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Antitumor effects of recombinant human interferon α-2a and hydroxyurea against chronic myelogenous leukemia

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만성 골수성 백혈병에 대한 유전자 재조합 인터페론 α-2a와 hydroxyurea의 항암효과

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Abstract : Prior to a clinical trial, the *in vitro* and *in vivo* antitumor effects of a new recombinant human interferon α-2a (rHu/IFN α-2a) with/without hydroxyurea (HU) were investigated using chronic myelogenous leukemia (CML)-derived cell lines (K562 and KU812F) and BALB/c nude mice transplanted with KU812F cells. The rHu/IFN α-2a (10^4-10^6IU/ml) strongly inhibited proliferation of both cell lines and the combined treatments with HU (10μg/ml) were more effective. In nude mice transplanted with KU812F cells, rHu/IFN α-2a(1×10^6IU) inhibited tumor growth by 42-65% at 15-21 days post-transplantation (DPT). The combined treatment of rHu/IFN α-2a (5×10^5IU) with HU (0.25mg/g b.w.) inhibited the tumor growth by 48-67% at 12-21 DPT. In addition, the treatment of rHu/IFN α-2a (5×10^6 IU or 1×10^7 IU) rejected tumor transplantation by 40%. These results suggest that the new rHu/IFN α-2a alone or with HU is effective on CML cell lines.

Key words : chronic myelogenous leukemia (CML), hydroxyurea, interferon α-2a, K562, KU812F, nude mice.

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Introduction

Interferons (INFs) are glycoproteins that exhibit antiproliferative activities against a variety of malignant cells and they are divided into three category according to the orgin of secreting cells; IFN-α (epithelial cell, leukocyte, lymphoblast), IFN-β (fibroblast), and IFN-γ (activated lymphocyte). INF-α has been demonstrated to be one of the most effective agents against hematological malignancies including chronic myelogenous leukemia (CML), hairy cell leukemia, and multiple melanoma[1-6]. IFN-α is also active against several human neoplasms including mammary tumor, kidney tumor, and non-Hodgkin's lymphoma[7-10]. The antitumor effects of INF-α may be partly due to induction of cell differentiation, inhibition of cell proliferation and modulation of host immunity by activating macrophage, natural killer cell, and cytotoxic T cell[11,12].

INF have been practically used for clinical therapies of tumor[1-10]. A DNA synthesis inhibitor, hydroxyurea (HU), has been successfully combined with INF-α to improve the efficacy of INF-α therapy for chronic myelogenous leukemia (CML). The combined therapy of INF-α with HU for CML allows rapid and effective hematological control and stimulates expression of INF-α receptors[13,14].

Prior to a clinical trial, the *in vitro* antitumor effects of a new recombinant human INF α-2a (rHu/IFN-α) produced in *E coli* by a Korean pharmaceutical company were evaluated using CML-derived cell lines (K562 and KU812F). The *in vivo* antitumor activities of rHu/INF-α were also investigated using nude mice transplanted with KU812F tumor cells.

Materials and Methods

Cells and animals : Two human leukemia cell lines, K 562 and KU812F, were obtained from Korean Research Institute of Bioscience and Biotechnology (Taejon, Korea) and grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum

One hundred and fifty female BALB/c nu/nu mice (4 weeks old) were purchased from Charls River Co. (Japan) and acclimatized for one week before experiment. The animals were housed in polycarbonate cages in the isolator with a hepafilter (Samkwang Co., Korea) and each mouse was recognized by ear punching and by tagging. Animal facility was maintained at 24±1.5℃, 55±10% humidity, 10-12/h ventilation, a 12-h light and dark cycle, and 150-200 lux. Food and water were autoclaved at 121℃ for 15 min and fed *ad libidum*. Caliper and scale were sterilized with 70% alcohol before use.

Interferon : Recombinant human IFN α-2a (Lot No. 3310003) was massively produced using *E coli* by Green Cross Pharmaceutical Co. Ltd. (Korea). The potency of lyophilized rHu/INF α-2a was 10^7 IU/ml per vial. The drug was stored at -20℃ until use. The drug was diluted in physiological saline when necessary

***In vitro* antitumor activity** : The tumor cells in RPMI 1640 medium were allocated to a 15-ml falcon tube and centrifuged. After removing the supernatant, the cells were resuspended with different concentrations of rHu/IFN α-2a and/or HU dissolved in RPMI 1640 medium. The mixtures of tumor cells ($4\text{-}5\times10^4$), rHu/IFN α-2a (1×10^6, 1×10^5, or 1×10^4 IU/ml), and/or HU (10μg/ml) were cultured for 24, 48, 72, 96 and 120 h at 37℃ with 5% CO_2 in 96-well plates. The treatment groups were shown in the legend of Fig 1. After collecting tumor cells at each time with 0.05% trypsin-0.02% EDTA, the viable tumor cells were stained with trypan blue and counted. This experiment was duplicated and the mean number was calculated. Adriamycin C (0.2μg/ml) was used as a positive control. The antitumor activity was calculated by the following formula ;

$$\text{Cell Survival}(\%) = \frac{(\text{NT} - \text{NoC})}{(\text{NC} - \text{NoC})} \times 100$$

NT = Cell density of the treated culture at each time.

NC = Cell density of the control culture at each time.

NoC = The initial cell density of the culture.

Tumor transplantation and measurement of tumor size : Two hundred microliters (2×10^6/ml) of KU812F cell subculture were subcutaneously injected into nude mice using a 1-ml syringe. After tumors were grown, the nude mice were killed by cervical dislocation. The tumors were removed together with the skin after disinfecting. Then, the

skin, connective tissue, and inner necrotic tissue of the tumors were removed. After washing the tumor mass three times with the medium on the ice, the tumor tissue was cut to small pieces ($3 \times 3 \times 3$mm). The pieces were inoculated into the subcutaneous tissue of the right flank using a transplantation needle, Remaining tissues were fixed in formalin for H & E stain. The tumor induction rates in mice by both cell lines and tissues were calculated.

When the size of the tumors was reached to 200-300 mm^3, five mice bearing tumors were randomly allocated to four experimental groups : saline (control), 1 ml; group I, INF α-2a (1×10^6 IU); group II, HU(0.5mg/g b.w.); group III, INF α-2a (5×10^5 IU)+HU (0.25mg/g b.w.). The dosages of rHu/INF α-2a were decided by comparing body weight of mouse to human from a clinical dose. The clinical administration route was applied to this study. rHu/INF α-2a and HU were daily injected at 15 : 00 of the clock subcutaneously and intraperitoneally, respectively, for 21 days. The length and the width of tumors with a sliding caliper were measured every three days after treatment. The tumor volume was calculated using the following formula; Tumor weight (mm^3, mg) = length (mm) $\times$ (width (mm))^2/2. Vtr/Vco values were also calculated by dividing the tumor weight of the treated group by that of the control group at each time.

General clinical signs such as anorexia, salivation, diarrhea, polyurea, anurea, fecal change, body weight, and mortality were recorded for evaluating toxic effects of the treatments.

Statistical analysis : Data were analyzed using SAS program for one-way analysis of variance. The least significant difference procedure was used to determine a significant difference at $p<0.05$ between the means of the groups.

Results

In vitro antitumor effect : In both K562 and KU812F tumor cells, rHu/INF α-2a alone or with HU (10μg/ml) strongly inhibited cell proliferation at 24-120 h after treatment (Fig 1). In K-562 tumor cells, the inhibition rate of cell proliferation by rHu/INF α-2a was markedly increased in a time- and dose- dependent manner (Fig 1a). In addition, when the cells were co-treated with rHu/INF α-2a and Hu, the growth inhibition was stronger than either only rHu/INF α-2a or HU treatment. The number of viable cells in the combined treatment of rHu/INF α-2a with HU was about half of that in rHu/INF α-2a alone (Fig 1a). The growth of KU812F

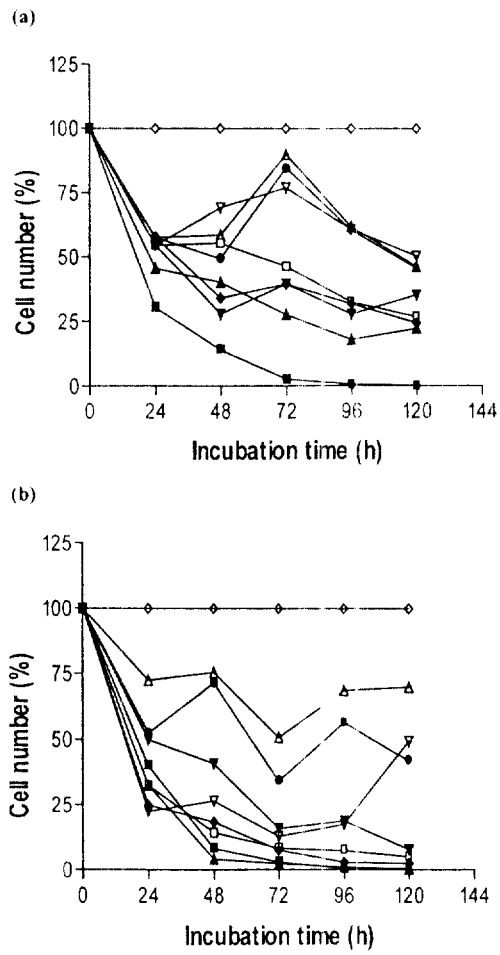

Fig 1. *In vitro* antitumor effect of IFN α-2a and/or HU to human CML cell lines, (a) K-562 cell line and (b) KU 812F cell line. A time- and dose-dependent cell growth inhibition by IFN α-2a was observed and the co-treatment of IFN α-2a with HU evidently increased its antitumor activity. Negative control (-◇-): RPMI 1640+ 10% FBS, Positive control (-■-): 0.2μg/ml adriamycin C, treatment 1 (-▲-): IFN 10^6 IU/ml + 10μg/ml HU, treatment 2 (-▼-): IFN 10^6 IU/ml, treatment 3 (-◆-): IFN 10^5 IU/ml + 10μg/ml HU, treatment 4 (-●-): IFN 10^5 IU/ml, treatment 5 (-□-): IFN 10^4 IU/ml + 10μg/ml HU, treatment 6 (-△-): IFN 10^4 IU/ml, treatment 7(-▽-): 10μg/ml HU.

cells was also inhibited in a time- and dose dependent-manner by the treatment of rHu/INF α-2a (Fig 1b). KU812F cell were much more sensitive to HU treatment than K562 cells (Fig 1). Adriamycin C (0.2μg/ml) as the positive control caused complete cell death at 120 h post-inoculation in both cell lines.

Tumor transplantation effect : Without treatment of INF α-2a, solid chronic myelogenous leukemia tumors were induced in nude mice with K562 and KU812F cell lines or with both cell lines-induced tumor tissues. There was a significant difference in the induction of chronic myelogenous leukemia tumors (Table 1). K562 cell line or tumor tissues induced only 20% (12/60) of chronic myelogenous leukemia tumors, while KU812F cell line or tumor tissues induced

Table 1. Tumor induction in nude mice after transplantation of K562 and KU812F cells and the cell lines-induced tumor tissues

Cell lines	No. of animals	Tumor induction rate (%)	
		Cell	Tissue
K562	60	20(2/10)	20(10/50)
KU812F	94	100(10/10)	100(84/84)

100% (94/94) of tumor.

However, an early administration of rHu/INF α-2a (1×10^5 or 1×10^6 IU) at tumor induction stage for 14 days (from the third day to the seventeenth day on the base) of initial tumor transplantation day) reduced the tumor induction rate by KU812F cells by 40%, corresponding to 40% transplantation rejection (Table 2). The tumor size varied from 13.5 to 3934.9 mm^3 (Table 2).

Table 2. Effect of early treatment of rHu/INF α-2a on tumor volume at 30 days after tumor post-transplantation (PT)

Dose of INF α-2a[a]	Tumor induction (No. of mice)	Tumor volume
5×10^6 IU	3/5(60%)	2257±2021
1×10^7 IU	3/5(60%)	1073±1286

[a] rHu/INF α-2a was treated for 14 days to KU812F cell-transplanted mice from 3 days post-transplantation (PT) to 17 days PT. At 30 days PT, the tumor size was measured (n = 3).

Clinical and histopathological changes : The induced and proliferated tumors were very solid and had black necrotic foci on the surface of the tumor. Tumor volume was decreased as the necrotic foci on the center spread out the whole surface. The tumors were microscopically identified as typical chronic myelogenous leukemia derived from

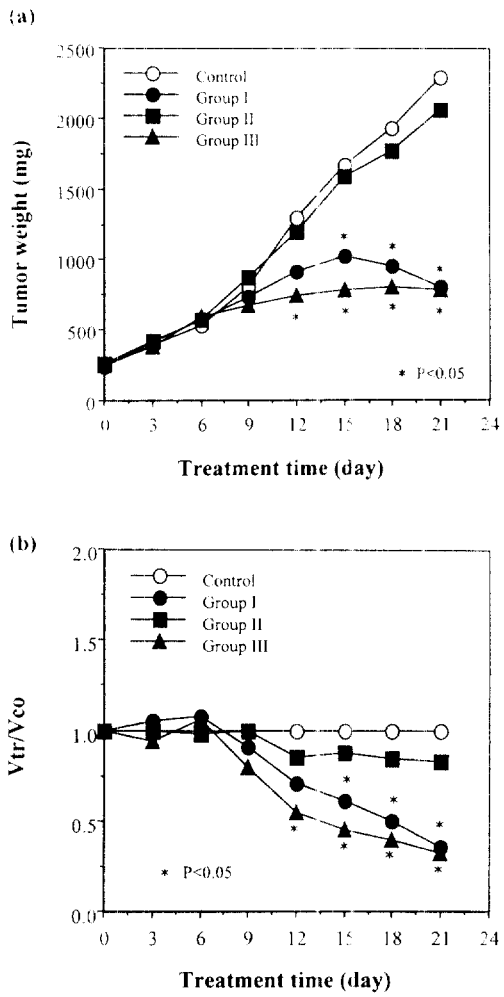

Fig 2. (a) Changes of tumor weight and (b) Vtr/Vco value in nude mice bearing KU812F cell-induced tumors. IFN and/or HU were administrated for 21b days after tumor volume was reached to 200-300 mm^3.
Control: Saline, 1 ml; Group I : IFN α-2a (1×10^6 IU); Group II : HU (0.5mg/g b.w.); Group III : IFN α-2a(5×10^5 IU) +HU (0.25mg/g b.w.).

mast cells. Tumor cells were circular or polygonal with profuse cytoplasm and the nuclei included many chromatins and 1-2 nucleoli.

All mice treated with HU (1 mg/g b.w.) or INF α-2a (1×10^6 IU) plus HU (0.5mg/g b.w.) died within the eighth day after the administration because of the toxicity of HU, and they were excluded from the experiment. The body weights of mice in treatment groups (Ⅰ, Ⅱ and Ⅲ) decreased, but not significantly different from the control (data not shown). No remarkable clinical signs were observed in all the groups.

In vivo antitumor effect : The starting average tumor volumes (weight) in each group were 240-260 mm^3(mg). Treatment of INF α-2a (1×10^6 IU) significantly reduced the tumor volume at 15-21 days PT (DPT) compared to the control (Fig 2a). In group Ⅱ, the ratio of Vtr/Vco at 21 DPT was 0.35, corresponding to the inhibition of 65% (Fig 2b). In group Ⅲ, the combined treatment of INF (5×10^5 IU) and HU (0.25mg/g b.w.) significantly decreased the tumor growth by 48-67% at 12-21 DPT, compared with the control (Fig 2b). However, there was no significant difference between Group Ⅱ and the control.

Discussion

Several basic experimental and clinical studies have been carried out in an attempt to improve the efficacy of INF-α therapy for CML. The combined use of HU and INF (500-1000mg daily) in INF-resistant cases facilitated maintenance of reduced leukocyte production or a reduction in the dose of INF[5]. In this study, rHu/INF α-2a or HU suppressed *in vitro* proliferation of CML-derived cell lines including KU 812F and K562, and the combined treatment of IFN and HU increased the antiproliferative activities. KU812F cells seemed to be more sensitive to the treatment of rHu/INF α-2a and/or HU than K562 cells, indicating a different sensitivity of CML cell lines to rHu/INF α-2a and/or HU. There are many reports that treatment of INF-α suppressed *in vitro* proliferatiopn of K562 and KU812F cell lines[15,16]. In K562 cells, INFs stimulate apoptosis and induce up-regulation of Spi-1/PU.1, a transcription factor for normal hematopoiesis[15,17].

In our study, the treatment of INF α-2a (1×10^6 IU) suppressed *in vivo* tumor growth in nude mice. The co-treatment of INF-α and HU, even with lower doses, showed a stronger antitumor effect. However, the treatment of HU (0.5 mg/g b.w.) alone had no antitumor effect in nude mice transplanted with KU812F cells. HU treatment is known to lead to apoptosis and accumulation of short DNA fragments which is directly correlated with its cytotoxicity[18]. However, HU alows rapid and effective hematological control and upregulation of INF-α receptors in CML patients[13,14]. Therefore, the combined therapy of INF and HU has been practically used in the clinical treatment of hematological malignancy[13].

Both K562 and KU812F cell lines produced solid tumors in nude mice. There was, however, a significant difference in the rate of tumor induction, even though the cell lines were derived from the same origin. K562 cell line showed only 20% tumor induction, compared to 100% with KU 812F cell line. It was known that KU812F cell line, as multipotential progenitors, was differentiated into either basophils or adult hematopoietic cells[19,20].

The use of nude mice as a host animal may be seriously limited because of the athymic condition of the immunodeficient animal. However, the used of this particular strain as a host of human tumor xenografts can partially help to elucidate putative direct, i.e. lymphocyte-function independent, effects of such treatment protocols on both the malignant parenchyma and supporting stroma of malignant tissues. From the result in this study, the successful tumor transplantation with KU812F cell line in nude mice may provide a good tool for an *in vivo* antitumor assay of certain chemicals.

IFNs show antitumor effects by directly inhibiting the growth of tumor and/or by activating the immune system of the host[21]. It is known that INF-α directly have an influence on the cell cycle distribution and phase distribution. In this study, the antitumor effect of rHu/INF α-2a in the athymic nude mice against CML seemed to be not due to its immunostimulatory activity but its direct antiproliferative activity. This result implies that rHu/INF α-2a may inhibit the

proliferation of tumor more potentially in human body with a synergistic effect of the host immune systems.

In conclusions rHu/IFN alone or with HU strongly inhibited the *in vitro* proliferation of CML cell lines and the *in vivo* transplantation and growth of tumors in nude mice. Prior to a clinical trial, a new rHu/INF α-2a has a strong antitumor activity against CML and even more with HU.

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