1 and SMKT-R2 cells more strongly than either IFN- α or sorafenib alone.

Effects of IFN- α and sorafenib on phosphorylation of ERK and MEK in several RCC cell lines

Although IFN- α alone increased the p-ERK levels in ACHN, Caki-1 and SMKT-R1 cells after 30 min of treatment, these increases were no longer observed after 2 h of treatment (Fig. 2). Sorafenib alone decreased the p-ERK levels in ACHN, Caki-1, SMKT-R1 and SMKT-R2 cells at either 0.5 or 2 h. On the other hand, sorafenib increased the p-ERK levels in Caki-2 cells at both 0.5 and 2 h. The combined treatment with IFN- α and sorafenib decreased

the p-ERK levels compared to treatment with the single agents in all RCC cell lines except Caki-2. In Caki-2 cells, the intensity of p-ERK for the combined treatment with sorafenib and IFN- α was comparable to that for treatment with sorafenib alone. The p-MEK levels showed a similar pattern to the p-ERK levels for the various treatments. The protein levels of ERK and MEK were not affected by treatment with IFN- α and/or sorafenib.

DISCUSSION

In the present study, we evaluated the antiproliferative effects of IFN- α and sorafenib on several RCC cell lines. The proliferation rates of all the RCC cell lines except Caki-2 were inhibited by IFN- α and sorafenib. The maximum inhibition by IFN- α alone was only 50% of the control value at the dose of 1000 IU/mL, even in the most sensitive cell line (ACHN), whereas sorafenib inhibited the proliferation of SMKT-R1 and SMKT-R2 by 80% at the dose of 10 μ M. The combination of IFN- α and sorafenib showed stronger antiproliferative activities toward all the RCC cell lines except Caki-2 compared with treatment with the single agents. However, the proliferation of Caki-2 cells was not influenced by either IFN- α or sorafenib, or their

Table 1 Effects of IFN- α and sorafenib on VEGF production by several RCC cell lines

		VEGF production (ng/mL)			
		Control mean $\pm$ SE	IFN- α mean $\pm$ SE	Sorafenib mean $\pm$ SE	IFN- α $\pm$ Sorafenib mean $\pm$ SE
ACHN	12 h	1.23 $\pm$ 0.03	0.97 $\pm$ 0.04**	0.59 $\pm$ 0.04**	0.59 $\pm$ 0.01 ^{##}
	24 h	1.24 $\pm$ 0.07	0.92 $\pm$ 0.04**	0.76 $\pm$ 0.02**	0.85 $\pm$ 0.01
Caki-1	12 h	1.16 $\pm$ 0.03	0.93 $\pm$ 0.05**	0.36 $\pm$ 0.02**	0.32 $\pm$ 0.02 ^{##}
	24 h	0.76 $\pm$ 0.07	0.81 $\pm$ 0.01	0.56 $\pm$ 0.05*	0.50 $\pm$ 0.02 ^{##}
Caki-2	12 h	1.03 $\pm$ 0.05	0.81 $\pm$ 0.02**	1.69 $\pm$ 0.01**	1.73 $\pm$ 0.05 ^{##}
	24 h	1.27 $\pm$ 0.02	1.28 $\pm$ 0.05	1.77 $\pm$ 0.04**	1.69 $\pm$ 0.04 ^{##}
SMKT-R1	12 h	3.04 $\pm$ 0.08	2.85 $\pm$ 0.04	2.56 $\pm$ 0.04**	2.16 $\pm$ 0.04 ^{###&}
	24 h	4.43 $\pm$ 0.22	3.78 $\pm$ 0.19	3.53 $\pm$ 0.10*	3.30 $\pm$ 0.05 [#]
SMKT-R2	12 h	2.49 $\pm$ 0.04	2.06 $\pm$ 0.10**	2.15 $\pm$ 0.02**	1.74 $\pm$ 0.02 ^{###&}
	24 h	3.88 $\pm$ 0.15	3.12 $\pm$ 0.03**	3.47 $\pm$ 0.04*	2.39 $\pm$ 0.07 ^{###&}
SMKT-R3	12 h	0.71 $\pm$ 0.08	0.62 $\pm$ 0.05	0.56 $\pm$ 0.03	0.58 $\pm$ 0.02
	24 h	1.60 $\pm$ 0.04	1.05 $\pm$ 0.05**	1.34 $\pm$ 0.06**	1.06 $\pm$ 0.08 ^{&}
SMKT-R4	12 h	0.54 $\pm$ 0.05	0.68 $\pm$ 0.01	0.62 $\pm$ 0.04	0.58 $\pm$ 0.08
	24 h	1.14 $\pm$ 0.05	1.13 $\pm$ 0.05	1.07 $\pm$ 0.04	1.17 $\pm$ 0.03

Seven RCC cell lines were seeded into 96-well plates. After 1 day of culture, the media were removed and exchanged for fresh media containing 1000 IU/mL IFN- α and/or 7 μ M sorafenib. After 12 or 24 h, the culture media were collected and their VEGF protein levels were determined by EIA. The effects of the single agents were analyzed by comparing control data with data for IFN- α or sorafenib alone (* P < 0.05; ** P < 0.01). Combined effects were analyzed by comparing data for the combined treatment with data for IFN- α alone ([#] P < 0.05; ^{##} P < 0.01) or sorafenib alone ([&] P < 0.05; ^{&&} P < 0.01). The significance of differences in each assay was assessed by one-way ANOVA, followed by the two-tailed Dunnett's test.

Fig. 2 Effects of IFN- α and sorafenib on phosphorylation of ERK and MEK in several RCC cell lines. Seven RCC cell lines were seeded into 6-well plates. After 4 days, the cells were treated with 0.1% DMSO as controls (C), 1000 IU/mL IFN- α (IFN), 7 μ M sorafenib (So) or a combination of 1000 IU/mL IFN- α and 7 μ M sorafenib (IFN+So). The cells were lysed after 0.5 or 2 h. (A) The p-ERK, ERK, p-MEK and MEK levels in the cell lysates were determined by western blot analysis. (B) Based on the band images in (A), the amounts of p-ERK were assessed as areas using Scion Image and the percentages relative to the control areas were calculated. Values are expressed as the mean $\pm$ SE. The effects of single agents were analyzed by comparing control data with data for IFN- α or sorafenib alone (* P < 0.05; ** P < 0.01). Combined effects were analyzed by comparing data for the combined treatment with data for IFN- α alone (** P < 0.01) or sorafenib alone ($\Delta\Delta P$ < 0.01). The significance of differences in each assay was assessed by one-way ANOVA, followed by the two-tailed Dunnett's test.

combination. Our data further showed that p-ERK expression and VEGF production by Caki-2 cells were not affected by IFN- α in the presence of sorafenib. It has been reported that Jak1, Tyk2 and Stat1 are phosphorylated in IFN- α -sensitive ACHN and Caki-1 cells by IFN- α , but not in IFN- α -resistant Caki-2 cells (21). These findings and our results suggest that the JAK-STAT pathway may be necessary to achieve the effects of the combination of IFN- α and sorafenib. IFN- α can induce apoptosis in cancer cells, but this effect is modified by anti-apoptotic factors. A recent study demonstrated that IFN- α induces the phosphorylation and expression of eukaryotic translation elongation factor 1A (eEF-1A), which counteracts IFN- α -induced apoptosis (9). Sorafenib antagonizes the eEF-1A increase induced

by IFN- α via inhibition of c-Raf activation, and enhances IFN- α -induced apoptosis. In RCCs, the enhanced antiproliferative effects by the combination of IFN- α and sorafenib may be related to eEF-1A expression determined by the balance of the effects of IFN- α and sorafenib.

VEGF is a key mediator in the pathogenesis of RCC. RCCs have been shown to express higher levels of VEGF mRNA and protein than normal kidney tissues (15, 23). IFN- α inhibits the growth of human neuroendocrine tumors and reduces their VEGF mRNA levels (24). In the present study, we determined the combined effects of IFN- α and sorafenib on VEGF secretion by RCC cell lines. IFN- α alone inhibited VEGF production by ACHN and SMKT-R2 cells, but its effects were weak. The

effects of sorafenib alone on VEGF production varied among the cell lines, similar to the results for the cell proliferation. Sorafenib inhibited VEGF production by ACHN and Caki-1 cells, but enhanced its production by Caki-2 cells. Interestingly, the combination of IFN- α and sorafenib showed prominent inhibitory effects on SMKT-R1 and SMKT-R2 cells exhibiting the highest secretion of VEGF among the analyzed cell lines. The effects of IFN- α and sorafenib on VEGF production showed a similar trend to their effects on cell proliferation.

Inhibition of the Raf-MEK-ERK pathway is expected to be a potential strategy in the treatment of advanced cancers, since ERK activation is required for tumor cell proliferation. Sorafenib binds to Raf and inhibits its kinase activity by maintaining it in an inactive configuration (25), thereby resulting in diminished MEK-1 and ERK-1 phosphorylation (26). IFN- α inhibits ERK activation in hepatomas and transformed T-cells and monocytoid cells (6, 17), but enhances its activation in HeLa and myeloma cells (1, 22). In the present study, we determined the phosphorylation levels of ERK and MEK in cells whose growth rates were affected by IFN- α and sorafenib. The p-ERK levels were increased in ACHN, Caki-1 and SMKT-R1 cells after 30 min of treatment with IFN- α alone, but returned to the control levels after 2 h. On the other hand, the responses to sorafenib alone varied among the cell lines. ERK and MEK phosphorylation was decreased in cell lines that were highly sensitive to sorafenib, such as ACHN, Caki-1, SMKT-R1 and SMKT-R2, but increased in cell lines showing low sensitivity to sorafenib, such as Caki-2, SMKT-R3 and SMKT-R4. The effects of sorafenib on ERK phosphorylation may be related to its effects on tumor proliferation and VEGF production. The p-ERK levels were comparable between the combined treatment and treatment with sorafenib alone in ACHN, Caki-1 and SMKT-R1 cells, and between the combined treatment and treatment with IFN- α alone in SMKT-R3 and SMKT-R4 cells. Previous studies have indicated that upregulation of VEGF secretion requires ERK activation (8, 11, 16). Our data showed that inhibition of ERK activation by both agents could be related to their antiproliferative activity and suppression of VEGF secretion. The p-MEK levels were similar to the p-ERK levels. The augmentation of antitumor effects by combined treatment with IFN- α and sorafenib may be expressed, at least partially, through the Raf-MEK-ERK pathway.

Abundant neovascularization and arteriovenous fistula formation are distinct characteristics of

RCCs. Tumor vascularity is related to the clinical outcomes because metastases are more likely in patients with highly vascularized RCCs. Since the serum VEGF levels in RCC patients are elevated compared with control subjects (20) and significantly correlated to both stage and grade (7), control of VEGF is essential for treating patients with RCC.

A clinical study demonstrated that IFN- α 2b therapy significantly decreased the serum VEGF level (29). On the other hand, sorafenib induced an increase in circulating VEGF levels and a decrease in soluble VEGF receptor levels (14). In the present study, VEGF production by SMKT-R1 and SMKT-R2 cells expressing higher levels of VEGF mRNA than other RCCs (23) was more strongly suppressed by the combination of IFN- α and sorafenib than by IFN- α or sorafenib alone. These results suggest that the targets for combined treatment with IFN- α and sorafenib could be tumors secreting high levels of VEGF. Moreover, examination of VEGF secretion and ERK phosphorylation may be useful for predicting the antitumor effects of combination therapy.

We have shown that combined treatment with IFN- α and sorafenib inhibited cell proliferation and VEGF production more strongly than treatment with each agent alone in several RCC cell lines. Our data could provide support for clinical application of IFN- α and sorafenib to patients with RCC.

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