Antiproliferative Effects of Interferon-αCon1 on Ovarian Clear Cell Adenocarcinoma *In vitro* and *In vivo*

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ABSTRACT

Purpose: We examined the antiproliferative effect of IFN-αCon1 and its mechanism on ovarian clear cell adenocarcinoma *in vitro* and *in vivo*.

Experimental Design: (*a*) The effects of IFN-αCon1 on growth, morphology, cell cycle, and type I IFN-α receptor (IFNAR-2) expression were examined on two ovarian clear cell adenocarcinoma cell lines (KOC-5C and KOC-7C) *in vitro*. (*b*) KOC-5C or KOC-7C cells were transplanted into nude mice, and changes in tumor volume, tumor weight, apoptosis, necrosis, and microvessel density were investigated. The expression of angiogenesis factors was examined in the serum and the developed tumors.

Results: Both cell lines expressed IFNAR-2 mRNA, but its protein was detected only in KOC-7C. In KOC-7C cells, antiproliferative effects were observed in a time- and dose-dependent manner and cell division was blocked at the S phase. The KOC-7C tumors showed decreases in tumor volume and weight; a decreasing tendency in basic fibroblast growth factor (bFGF), vascular endothelial growth factor, and interleukin (IL)-8 protein expression in the tumor; a significant decrease in bFGF and IL-8 protein expression in the serum, and of microvessel density; and significant increase in apoptosis and necrosis in the tumor. In the KOC-5C tumors, these *in vitro* and *in vivo* changes were not apparent, and the antiproliferative effects of IFN-αCon1 were not obvious.

Conclusions: IFN-αCon1 suppresses tumor proliferation by inducing apoptosis, blocking the cell cycle, and inhibiting tumor angiogenesis. Our findings show that the clinical efficacy of IFN-αCon1 can be predicted by examining IFNAR-2 expression on tumor cells, and the efficacy of IFN-αCon1 treatment can be evaluated by measuring serum bFGF and IL-8 levels.

INTRODUCTION

The frequency of ovarian carcinoma in Japan has increased remarkably from 0.8 in 100,000 females in 1950 to 5.4 in 1990. Ovarian carcinoma is quite malignant, and the death rate is the highest among malignant gynecologic tumors. Diagnosis and therapeutic treatment are also difficult because they vary in malignancy and histologic type. Major histologic types of ovarian carcinoma are serous, mucinous, endometrioid, and clear cell. Sugiyama *et al.* (1) reported that clear cell carcinoma presents biological features different from other ovarian carcinomas. Clear cell carcinoma is characterized with poor prognosis that is worse than the other types, high detection rate at stage I, resistance to chemotherapy (mainly cisplatin), and frequent association with endometriosis. The frequency of clear cell adenocarcinoma (CCA) is thought to be 5–10% of all ovarian carcinoma in Western countries, and it is higher in Japan. Currently, a combination of paclitaxel and carboplatin is well known as a postoperative chemotherapy for ovarian carcinoma, but this therapy is not efficacious for CCA (2); thus, researchers have been seeking other remedies.

Recent studies reveal the involvement of angiogenesis in the infiltration and proliferation of ovarian carcinoma, and the clinical application of IFN, which is an angiogenesis prohibitor for ovarian carcinoma, has been studied since 1981 (3–12). However, the results and reported mechanisms of action vary widely, and consensus on clinical efficacy has not yet been reached. More recently, the suppressive effect of IFN on the proliferation of cultured cell lines and of transplanted tumor cells on animals has been reported, and the efficacy of IFN in combination with other medicines is indicated (7, 12–16).

IFN was found by Nagano and Kojima in 1954 as a virus-inhibiting factor (17), and showing virus interference is a phenomenon discovered by Isaac and Lindenmann in 1957 (18). Among IFNs, IFN-α possesses various biological activities in addition to antiviral effects (*e.g.,* antiproliferative activity and immunoregulatory activity; ref. 19), and it is clinically indicated for renal cancer and leukemia (20–22). Reported mechanisms of the antitumor effects of IFN-α are the direct suppressive effect on tumor proliferation and indirect effects such as the enhancement of tumor killing through the activation of immune cells and inhibition of tumor growth through the suppression of angiogenesis (23–27).

Consensus interferon (IFN-αCon1) is a wholly synthetic Type 1 IFN, developed by scanning several IFN-α nonallelic

subtypes and assigning the most frequently observed amino acid in each position (28). Compared with other IFN-α species, IFN-αCon1 shows more potent antiproliferative effects and NK cell activation activity (29), and it is expected to be efficacious in cancer treatment. This study investigates (*a*) its antiproliferative effects on ovarian CCA *in vitro* and *in vivo*, (*b*) the mechanism of its antiproliferative action, (*c*) the expression of IFN-α receptors, and (*d*) the relationship between the expression of the angiogenesis factor and IFN-αCon1.

MATERIALS AND METHODS

Ovarian CCA Cell Lines and Culture Conditions. The human ovarian CCA cell lines KOC-5C and KOC-7C were established in the culture as described elsewhere (30). These cell lines were grown in DMEM (Nissui Seiyaku Co., Japan) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Bioserum, Victoria, Australia), 100 units/mL penicillin, 100 μg/mL streptomycin (Life Technologies, Inc., Gaithersburg, MD) and 12 mmol/L sodium bicarbonate, in a humidified atmosphere in 5% CO_2 in air at 37°C.

Reagents. IFN-αCon1 (Advaferon) was kindly provided by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Specific activity of the IFN-αCon1 was 1×10^9 IU/mg protein. Monoclonal antihuman type II IFN receptor (IFNAR-2; IgG, clone ANOC4866) was kindly provided by Cellular Technology Institute, Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). The other antibodies were purchased from the following manufacturers: (*a*) fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin and anti-BrdUrd antibody, Becton Dickinson Immunocytometry System (San Jose, CA); (*b*) control normal mouse IgG1 antibody, DAKO (Glostrup, Denmark); (*c*) monoclonal antihuman vascular endothelial growth factor (VEGF) antibody and monoclonal antihuman interleukin (IL)-8-fluorescein antibody, R&D System (Minneapolis, MN); and (*d*) antihuman basic fibroblast growth factor (bFGF) monoclonal antibody, Wako Pure Chemical Industries, Inc (Tokyo, Japan).

Analysis of bFGF, IL-8, VEGF, and IFNAR-2 mRNA Expression by Reverse-Transcription-PCR. Reverse transcription-PCR analysis for bFGF, IL-8, VEGF, and IFNAR-2 were described in our previous reports (31–35) in detail. The *IFNAR-2* gene has been reported to produce four different transcripts that encode three different forms of *IFNAR-2* (36). The specific primers reported by Lutfalla *et al.* (36) were used to amplify *IFNAR-2a* and *IFNAR-2c* cDNAs, and one of the two *IFNAR-2b* cDNAs (31, 32). The PCR product (5 μL) was electrophorased with a 2% NuSieve agarose gel (FMC Bioproducts, Rockland, ME) that contained 0.5% ethidium bromide, and specific DNA bands were examined under a UV transilluminator.

Flow Cytometric Analysis for bFGF, IL-8, VEGF, and IFNAR-2. Flow cytometric analysis for bFGF, IL-8, VEGF, and IFNAR-2 was done as described in our previous reports (31–35). Briefly, after cell fixation and permeabilization, cell suspension (4×10^5 cells/tube) was reacted with monoclonal anti-bFGF antibody, anti-IFNAR-2 antibody (final concentration, 10 μg/mL), monoclonal anti-IL-8 antibody, anti-VEGF antibody (final concentration, 50 μg/mL), or an appropriate control antibody, except for the IL-8 samples, the cells of which

were reacted with FITC-conjugated goat antimouse IgG. All samples were analyzed with a FACScan (Becton Dickinson Immunocytometry System).

Observation of Morphological Changes. For light-microscopic observation, cells were seeded on Lab-Tek Tissue Culture Chamber Slides (Nunc, Inc., Roskilde, Denmark), cultured with IFN-αCon1 (0, 1,000, or 4,000 units/mL) for 72 hours, fixed in Carnoy's solution for 10 minutes, and then stained with H&E.

Effect of IFN-αCon1 on the Proliferation of Ovarian CCA Cell Lines. The effect of IFN-αCon1 on the proliferation of cells was investigated with colorimetric assays with MTT [3-(4,5-dimethylthiazol-2yl-yl)-2, 5-diphenyltetrazolium bromide] cell growth assay kits (Chemicon International, Inc., Temecula, CA) as described in our previous report (31, 37). Briefly, cells (5×10^3 cells/well) were seeded on 96-well plates (Nunc), cultured for 24 hours, and then the medium was replaced into a fresh 100 μL medium alone or a medium containing IFN-αCon1 (0, 250, 1,000 or 4,000 units/mL). After 24, 48, 72, or 96 hours, viable cell numbers were counted. Eight samples were used in each experiment, and each experiment was repeated at least 3 times.

Cell Cycle Analysis. Cells were cultured with or without IFN-αCon1 (1000 or 4000 units/mL) for 24, 48, 72, or 96 hours, labeled with 10 μmol/L BrdUrd for 30 minutes, fixed in 70% cold ethanol at 4°C overnight, and stained with anti-BrdUrd antibody and propidium iodide (Sigma Chemical Co., St. Louis, MO) with the technique described elsewhere (37). The stained cells were analyzed with a FACScan.

Effect of IFN-αCon1 on Ovarian CCA Cell Proliferation in Nude Mice. The ovarian CCA cells KOC-5C and KOC-7C (1×10^7 cells/mouse) were transplanted subcutane-
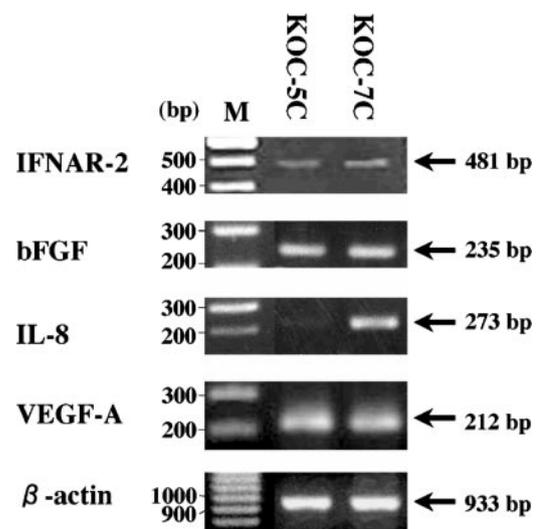

Fig. 1 Reverse transcription-PCR analysis of the expression of IFNAR-2, bFGF, IL-8, and VEGF-A mRNAs in the ovarian CCA cell lines KOC-5C and KOC-7C. The PCR products were electrophoresed in a 2% (for bFGF, IL-8 and VEGF-A) or 4% (for IFNAR-2) NuSieve agarose gel and were stained with ethidium bromide. *Lane M* shows a 100 bp ladder, which was used as the DNA molecular-weight marker. The positions of the expected size of intact products are indicated.
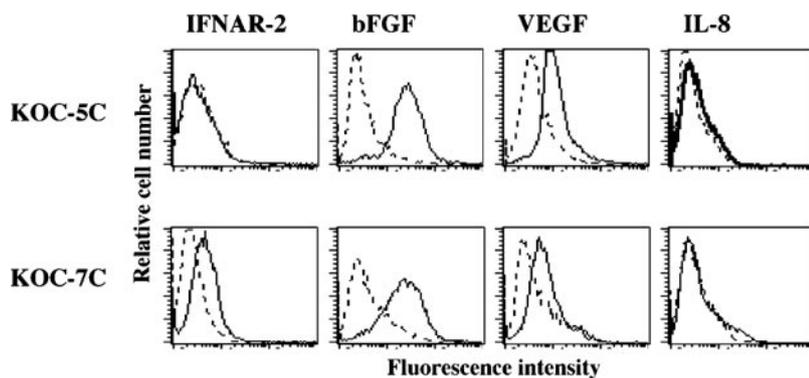
Fig. 2 Flow cytometric analysis of IFNAR-2, bFGF, IL-8, and VEGF-A. These factors were expressed on the ovarian CCA cell lines KOC-5C and KOC-7C. Staining with specific antibodies or the control antibody is shown by the *solid and the dotted line*, respectively. IFNAR-2 expression on the cell surface was undetectable in KOC-5C but detectable at a low level in KOC-7C (positive cell rate, 4.1%). The rate of cells that were positive in KOC-5C for bFGF, VEGF, and IL-8 was 66.8, 12.0, and 3.0%, respectively, and 33.4, 6.3, and 3.7% in KOC-7C.

ously into 5-week-old female BALB/c athymic nude mice (Clea Japan, Inc., Osaka, Japan). On the 18th post-transplantation day when tumor formation was confirmed, the mice were divided into four groups (*n* = 5–7 each) in a manner to equalize the mean tumor diameter of every group. Each mouse received a subcutaneous injection of 0.1 mL of PBS containing either 0, 1×10^4, 1×10^5, or 1×10^6 IU of IFN-αCon1 for 14 consecutive days. The lowest concentration (1×10^4 IU/mouse) was about 1.3 times greater than the clinical dose for chronic hepatitis C. Tumor size was measured in two directions using calipers 3 times per week, and tumor volume (mm³) was estimated as "length × (width)² × 0.5." The tumors were resected 2 weeks after the initial injection. After measuring the weight, the tumors were used for morphological studies (*e.g.,* H&E staining and immunohistochemistry) and ELISA analysis. The number of apoptotic cells was counted in ten 0.25 mm²-areas within an H&E-stained specimen, and the average number per area was obtained. In the counting, necrotic areas were excluded. In addition, the appearance of the apoptotic cells was confirmed with the TUNEL [terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling] technique (Apoptag

Peroxidase *In Situ* Apoptosis Detection kit, Chemicon International Inc.). The area of necrosis in each specimen was quantified with an NIH image 1.61 software program. Each animal experiment was repeated at least twice.

The animals were treated in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH, publication no. 86–23, revised 1985).

Quantification of Microvessel Density. Double immunohistochemical staining was done with antimouse endothelial cell (anti-CD34) antibody, antihuman α smooth muscle actin antibody, Histofine simple stain mouse MAX-PO (Rat) kits (Nichirei, Tokyo, Japan) and HistoMouse-plus kits (Zymed Labs, Inc., San Francisco, CA). Histofine simple stain mouse MAX-PO (Rat) kits were used to first immunostain the tumor sections with antimouse endothelial cell antibody, and they were reacted with a mixture of 3,3-diaminobenzidine tetrahydrochloride. We conducted the second staining with antihuman α-smooth muscle actin antibody by making the section react to the primary antibody, α-SMA, at 4°C overnight, and by using the HistoMouse-plus kits. The sections were developed with the mixture of a commercial chromogen (VIP) and hydrogen per-
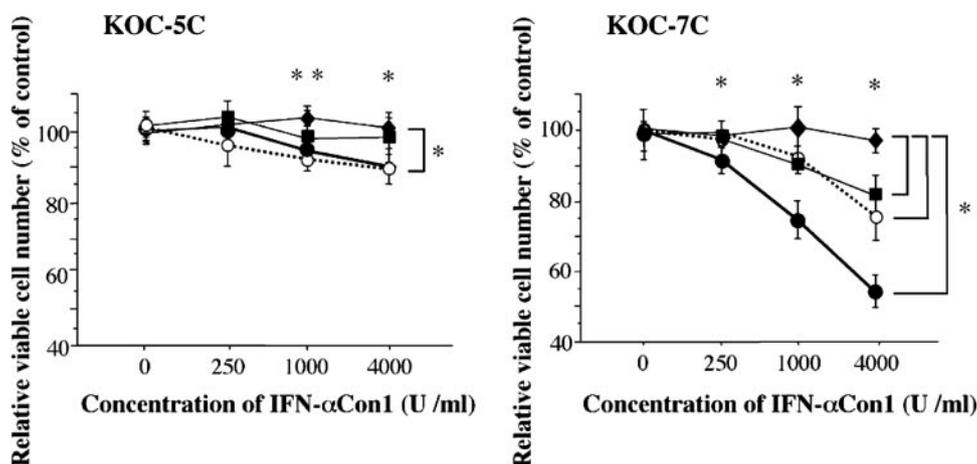
Fig. 3 Effect of IFN-αCon1 on the growth of ovarian CCA cell lines. Cells (5,000 cells/well) were seeded on 96-well plates and incubated in a medium with or without IFN-αCon1 (250–4000 units/mL) for 24 to 96 hours. The relative viable cell number was calculated as a percentage of the viable cell number per experimental well to the number in the corresponding control culture. Significant differences between the values for the experimental wells and their corresponding control wells are as follows: ∗*P* < 0.001, ∗∗*P* < 0.05. The *error bar* represents SD. The *arrows* indicate the concentration of IFN-αCon1 (units/mL). ◆, 24 hours; ■, 48 hours; ○, 72 hours; ●, 96 hours.

Fig. 4 Time course of changes in the cell cycle of the ovarian CCA cell line (KOC-7C) cultured with 1,000 or 4,000 units/mL of IFN-αCon1 or with medium alone (control). The cells were labeled with BrdUrd (30 minutes, 37°C), fixed, stained with anti-BrdUrd antibody and propidium iodide, and analyzed with a FACScan. The contour plots show the effect of IFN-αCon1 on the cell cycle of KOC-7C. The data represent the percentages of the cells in the S phase.
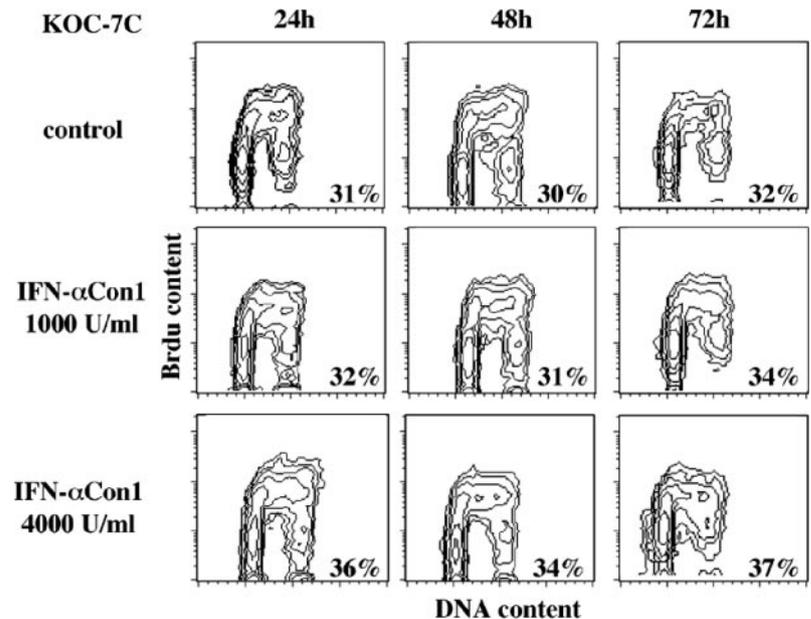
oxide (Vector VIP Substrate kit, Vector Labs, Inc., Burlingame, CA), and counterstaining was done with hematoxylin. The number of microvessels in a unit area (one mm^2) was counted on every section, and the mean number of the group was obtained.

ELISA. The tumors were cut into pieces, and a pellet pestle was used to homogenize an appropriate amount in 500 μL of ice-cold Ca^{2+}- and Mg^{2+}-free PBS containing 100 μL/mL phenylmethylsulfonyl fluoride. The mixture was centrifuged for 10 minutes (12,000 rpm, 4°C), and the supernatant was stored at −20°C until use. The amount of bFGF, IL-8, and VEGF in the supernatant and serum was measured with the Quantikine ELISA kit (bFGF and IL-8, Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom; VEGF, TECHNE Corporation, Minneapolis, MN). The amount of tissue protein

was determined with a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

Statistical Analysis. The differences in tumor weight and tumor volume, the results of the MTT assay and ELISA and microvessel density (MVD) among the groups were compared by ANOVA.

RESULTS

Expression of IFNAR-2, bFGF, IL-8, and VEGF mRNAs. The two cell lines expressed a 481 bp band. This corresponds to IFNAR-2c, which is the binding subunit and is necessary for IFN-α to express its effects. The cell lines did not express the 350 and 713 bp bands that correspond to the PCR products of
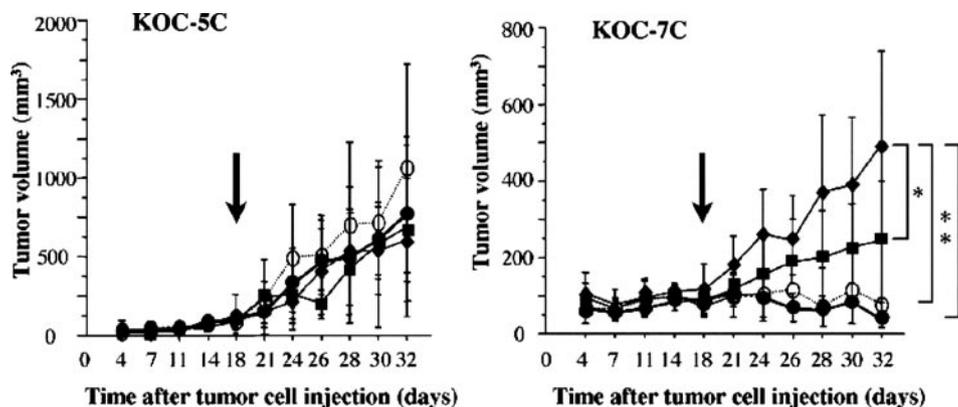
Fig. 5 Changes in estimated tumor volume. KOC-5C and KOC-7C cells were transplanted subcutaneously to nude mice, and 0.1 mL of IFN-αCon1 diluted with PBS into 1×10^4, 1×10^5, or 1×10^6 units/mouse or 0.1 mL of PBS was injected subcutaneously for 14 consecutive days from the 18th post-transplantation day (*arrow*) when tumor formation was confirmed. The mice were sacrificed on the 32nd day. *$P < 0.05$, **$P < 0.001$, and ***$P < 0.0001$ by ANOVA. The *error bar* represents SD. The *arrows* indicate the days. ♦, 0 units/mL; ■, 1×10^4 units/mL; ○, 1×10^5 units/mL; ●, 1×10^6 units/mL. Each animal experiment was repeated at least twice, and almost the same results were obtained. The figures show representative results.

Table 1 Treatment of human ovarian CCA that was growing subcutaneously in nude mice

Dosage of IFN-αCon1	Number	Frequency*	Tumor weight (g)†	
KOC-5C				
Saline (control)	5	5/6	0.331	(1.01–0.249)
1×10^4 units	5	5/6	0.394	(0.996–0.184)
1×10^5 units	5	5/6	0.726	(1.656–0.133)
1×10^6 units	5	5/7	0.441	(0.570–0.217)
KOC-7C				
Saline (control)	7	7/7	0.253	(0.396–0.087)
1×10^4 units	6	6/6	0.137	(0.227–0.021)‡
1×10^5 units	6	6/6	0.107	(0.293–0.0507)§
1×10^6 untis	6	6/6	0.047	(0.079–0.017)¶

NOTE. KOC-5C or KOC-7C human ovarian CCA cells (1×10^7) were subcutaneously transplanted into nude mice. From the 18th day after the transplantation, mice in each group received IFN-αCon1 or saline injection for 14 consecutive days. All mice were sacrificed on the 32nd day.

* Number of mice that developed tumor/number of mice that received transplantation.

† Median and range.

‡ $P < 0.05$, *versus* control.

§ $P < 0.01$, *versus* control.

¶ $P < 0.001$, *versus* control.

IFNAR-2a and IFNAR-2b, respectively. bFGF and VEGF-A (VEGF165) were expressed in both of the cell lines. The expression of IL-8 was more apparent in KOC-7C (Fig. 1).

IFNAR-2 expression on the cell surface was undetectable on KOC-5C cells, whereas IFNAR-2 expression was detectable at a low level on KOC-7C cells (positive cell rate, 4.1%). The cells that were positive for bFGF and VEGF accounted for 66.8% and 12.0%, respectively, in KOC-5C, and the rates in KOC-7C were 33.4% and 6.3%, respectively. IL-8 expression was detected at a very low level, *i.e.,* 3.0% in KOC-5C and 3.7% in KOC-7C (Fig. 2).

Effects of IFN-αCon1 on Apoptosis, Proliferation, and the Cell Cycle. Seventy-two hours after the addition of IFN-αCon1 to the culture medium, no remarkable morphological changes were observed on KOC-5C, whereas KOC-7C presented apoptosis-specific changes such as shrinkage of the cy-

toplasm, nuclear concentration, chromatin condensation, and nuclear fragmentation in a dose-dependent manner (data not shown).

The proliferation of both KOC-5C and KOC-7C was suppressed with IFN-αCon1, and it was more remarkable in KOC-7C. In KOC-5C, suppression was observed 48 hours or later after the addition of IFN-αCon1, and suppression at the 72nd hour was substantial compared with the 24th hour ($P < 0.001$). The cell number decreased by 16% at maximum in the group that had 1,000 or 4,000 units/mL of IFN-αCon1 compared with the control ($P < 0.05$, $P < 0.001$). In KOC-7C, suppression was observed 24 hours or later after the addition of IFN-αCon1, the effects increased in a time- and dose-dependent manner, and the cell number decreased by 45% at maximum ($P < 0.001$; Fig. 3).

In KOC-7C, the ratio of cells in the S phase tended to increased, and that in the G_1 phase tended to decreased slightly under the IFN-αCon1 treatment compared with the control at any time point during the 24–72 hours after the addition of 1,000 or 4,000 units/mL of IFN-αCon1 to the cultures (Fig. 4). The experiment was done twice, and almost the same results were obtained.

Effects of IFN-αCon1 on Proliferation of Ovarian CCA in Nude Mice. Figure 5 summarizes the chronological changes in estimated tumor volume. The volume of the KOC-5C tumors decreased slightly (although not significantly) when 1×10^6 units/mouse of IFN-αCon1 was administered. The volume of the KOC-7C tumors decreased markedly in a time- and dose-dependent manner, and the difference in tumor volume among the groups became apparent from the 8th day of IFN-αCon1 treatment ($P < 0.05$, between the control and each IFN-αCon1 group, and between 1×10^4 units/mouse and 1×10^6 units/mouse).

The weight of the KOC-5C tumors increased in the 1×10^5 units/mouse group, but there were no changes in the other dosage groups. The weight of the KOC-7C tumors decreased dramatically in a dose-dependent manner, and the weight of the 1×10^6 units/mouse group decreased by approximately 81% compared with the control ($P < 0.05$, between every IFN-αCon1 group and the control, Table 1).

H&E staining revealed that the appearance of apoptotic

KOC-7C

Control　　　**IFN-αCon1**　　　**TUNEL staning**
　　　　　　　　1×10^6 U

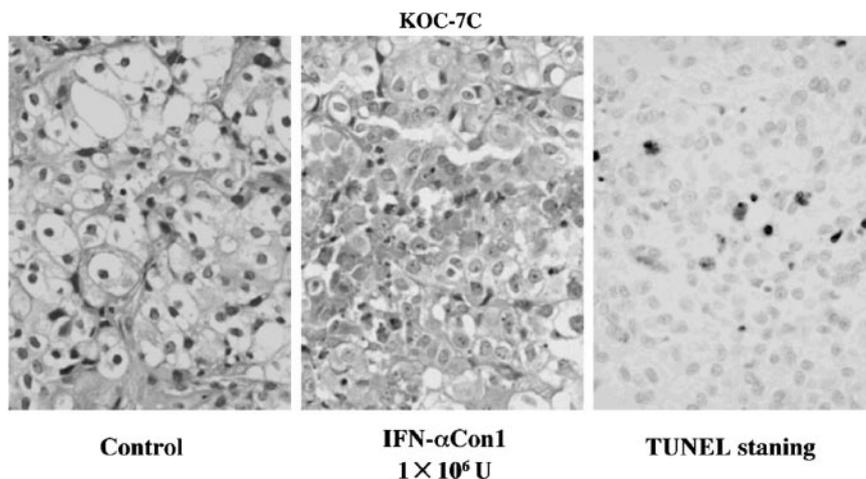*Fig. 6* Apoptosis and necrosis (H&E staining, ×40). On the 32nd day, tumors of the KOC-5C and KOC-7C cells were resected, fixed in formalin, prepared in paraffin sections, and underwent H&E staining. In the KOC-7C tumors, the number of apoptotic cells and necrosis decreased significantly with 1×10^6 units of IFN-αCon1. The TUNEL technique was used to confirm the appearance of apoptotic cells.

Table 2 Apoptosis and necrosis in human ovarian CCA that was growing subcutaneously in the control and IFN-αCon1 (1×10^6 units) treated nude mice

Dosage of IFN-αCon1	Apoptosis (mean ± SD)*	Necrosis (%, mean ± SD)†
KOC-5C		
Saline (control)	3.30 ± 0.822	11.25 ± 2.500
1×10^4 units	3.86 ± 1.808	7.00 ± 2.739
1×10^5 units	3.90 ± 1.430	12.00 ± 4.472
1×10^6 units	3.70 ± 1.056	7.00 ± 2.739
KOC-7C		
Saline (control)	4.27 ± 0.939	2.95 ± 2.502
1×10^4 units	4.42 ± 1.542	3.59 ± 3.177
1×10^5 units	8.20 ± 3.332	19.57 ± 16.768
1×10^6 units	19.07 ± 4.745‡	30.16 ± 22.847§

NOTE. The KOC-5C or KOC-7C cells (1×10^7) were subcutaneously transplanted into nude mice. From the 18th day after the transplantation, mice in each treatment group received IFN-αCon1 or saline for 14 consecutive days. Tumor was obtained on the 32nd day.

* The number of apoptotic cells per areas in each section was counted, and the mean of each group was obtained.

† The area of necrosis in each specimen was quantified with an NIH image 1.61 software program.

‡ $P < 0.001$, *versus* control.

§ $P < 0.01$, *versus* control.

cells and necrosis in the KOC-5C tumors of the groups with IFN-αCon1 treatment was no different from that of the control group. On the other hand, in the KOC-7C tumors, the number of apoptotic cells as well as the number of necrotic lesions increased significantly in a dose-dependent manner ($P < 0.01$; Fig. 6; Table 2).

MVD in a unit area (one mm^2) of the KOC-5C tumors did not show significant differences between the groups with or without IFN-αCon1, whereas MVD within the KOC-7C tumors decreased significantly with 1×10^6 units of IFN-αCon1 ($P < 0.005$). MVD on the margin of the KOC-7C tumors did not decrease (Table 3).

Detection of bFGF, IL-8, and VEGF *In vivo* with ELISA. The expression of bFGF, VEGF, and IL-8 in the KOC-5C tumors did not change among the groups with or without IFN-αCon1 treatment, whereas the KOC-7C tumors presented a decreasing tendency in the expression of bFGF and IL-8, and there was a substantial decrease in VEGF ($P < 0.005$, Table 4). The serum levels of the three angiogenesis factors were not affected by IFN-αCon1 in the nude mice with KOC-5C

tumors, whereas in the mice with KOC-7C tumors, IL-8 decreased significantly in the groups that received 1×10^5 ($P < 0.05$) or 1×10^6 units/mouse ($P < 0.01$), and bFGF decreased significantly in all dosage groups ($P < 0.01$ for 1×10^4, $P < 0.05$ for 1×10^5, and $P < 0.001$ for 1×10^6; Table 4).

DISCUSSION

In our *in vitro* experiment, IFN-αCon1 suppressed the proliferation of both KOC-5C and KOC-7C, and suppression was more obvious for KOC-7C. Previous studies have shown that the mechanisms of growth inhibition by IFN-α are the induction of apoptosis and the inhibition of cell cycle progression at the S, G_2-M, or G_1 phase (31, 38, 39). We also examined the mechanism of growth inhibition and found morphological evidence (*i.e.,* in KOC-7C cells) of apoptotic cells increasing in number with the increase in IFN-αCon1 level. Cell cycle analysis with the double-staining technique with anti-BrdUrd antibody and propidium iodide showed that the ratio of cells in the S phase tended to increase in a dose- and time-dependent manner. These findings suggest that IFN-αCon1 directly inhibits the proliferation of KOC-7C by inducing apoptosis, necrosis, and blockage of cell cycle progression at the S phase. However, these changes were not obvious in KOC-5C.

In our *in vivo* experiment, IFN-αCon1 induced a remarkable decrease in estimated tumor volume as well as actual tumor weight in the KOC-7C tumors in a dose-dependent manner, but the nude mice with KOC-5C tumors did not show significant differences between the control and the IFN-αCon1–treatment groups. The mechanism of the *in vivo* antiproliferative effect was examined by monitoring angiogenesis, the expression of angiogenesis factors, and apoptosis. Well-known angiogenesis factors relating to cancer metastasis and invasion are bFGF (40, 41), IL-8 (42, 43), VEGF (34, 44), and matrix metalloproteinase (45–48). IFN-α is reported to suppress angiogenesis and thus tumor proliferation by decreasing the expression of these factors, mainly of bFGF and IL-8 (40–43). We examined this point by measuring the amount of angiogenesis factors expressed in the subcutaneous tumor using ELISA and found a decreasing tendency in bFGF, VEGF, and IL-8 with IFN-αCon1 treatment. The serum levels of the factors were also measured, and IL-8 and bFGF decreased significantly although VEGF did not. IL-8 decreased in a dose-dependent manner, and bFGF decreased significantly with both a high dose and a low dose of IFN-αCon1 to a similar degree. These results show that the effects of

Table 3 Microvessel density in human ovarian CCA that was growing subcutaneously in the control and IFN-αCon1 (1×10^6 units) treated nude mice

Cell line	Treatment	MVD (mean ± SD, pg/ml)*		
		Inside tumor	Margin	Total no. of microvessels
KOC-5C	Saline (control)	19.7 ± 8.0	9.8 ± 4.0	22.3 ± 9.0
	IFN-αCon1 (1×10^6 units)	23.6 ± 14.0	9.0 ± 9.0	26.3 ± 15.0
KOC-7C	Saline (control)	27.0 ± 11.0	2.7 ± 1.0	29.7 ± 12.0
	IFN-αCon1 (1×10^6 units)	12.5 ± 4.0†	4.5 ± 1.1	17.0 ± 4.0†

NOTE. The KOC-5C or KOC-7C cells (1×10^7) were subcutaneously transplanted into nude mice. From the 18th day after the transplantation, mice in each treatment group received IFN-αCon1 or saline for 14 consecutive days. Tumor was obtained on the 32nd day.

* The number of microvessels per unit area in each section was counted, and the mean of each group was obtained.

† $P < 0.05$, *versus* control.

Table 4 ELISA of human ovarian CCA that was growing subcutaneously in nude mice

Dosage of IFN-αCon1	Serum level (pg/ml)			Levels in the tumor lysate (pg/ml)		
	BFGF	IL-8	VEGF	BFGF	IL-8	VEGF
KOC-5C						
Saline (control)	77.5 ± 32.0	17.0 ± 4.0	236.9 ± 38.0			
1×10^4 units	46.7 ± 24.0	15.5 ± 4.0	272.5 ± 43.0			
1×10^5 units	74.9 ± 45.0	30.2 ± 13.0	288.8 ± 31.0			
1×10^6 units	73.4 ± 20.0	28.8 ± 13.0	205.1 ± 39.0			
KOC-7C						
Saline (control)	81.3 ± 14.0	23.2 ± 4.0	229.0 ± 50.0	341.3 ± 123.0	1348.4 ± 1722.0	858.3 ± 272.0
1×10^4 units	34.5 ± 9.0 †	22.0 ± 3.0	231.1 ± 39.0			
1×10^5 units	47.4 ± 22.0*	17.1 ± 4.0*	262.3 ± 28.0			
1×10^6 units	31.6 ± 11.0‡	15.0 ± 5.0†	215.5 ± 24.0	277.3 ± 122.0	120.7 ± 58.0	565.1 ± 138.0*

NOTE. The KOC-5C or KOC-7C cells (1×10^7) were subcutaneously transplanted into nude mice. From the 18th day after the transplantation, mice in each group received IFN-αCon1 or saline for 14 consecutive days. All mice were sacrificed on the 32nd day.

* $P < 0.05$, *versus* control.
† $P < 0.01$, *versus* control.
‡ $P < 0.001$, *versus* control.

IFN-αCon1 vary among the factors and indicate that IFN-αCon1 reduced the expression of IL-8 and bFGF on tumor cells, thus suppressing angiogenesis in the tumor, having an antiproliferative effect.

MVD in the KOC-7C tumors decreased significantly with high-dose IFN-αCon1 administration. In this respect, IFN-αCon1 does not directly inhibit angiogenesis by inducing growth inhibition or apoptosis in mouse endothelial cells because human IFNα-Con1 does not act on mouse cells. This suggests that IFN-αCon1 indirectly suppresses angiogenesis by inhibiting the expression of angiogenesis factors in tumor cells. In addition, an increase in apoptosis and necrosis in tumor was morphologically confirmed, and this indicates that IFN-αCon1 directly induces apoptosis, and indirectly induces necrosis, by suppressing tumor angiogenesis. On the other hand, the KOC-5C tumors did not show substantial changes in the expression of angiogenesis factors or the appearance of apoptosis and necrosis.

The effects of IFN-α are mediated through interaction with the specific cell-surface receptor, *i.e.,* type I IFN receptor (36, 49, 50). The receptor consists of at least two chains (*i.e.,* AR-1 and AR-2). AR-1 is essential for the expression of a high affinity receptor, and AR-2 is the subunit to which IFN binds. AR-2 also consists of three forms, *i.e.,* short (AR-2b) and long (AR-2c) forms that are *trans*-membrane proteins and a soluble form (AR-2a) that is secreted outside the cell. These forms are probably produced by exon skipping, alternative splicing of the same gene, or the use of a different polyadenylation position. AR-2c is the most important form for the expression of IFN action as well as for normal IFN binding (36, 49). Reverse transcription-PCR showed the expression of IFNAR-2c mRNA, but not of IFNAR-2a or IFNAR-2b mRNAs, on the two ovarian CCA cell lines, and this indicates that the expression levels of AR-2a and AR-2b are low. Flow cytometric analysis revealed the protein expression of AR-2c on KOC-7C but not on KOC-5C. This suggests that IFN-αCon1 cannot bind with its receptor on KOC-5C because there is little expression of IFNAR-2, and the signal transduction pathway of IFN-αCon1 is not activated in the cells. This may result in the apparent difference between the two cell lines on the antiproliferative effects of IFN-αCon1 and its suppression of angiogenesis factors. It can be argued that it is difficult to understand how KOC-7C cells respond to IFN-α if only 4.1% of the cells expressed IFNAR-2. In our previous study (31), however, the proliferation of several hepatocellular carcinoma cell lines expressing a comparable level of AR-2c was also suppressed significantly by IFN-α.

Our findings show that the clinical efficacy of IFN-αCon1 can be predicted by examining IFNAR-2 expression on tumor cells, and the efficacy of IFN-αCon1 treatment can be evaluated by measuring serum bFGF and IL-8 levels.

Our study shows the antiproliferative effect of IFN-αCon1 on ovarian carcinoma; however, IFN treatment for ovarian carcinoma has not yet fully established, and there are only few reports using animal experiments or on the clinical application of CCA. In recent trials, IFN-α has been used with IFN-γ, retinoid (7, 16), IL-2 (20), or chemotherapy such as 5-fluorouracil (21) and paclitaxel (13). To fully use the action of IFN-αCon1, additional studies on combination therapy as well as the suppression of tumor proliferation on other histologic types of ovarian carcinoma are needed. The latter is currently under way using three ovarian serous adenocarcinoma cell lines. In addition, we plan to inject our ovarian carcinoma cell lines into various sites in nude mice, such as the ovary (orthotopic site) and the peritoneal cavity and to examine the antitumor effect of IFN-αCon1 in different environments.

ACKNOWLEDGMENTS

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Clin Cancer Res 2004;10:7418-7426.

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