Type I interferon prolongs cell cycle progression via p21$^{WAF1/CIP1}$ induction in human colon cancer cells

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Received April 2, 2007; Accepted May 30, 2007

Abstract. Type I interferon (IFN) was originally identified as an immunomodulatory cytokine because of its antiviral activity. Further characterization of its biological effects revealed a prominent role in the direct control of cell growth and potent immunomodulatory and antiangiogenic actions. IFN-α and IFN-ß had both been classified as type I IFN, but differences in their antitumor activities were reported. We confirmed the difference in the antiproliferative activities of IFN-α2b and IFN-ß toward HT29 and SW480 cells. IFN treatment was observed to prolong cell cycle progression; in particular, the accumulation of S-phase population was one of the most characteristic changes. The prolongation of S-phase progression and transition into G2/M-phase was suggested to be a crucial action of type I IFN on colon cancer. Additionally, IFN activated the p21 promoter gene and induced p21$^{WAF1/CIP1}$ expression. Furthermore, the cell cycle prolongation effect of IFN was suppressed when p21 expression was downregulated. Therefore, we confirmed that p21$^{WAF1/CIP1}$ was a crucial target molecule for the effects of IFN on the cell cycle. Additionally, the ability of p21 induction differed between IFN-α2b and IFN-ß and correlated with their inhibitory activities toward cell growth. We conclude that type I IFN prolongs cell cycle progression by p21$^{WAF1/CIP1}$ induction in human colon cancer cells.

Introduction

Colorectal cancer is one of the most frequent malignancies worldwide. Surgical resection is generally conducted as a major treatment for patients with colorectal cancer, but approximately 30% of postoperative patients experience a recurrence within 5 years (1). For prognostic improvement of colorectal cancer, postoperative adjuvant therapy is one of the most important factors. Various regimens of chemotherapy include 5-fluorouracil (5-FU), irinotecan (CPT-11) and so on, (2,3) but the results have not been satisfactory yet. Under this situation, some studies reported that chemotherapies with interferon (IFN)-ß were effective for patients with advanced colorectal carcinoma (4-6).

IFN-α and IFN-ß are classified as type I IFN, and both interact with the IFN-α receptor (IFNAR), but some studies have reported the difference between their antitumor activities (7,8) Type I IFN was originally identified as an immunomodulatory cytokine because of its antiviral activity. Further characterization of its biological effect revealed a prominent role in the direct control of cell growth as well as potent immunomodulatory and antiangiogenic actions (9-11). Indeed, the antiproliferative effects of IFN have been reported in many malignancies such as leukemia (12) and hepatocellular carcinoma (HCC) (7). Some studies reported that the cell growth inhibition effect of IFN resulted from the control of cell cycle progression, including G1-phase arrest, S-phase prolongation, and blockage of transition into G2/M phase (7,13,14). Others reported that the cell growth inhibition was related with the induction of apoptosis (15,16).

The antiproliferative mechanisms of type I IFN is not well understood. A detailed investigation of the molecular mechanism could redefine the potential therapeutic benefit of IFN. Additionally, understanding the target molecule of its growth inhibitory pathways might be important in tumor treatment. In the present study, we confirm the cell growth inhibitory effect of type I IFN on colon cancer cell lines and elucidate the mechanism and the target molecule for cell growth control.

Materials and methods

Cells. The colon cancer HT29 cells were obtained from the American Type Culture Collection (Rockville, MD); SW480 cells were a kind gift from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (D-MEM/F12) and

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Key words: interferon, p21, cell cycle

phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS), trypsin/EDTA, penicillin, and streptomycin were obtained from Gibco BRL/Life Technologies, Inc. (Gaithersburg, MD). Both HT29 and SW480 cells were grown in a culture medium consisting of D-MEM/F12 with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at a temperature of 37˚C in a humidified atmosphere of 5% CO_2 in air.

Chemicals. Recombinant human IFN-α2b was kindly provided by Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan); natural human IFN-ß by Toray Co. Ltd. (Tokyo, Japan). The antibodies for Cyclin A, Cyclin B, Cdk1, Cdk2, p19, p21, and p27 were obtained from BD Bioscience (San Diego, CA); the antibody for ß-actin, from Sigma Chemical Co. The antibodies for human IFNAR-1 and human IFNAR-2 were kindly provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). All secondary antibodies were purchased from Amersham Biosciences (Braunschweig, Germany).

Growth inhibition assay. A collagen solution was prepared using a collagen gel culture kit (Nitta Gelatin Inc., Osaka, Japan). The viable cells were collected and embedded into collagen gel droplets (30 μl per drop) according to the method of collagen gel droplet embedded-culture drug sensitivity test (CD-DST) (17) and cultured in serum-added medium for 24 h. IFN was then added to the medium at the concentrations of 0, 50, 500, or 5,000 IU/ml, and the mixture was incubated for periods of 48, 96, or 144 h. The cells were then transferred to an IFN-free medium and cultured until the total incubation period reached 144 h. At the end of the incubation, neutral red was added to each well to a final concentration of 50 μg/ml. The cells were fixed with 10% formalin for 45 min and washed in water. After drying the collagen gel droplets, the total volume of the tumor colonies was calculated with the help of the Scion Image computer program (Scion Corp., Frederick, MD). *In vitro* sensitivity is expressed as the percentage T/C ratio, where T is the total volume of tumor colonies in the treated group, and C is that of tumor colonies in the control group.

Flow cytometry. After trypsination, ~1x10^6 cells were collected by centrifugation at 1,500 rpm for 3 min. The cells were then washed in PBS followed by resuspension and fixation in 70% ethanol for 2 h. Next, they were briefly centrifuged, washed in PBS, and resuspended in 500 μl PBS containing 100 μg RNase and incubated for 30 min. Cellular DNA was then stained by the addition of 10 μg propidium iodide, and a total of 10,000 cells per condition were analyzed using FACScan (Beckman Coulter, Fullerton, CA).

Western blot analysis. Cells were rinsed twice with ice-cold PBS and lysed in lysis buffer (CelLytic™ -M reagent; Sigma Chemical Co.) containing protease and phosphatase inhibitors (Sigma Chemical Co.). The lysed cells were collected using a scraper and centrifuged at 12,000 rpm and 4˚C for 15 min to obtain a pellet of the cellular debris. After removing the protein-containing supernatant, the samples were divided into aliquots and kept at -80˚C.

After the sample buffer [60 mM Tris/HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue] was added, the samples were boiled for 5 min. The protein sample (20 μg) was subjected to electrophoresis, transferred onto polyvinylidene difluoride membranes, and blocked in PBS-T containing 5% non-fat dry milk for 2 h at room temperature. Incubation with the primary antibody was carried out overnight at 4˚C. After washing in PBS-T, the membranes were incubated with horseradish peroxidase-conjugated second antibody for 1 h, followed by washing. Bands were visualized using the ECL system (Amersham Pharmacia Biotech, UK). Hyperfilm ECL was exposed to the membrane, and the intensity of the specific bands was calibrated by Scion Image.

Plasmids. The luciferase reporter plasmid containing the full-length promoter of the mouse $p21^{WAF1/CIP1}$ gene, pGL3b-4542, was described previously (18,19). The thymidine kinase promoter-driven Renilla luciferase plasmid pRL-TK was obtained from Promega (Madison, WI).

Luciferase reporter assay of p21 promoter activity. The cells were plated on 12-well plates at a density of 5x10^5 per well and were transfected using the FuGENE6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) with the luciferase reporter plasmid (1 μg/well) and with the thymidine kinase promoter-driven Renilla luciferase plasmid pRL-TK (20 ng/well). After 48 h, the cells were lysed, and the luciferase activity was measured with a Dual Luciferase Assay Kit (Promega). The activity of firefly luciferase was normalized by that of Renilla luciferase.

RNA interference. The RNA interference (RNAi) technique is used for downregulating the expression of a specific gene in living cells by introducing a homologous double-stranded RNA (dsRNA), and 21-base small interfering RNAs (siRNAs) are potent mediators of the RNAi effect in mammalian cells. The p21 siRNA (silencer validated siRNA) was purchased from Ambion (Foster City, CA). The targeting sequences of dsRNA and complimentary dsRNA were 5'-GGAGUCAGA CAUUUUAAGAtt-3' and 5'-UCUUAAAAUGUCUGACUC Ctt-3'. The dsRNAs were transfected into SW480 cells for 48 h using the FuGENE6 reagent by following the protocol provided by the manufacturer.

Results

Growth inhibitory effects of IFN-α2b and IFN-ß for colon cancer cell lines. We used two human colon cancer cell lines, HT29 and SW480, and first assessed their expressions of IFNAR. As seen in the results shown in Fig. 1, the expressions of IFNAR-1 (110 kDa), IFNAR-2(b) (52 kDa), and IFNAR-2(c) (102 kDa) were detected in both cell lines.

Next, we investigated the cell growth inhibitory activities of type I IFN by using CD-DST methods. IFN-α2b and IFN-ß showed cell growth inhibitory activities in both cell lines, but the effect of IFN-ß was significantly higher than that of IFN-α2b at every concentration ($P<0.05$) (Fig. 2A). In the SW480 cells, in particular, the growth inhibitory effect of IFN-ß increased in a dose-dependent manner ($P<0.05$), but

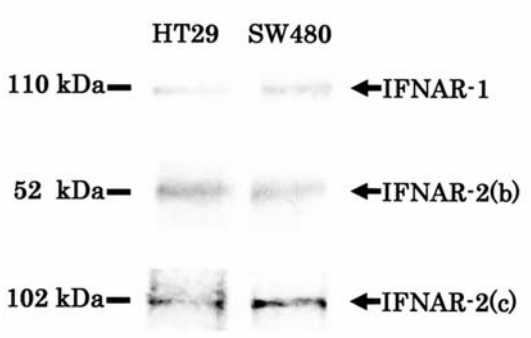

Figure 1. Expressions of IFNAR-1 and IFNAR-2 in colon cancer cell lines. Protein sample (20 μg) was subjected to electrophoresis and transferred onto polyvinylidene difluoride transfer membranes. The membranes were incubated overnight with anti-human IFNAR-1 or anti-human IFNAR-2 polyclonal antibodies at 4˚C, washed with PBS-T, and then incubated with HRP-conjugated second antibody for 1 h. Bands were visualized using the ECL system. Hyperfilm ECL was exposed to the membrane. IFNAR-1 protein; IFNAR-2 protein [IFNAR-2(b) and IFNAR-2(c)]. Lane 1, HT29; lane 2, SW480.

the relationship between exposure time and the inhibitory effect of IFN was not clear (Fig. 2B).

Induction of apoptosis by IFN. To investigate whether IFN induced apoptosis in these cell lines, we evaluated apoptosis as DNA fragmentation of cells by using gel ladder analysis. We could not detect any difference between the effects on controls and IFN-treated cells (data not shown).

Effect of IFN on cell cycle distribution. Next, we tested for change in the cell cycle distribution in response to the IFN treatment by flow cytometry (Fig. 3A). The G0/G1-phase population of control cells began to decrease immediately after release from serum starvation, reached a minimum level after 16 h, and then increased slowly. On the other hand, the cell cycle in IFN-treated cells progressed so slowly that the decrease in the G0/G1-phase population was prolonged until 32 h (Fig. 3B). Synchronously, the increase in the S-phase and G2/M-phase populations was also delayed and prolonged in IFN-treated cells as compared with control cells. The accumulation of the S-phase population was one of the most characteristic changes induced by the cell cycle effects of IFN, and this change was more potent in IFN-ß-treated cells than in IFN-α2b-treated cells.

Effect of IFN on the expressions of S-phase regulators. The progression through S-phase and transition into G2/M-phase are regulated by the sequential activation of cyclin A-Cdk1, cyclin A-Cdk2, and cyclin B-Cdk1 complexes. To elucidate the mechanism of cell cycle prolongation, we analyzed whether the expressions of S-phase regulators would change in response to IFN treatment. In control cells, the expressions of cyclin A and cyclin B began to increase immediately after release from serum starvation, reached a maximum level after 16 h, and then decreased slowly (Fig. 4). In IFN-treated cells, the change in the expressions of these cyclins was slow, and the maximum expression level was higher than that observed in control cells. The expression pattern of cyclin A and cyclin B

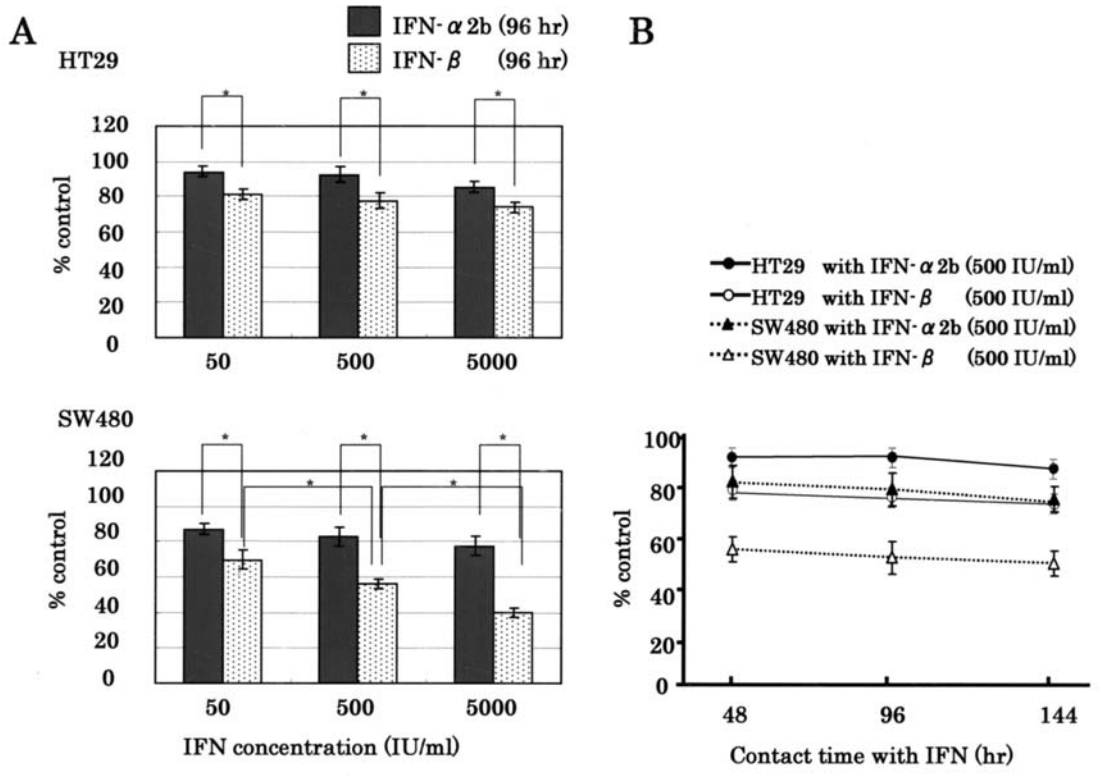

Figure 2. Growth inhibitory effects of IFN-α2b and IFN-ß on colon cancer cell lines. (A) HT29 and SW480 cells were incubated in the absence (control) or presence of IFN (IFN-α2b or IFN-ß; 50, 500, or 5000 IU/ml) for 96 h, and (B) both cell lines were incubated with IFN (500 IU/ml) for the indicated times (48, 96 and 144 h). The growth inhibitory effects are determined as the percentage T/C ratio (% control), as described in Materials and methods. The data shown are mean ± SD of 3 experiments. *P<0.05; statistical significance was determined by Student's t-test.

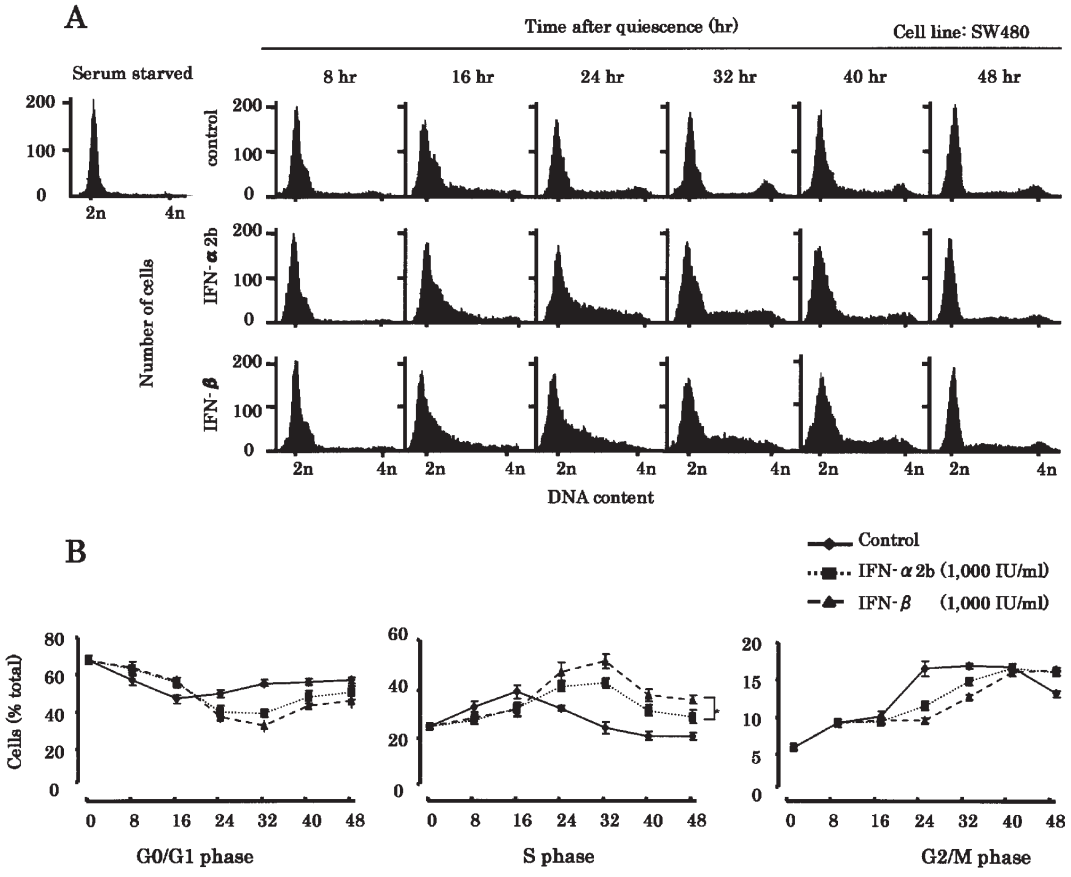

Figure 3. Effect of IFN on cell cycle distribution. (A) Cell cycle progression of synchronized SW480 cells grown in the absence (control) or presence of IFN (IFN-α2b or IFN-ß; 1,000 IU/ml) at the indicated periods after quiescence. (B) A summarized time course of cell cycle distribution in SW480 cells (●, control; ■, IFN-α2b; ▲, IFN-ß). The data shown are mean ± SD of 3 independent experiments. (*P<0.05).

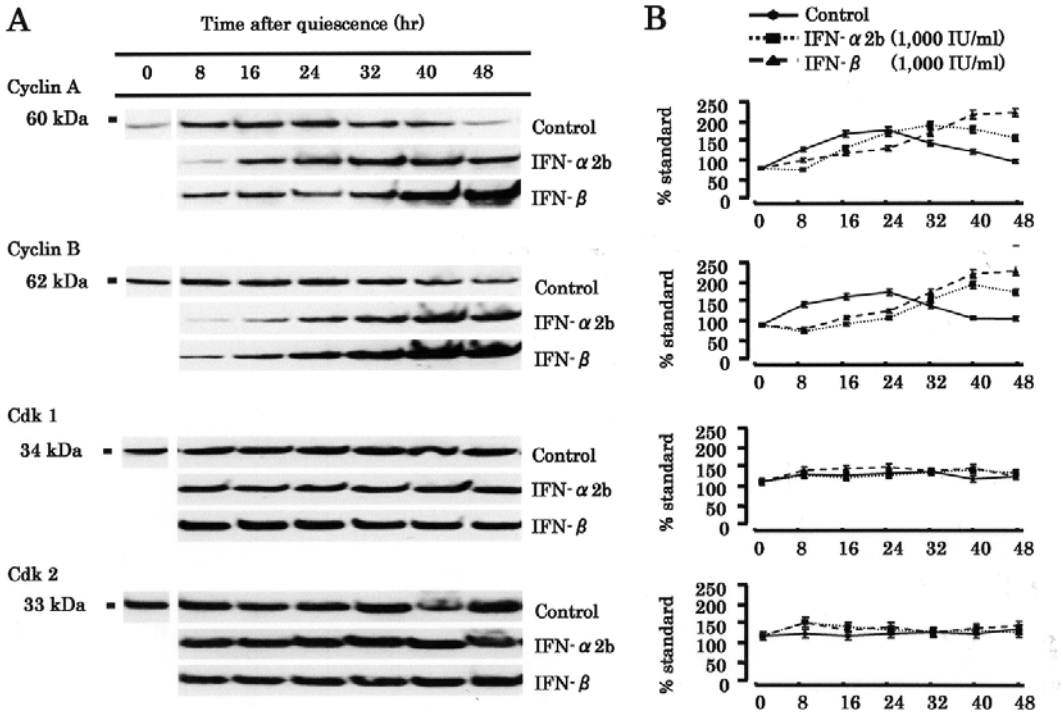

Figure 4. Effect of IFN on the expressions of S-phase regulators. Whole cell lysates were isolated from synchronized cells, which were grown in the absence (control) or presence of IFN (IFN-α2b or IFN-ß; 1,000 IU/ml), at the indicated periods after quiescence and subjected to Western blot analysis. The band intensity was determined by densitometry in 3 independent experiments. The values obtained for controls and IFN-treated cells are expressed as the percentage of the values obtained when using time-matched ß-actin as an internal standard (data not shown) (●, control; ■, IFN-α2b; ▲, IFN-ß). The data shown are mean ± SD of 3 independent experiments.

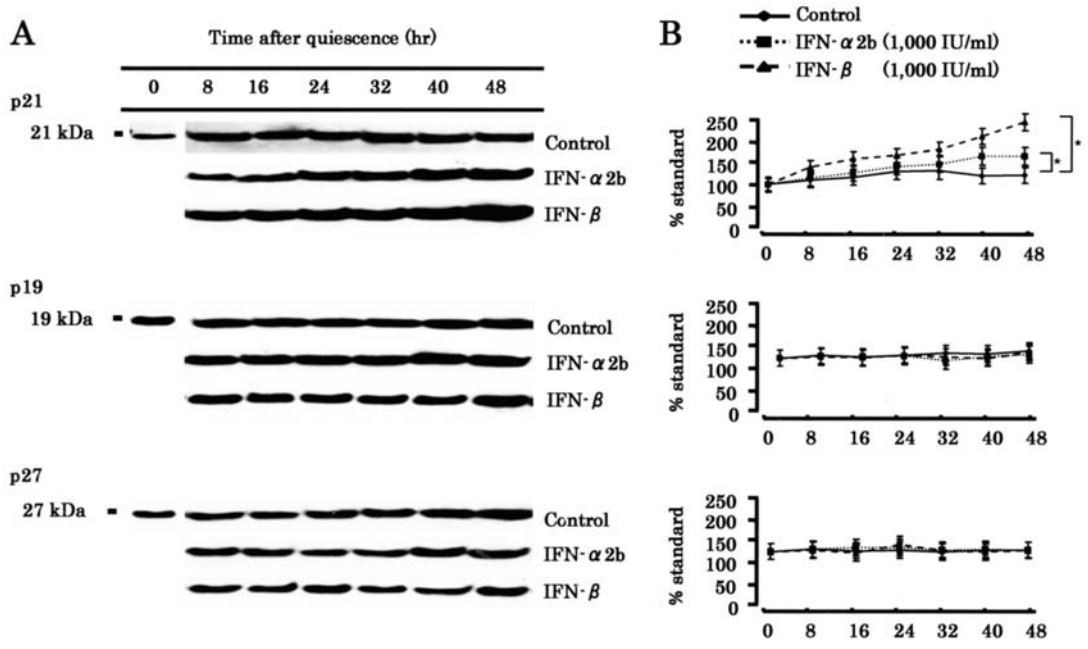

Figure 5. Effect of IFN on the expressions of CKIs. Whole cell lysates were isolated from synchronized cells, which were grown in the absence (control) or presence of IFN (IFN-α2b or IFN-ß; 1,000 IU/ml), at the indicated periods after quiescence and subjected to Western blot analysis. The band intensity was determined by densitometry in 3 independent experiments. The values obtained for controls and IFN-treated cells are expressed as the percentage of the values obtained when using time-matched ß-actin as an internal standard (data not shown) (●, control; ■, IFN-α2b; ▲, IFN-ß). The data shown are mean ± SD of 3 independent experiments.

was found to be correlated with the change in the S-phase population on flow cytometric analysis. On the other hand, the expressions of cyclin-dependent kinases (Cdk) were invariable throughout the whole experiment in all groups.

Effect of IFN on the expressions of Cdk inhibitors. Cdk inhibitors (CKIs) are well known to bind to cyclin-Cdk complexes and block their activities. We examined whether the expressions of CKIs ($p21^{WAF1/CIP1}$, $p27^{Kip1}$, and $p19^{Ink4}$) would change in response to IFN treatment. As seen in the results shown in Fig. 5, in control cells, the expression of p21 did not change throughout the whole experiment; however, in IFN-treated cells, the p21 expression kept increasing until 48 h. Moreover, the increase in p21 expression was significantly higher in IFN-ß treated cells than in IFN-α2b-treated cells ($P<0.05$). Further, densitometric analysis showed that the expression of p21 in the IFN-ß group was ~2.5 times the intensity of the control at 48 h, whereas that in the IFNα2b group was ~1.5 times this intensity. On the other hand, we did not detect any change in p27 and p19 expressions on IFN treatment.

Effect of IFN on p21 promoter activity. We analyzed p21 promoter gene activity by performing the luciferase reporter assay. The transactivity of the p21 promoter gene in IFN-treated cells was approximately twice the increase observed in the control at 24 h ($P<0.05$), and the activity was higher in the IFN-ß group than in the IFN-α2b group (Fig. 6).

Effect of IFN on cell cycle distribution in p21 knockdown cells. To investigate whether the induction of p21 was required for IFN-mediated cell cycle effects, we examined the change in

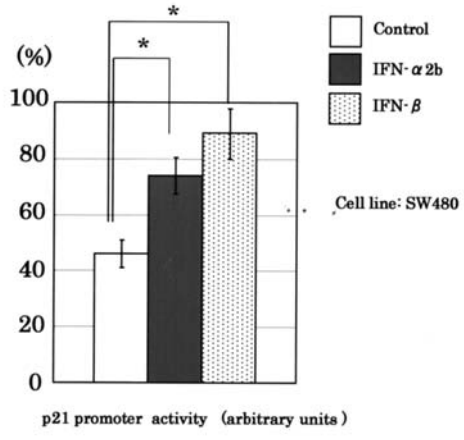

Figure 6. Effect of IFN on p21 promoter activity. SW480 cells were subjected to transient transfection with the luciferase reporter plasmid containing the full-length promoter of the mouse $p21^{WAF1/CIP1}$ gene (pGL3b-4542) and with the thymidine kinase promoter-driven Renilla luciferase plasmid pRL-TK. Following transfection, the cells were cultured in the absence (control) or presence of IFN (IFN-α2b or IFN-ß; 1,000 IU/ml) for 24 h. Subsequently, the cells were lysed, and the luciferase activity was measured with a Dual Luciferase Assay Kit. The activity of firefly luciferase was normalized to that of Renilla luciferase. The data shown are mean ± SD of 3 independent experiments.

cell cycle distribution in p21 knockdown cells by using RNAi. First, we confirmed that p21 expression was downregulated by RNAi throughout the experimental period (Fig. 7A). Subsequently, we analyzed the cell cycle distribution in p21 knockdown cells, which were grown in the absence or presence of IFN. In p21 knockdown cells, the cell cycle prolongation effect of IFN was reduced, and we could not observe any

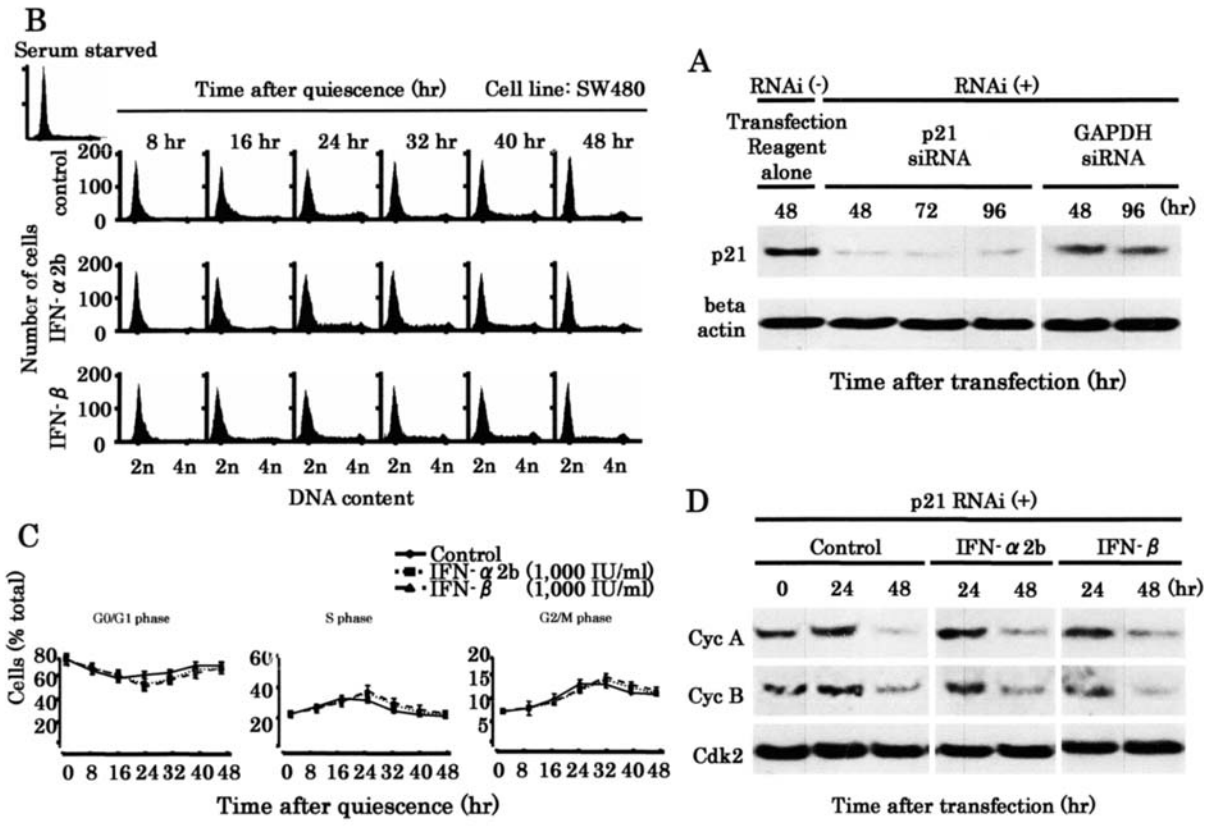

Figure 7. Effect of IFN on cell cycle distribution for p21 knockdown cells. (A) SW480 cells were exposed to a transfection reagent alone as RNAi(-) negative control and were transfected with GAPDH siRNA as RNAi(+) positive control or with p21 siRNA for 48 h. The expression of p21 was assessed at the indicated periods after transfection by Western blot analysis. (B) After transfection with p21 siRNA, cells were synchronized by serum starvation for 24 h. They were then released with serum and cultured in the absence (control) or presence of IFN (IFN-α2b or IFN-ß; 1,000 IU/ml). The cells were collected at the indicated periods after quiescence, stained with propidium iodide, and analyzed for cell cycle distribution by flow cytometry. Similar results were observed in 3 independent experiments. (C) A summarized time course of the cell cycle distribution in SW480 cells in which the expression of p21 was suppressed by RNAi [●, RNAi(+) positive control; ■, IFN-α2b; ▲, IFN-ß]. The data shown are mean ± SD of 3 independent experiments. (D) After transfection with p21 siRNA and synchronization, the cells grown in the absence or presence of IFN (IFN-α2b or IFN-ß; 1,000 IU/ml) were collected at the indicated periods after quiescence and subjected to Western blot analysis.

difference in cell cycle distribution among IFN-α2b-treated, IFN-ß-treated, and control groups (Fig. 7B and C). The change in the expressions of cyclin A and cyclin B also did not differ among these three groups (Fig. 7D).

Discussion

In the present study, we confirmed that type I IFN (IFN-α2b and IFN-ß) possessed cell growth inhibitory activity toward colon cancer cell lines. In addition, we concluded that the antiproliferative activity of IFN resulted from the prolongation of cell cycle via the induction of $p21^{WAF1/CIP1}$.

In regard to the effect of IFN on cell cycle in normal and various tumor cells, the induction of G1 arrest has been well reported (12,13,20-22). Additional effects reported include the S-phase prolongation (13,23,24), and G2/M arrest (25). The various immunobiological activities of IFN were classified according to tumor types. The G1 arrest is reported largely for hematopoietic tumor cells, including Burkitt's lymphoma and leukemia. On the other hand, the S-phase prolongation and blockage of transition into G2/M-phase are characteristic changes in solid tumor cells, including HCC, prostate cancer and breast cancer (12,13,23,24,26). In this study using colon cancer cell lines, type I IFN (IFN-α2b and IFN-ß) induced the prolongation of cell cycle progression with the accumulation of the S-phase population. Qin *et al* (13) described that the accumulation of the S-phase population occurred due to the loss or inactivation of the pRb-responsive G1 checkpoint and the subsequent failure of S-phase cells to enter into G2/M-phase. However, Hobeika *et al* (26) and Sangfelt *et al* (12) reported the induction of G1 arrest in cells without a normal Rb gene. In this study, no abnormality of pRb expression was reported in HT29 cells (27). Therefore, we inferred that not only pRb but also another abnormality in the molecules is involved in the regulation of cell cycle.

To gain insight into the mechanism of S-phase prolongation, we analyzed the expressions of cell cycle regulators in S-phase, including cyclin A and cyclin B. Theoretically, when cyclin A and cyclin B are induced, they bind to form cyclin-Cdk2 complex immediately and promote progression through S-phase (28). However, in the present study, the progression through S-phase could not be promoted efficiently despite their expressions. The change in their expressions was extremely slow and barely correlated with the change in the S-phase population. Moreover, in p21 knockdown cells, the difference in the expressions of cell cycle regulators as well as the S-phase population was diminished between IFN-treated cells and control cells. This result indicated that

the expressions of cyclin A and cyclin B were not regulated directly by IFN, but reflected the amount of the S-phase population.

Further, this result allowed us to infer a relationship between IFN and CKIs, which bound to cyclin-Cdk complexes and suppressed their activities. We examined the expressions of CKIs ($p21^{WAF1/CIP1}$, $p27^{Kip1}$, and $p19^{Ink4}$) in cells that were incubated in the presence or absence of IFN. The expression of p21 was increased with IFN treatment, whereas that in control cells did not show any change during the experiment. Additionally, the increase in the expression of p21 resulted from the transactivation of the p21 promoter gene by IFN. Moreover, we demonstrated that the effects of IFN on cell cycle were suppressed under the conditions in which the p21 expression was downregulated. Therefore, we identified $p21^{WAF1/CIP1}$ as a crucial target molecule to study the effects of IFN on the cell cycle.

It is well known that IFN causes the cell cycle to progress to G1 arrest, and the mechanism has been reported to depend on G1 checkpoint functions such as the p21-mediated inactivation of cyclin E- and cyclin D1-Cdk2 complexes, suppression of Rb phosphorylation, lowered E2F-DNA-binding activity, and suppression of c-myc expression (12,20-22,26,29-32). However, cells lacking the G1 checkpoint function cannot remain in the G1-phase after IFN treatment and may progress into the next phase autonomously. Qin *et al* (13) reported that when the G1 checkpoint was lost, IFN induced accumulation of the S-phase population. However, we inferred that the accumulation of S-phase population probably resulted from not only the loss of G1 checkpoint but also another cell cycle regulation such as the prolongation through S-phase and blockage of transition into G2/M-phase. This is because HT29 and SW480 cells have a normal G1 checkpoint function.

Dulic *et al* (14) described that the inactivation of cyclin A-Cdk2 complex due to the induction of p21 inhibited efficient G2/M-phase transition. Indeed, in the present study, we inferred that the inactivation of cyclin A-Cdk2 complex was due to the induction of p21. Because Cyclin A simply reflected the amount of S-phase cells and because the amount of Cdk2 protein did not change after IFN treatment, the results suggested that p21 might not reduce the amount of cell cycle regulators, but functionally inhibit the kinase activity of Cdk2. Furthermore, Hobeika *et al* (26) had reported that the induction of p21 inhibited the activity of cyclin E-Cdk2 complex. Therefore, we considered that p21 may play important roles in the biological activities of IFN, such as not only G1-arrest but also prolongation of S-phase and blockage of transition into G2/M-phase.

We demonstrated significant differences in the cell growth inhibitory activities of IFN-α2b and IFN-ß on two colon cancer cell lines. It may be unreasonable to use 'Unit' as a scale for the comparison of antiproliferative activities of two IFNs because 'Unit' was established based on antiviral activity. However, another study with 'molecular weight' as a scale has also reported the difference in the cell growth inhibitory activities of these two IFNs (7). Some studies have reported that the difference in the biological activities of the two IFNs result from differences in intracellular signaling (33), the abilities to interact with receptor chains (34), and the abilities to induce apoptosis (8). In the present study, the difference in the antiproliferative activities was found to be correlated with the difference in the ability to induce the expression of $p21^{WAF1/CIP1}$. This result confirmed the importance of p21 for the antiproliferative activity of IFN.

In conclusion, that the antiproliferative activities of type I IFN (IFN-α2b and IFN-ß) could be attributed to the prolongation of cell cycle, particularly in S-phase, and $p21^{WAF1/CIP1}$ was one of the most important molecules mediating the effect of IFN on the cell cycle. Therefore, $p21^{WAF1/CIP1}$ could be a potential therapeutic target and predictor in IFN therapy for human colon cancer.

Acknowledgements

We thank Dr Ken-ichi Isobe (Nagoya University, Nagoya, Japan) for providing p21-promoter plasmid; Yoshiaki Yanai (Hayashibara Biochemical Laboratories, Okayama, Japan), for technical advice on the expression of IFNAR.

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