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Efficacy of in vitro Treatment of Chronic Myelogenous Leukemia Cell Line, K562 Cells, using 4-Hydroperoxycyclophosphamide, α-Interferon and γ-Interferon*

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α-Interferon(α-IFN) and γ-interferon(γ-IFN) are highly effective biologic agents in the treatment of chronic myelogenous leukemia(CML). 4-hydroperoxyclophosphamide(4-HC) is the most commonly used chemotherapeutic drug when purging bone marrow contaminated with malignant cells. To investigate the efficacy of α-IFN, γ-IFN and 4-HC as purging agents in CML patients, K562 cells, a human CML cell line, were treated in vitro with these drugs singly or in combination. The cytotoxic effect was evaluated by MTT assay. Data were analyzed for synergism by the median effect principle of Chou and Talalay. Using Southern blot analysis, the effect of purging on the suppression of bcr gene rearrangement was also evaluated. α-IFN, γ-IFN and 4-HC all showed good concentration-dependent cytotoxic effect. Median cytotoxic doses of α-IFN, γ-IFN and 4-HC were 0.03 μM, 0.20 μM and 1.97 μM respectively, and molar ratios of median doses of α-IFN to 4-HC, γ-IFN to 4-HC and α-IFN to γ-IFN were 1 : 80, 1 : 10 and 1 : 8, respectively. The combination treatment of α-IFN and 4-HC, and γ-IFN and 4-HC showed significant synergistic cytotoxic activities, but combination of α-IFN and γ-IFN was antagonistic. Status of bcr gene rearrangements in K562 cells was not changed after the treatments. Our results appear to provide an efficient method for removing CML cells from the bone marrow of CML patients.

Key Words: *α-Interferon(α-IFN), γ-Interferon(γ-IFN), 4-hydroperoxycyclophosphamide(4-HC), K562 cells, Chronic myelogenous leukemia(CML), MTT assay*

INTRODUCTION

Autologous bone marrow transplantation has been widely employed for the treatment of acute leukemia, non-Hodgkin's lymphoma, Hodgkin's disease and a variety of other malignancies(Frei et al., 1989, Santos et al., 1989, Stuart, 1993, Coiffier et al., 1994). The ideal candidates for a successful therapeutic outcome of autologous bone marrow transplantations are a

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malignancy that is responsive to cytoreductive therapy with a dose-response relationship, an effective cytoreductive therapy the limiting toxicity of which is marrow failure, performance of the transplantation when there is minimal tumor burden, and a source of hematopoietic stem cells free of clonogenic tumor cells. Unfortunately, a potential obstacle to the optimal utilization of this procedure is the contamination of autologous grafts with tumor cells(Bortin et al.,1988, Barrett et al., 1989), so the emphasis has been on the efficacy of removal of residual tumor cells from the marrow without affecting hematopoietic stem cells.

Various techniques have been devised for bone marrow purging(Charak et al., 1991, Becker et al., 1993, Skorski et al., 1993, Passos-Coelho et al., 1994). 4-hydroperoxycyclophosphamide(4-HC) can eliminate or purge leukemic cells from suspensions of normal hematopoietic stem cells and tumor cells without affecting hematologic reconstitution, and presently is the most commonly used purging agent (Jones et al.,1987, Areman et al., 1990). α-Interferon (α-IFN) has been proven to be very effective in the treatment of chronic myelogenous leukemia(CML). Complete hematologic remissions are achieved in 70 % to 80 % of patients and major cytogenetic responses in 40 % to 60 %(Talpaz et al., 1986, Talpaz et al., 1991, Kantarjian et al.,1993). γ-Interferon(γ-IFN) has also shown definite but modest activity in CML with complete hematologic remission rates of 20 % to 30 %(Kurzrock et al., 1987). Combination of α-IFN with other cytotoxic drugs was associated with significantly higher complete hematologic remission rates, a better cytogenetic response rate, and with significantly longer survival(Kantarjian et al., 1992).

We therefore have investigated the in vitro sensitivity of K562 cells, a human CML cell line, to α-IFN, γ-IFN and 4-HC, and have also examined the possibility of synergism when these agents were used in combination, using MTT assay and Southern blot analysis. The data were analyzed by the median effect principle of Chou and Talalay(1984).

MATERIALS AND METHODS

Drugs and reagents

IFN-α(specific activity : 2.2×10^8 IU/mg, molecular weight : 18,520 dalton on SDS-PAGE) and IFN-γ (specific activity : 3.0×10^7 IU/mg, molecular weight : 17,200 dalton on SDS-PAGE) were generously provided by LUCKY Biotech's.(Seoul)

MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide], 4-HC, and dimethyl sulfoxide (DMSO) were obtained from SIGMA.(St. Louis) 5' bcr specific 1.95 kbp BglII-HindIII fragment was obtained from American Type Culture Collection (ATCC). BglII was purchased from Promega Biotech. (Madison)

Cell line

The K562 cell line, obtained from ATCC was grown in RPMI-1640 medium supplemented with 10 % fetal bovine serum, gentamycin and 24mM sodium bicarbonate and maintained in a humidified atmosphere 5 % CO_2 at 37℃. In all studies, cells in a logarythmic growth phase were used.

MTT assay

K562 cells maintained in an exponential growth phase were adjusted to 3×10^5 cells/ml. 100 μl alliquots of the single cell suspensions were distributed into the individual wells on 96-well tissue culture plates. α-IFN, γ-IFN and 4-HC at different concentrations(α-IFN and γ-IFN : 10^4IU/ml, 10^5IU/ml, 2×10^5IU/ml, 5×10^5IU/ml, 1×10^6IU/ml, 3×10^6IU/ml, 6×10^6IU/ml, and 4-HC : 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 5 μg/ml, 10 μg/ml, 15 μg/ml, 30 μg/ml) were added to each well in a volume of 20 μl, then the plates were incubated at 37℃ for 4 days. After 4 days of culture, with or without drugs, 0.1mg(50 μl of 2mg/ml) of MTT was added to each well and incubated for another 4 hours. After incubation, plates were centrifuged, and the medium was removed. 150 μl of DMSO was added, and the plates were vigorously shaken to solubilize the MTT-formazan products. Absorbance at 540nm was measured with a microplate ELISA reader.

Data analysis

Data were collected as replicates of six wells and the cytotoxic effects of α-IFN, γ-IFN and 4-HC on K562 cells were defined as survival fractions of cells, which were determined by the formula :

Survival fraction=OD(%) of treated cells/OD(%) of untreated cells(OD ; optic density)

Concentration-effect curves were generated for each single drug and for each combination. We used the median effect principle of Chou and Talalay(1977)

Table 1. Median dose values(ID50) for cytotoxic effects of α-IFN, γ-IFN and 4-HC against K562 cells.

Drug	Median Dose			
	Single	Combined drugs		
		4-HC	IFN-α	IFN-γ
IFN-α	1×10^5IU/ml (0.03 μM)	2.5×10^4IU/ml (0.01 μM)		5×10^5IU/ml (0.12 μM)
IFN-γ	1×10^5IU/ml (0.2 μM)	3.3×10^4IU/ml (0.07 μM)	5×10^5IU/ml (1.00 μM)	
4-HC	0.51 μg/ml (1.97 μM)		0.13 μg/ml (0.50 μM)	0.17 μg/ml (0.67 μM)

Values in parenthesis expressed by different units are equivalent doses.

to determine the concentration ratio of one drug to the other that allows for the calculation of the interaction between both drugs. The ratio between each drug were based on the ID50 concentration estabilished from the concentration-effect curve. Synergism, additism, or antagonism of the drug effects were assessed with the multiple drug effect analysis developed by Chou and Talalay(1984) that quantitates the interactions of two drugs. A computerized program based on the median effect plot and the combination index equations were used for the data analysis.

Southern blot analysis

We centrifuged IFN-treated K562 cells at 100× g for 5 minutes and washed with PBS, and then incubated at 65℃ for 15 min with 9 volumes of proteinase K buffer. Genomic DNA was isolated using phenol/chlorform(1 : 1) extractions of lysates with 1/10 volume sodium bicarbonate(3M) and 2.5 volume ethanol precipitation. Thereafter, the DNA was washed in 80 % ethanol and dissolved in TE buffer, and then RNase was added and the mixture was incubated at 37℃ for 30minutes. A 5' bcr specific 1.95kbp BglII-HindIII fragment from ATCC was used as probe and labelled with α^{32}P-dCTP by the Nick translation method(Ausubel et al., 1992). Digestion with BglII obtained from Promega Biotech was performed, and restriction fragments were separated on 0.8 % agarose gels for 12 hours at 30V, and then transferred to nitrocellulose paper in 10×SSC transfer buffer. Prehybridization was done in 50 % formamides, 6×SSC, 100 μg/ml ssDNA, 1×Denhardt's solution at 42℃ for 12 hours, and prehybridization buffer was removed. the denatured probe(specific activity : 3×10^7cpm/μg) and hybridization buffer(50 % dextran sulphate : prehybidization solution=1 : 4) were added and incubated at 42℃ for 24 hours. After hybridization, the nitrocellulose paper was washed with 2×SSC, 0.1 % SDS at room temperature for 5 minutes four times, followed by 0.1 % SSC, 0.1 % SDS, at 52℃ for 20 minutes two times, and the nitrocellulose paper was dried and exposed to X-ray film(Davis et al.,1994, Ausubel et al., 1992).

RESULTS

Cytotoxic effect of α-IFN, γ-IFN and 4-HC

The median dose values for α-IFN, γ-IFN and 4-HC are shown in Table 1. The concentration-cytotoxic effects of α-IFN, γ-IFN, 4-HC, and combinations of the drugs are shown in Fig. 1, 2, and 3.

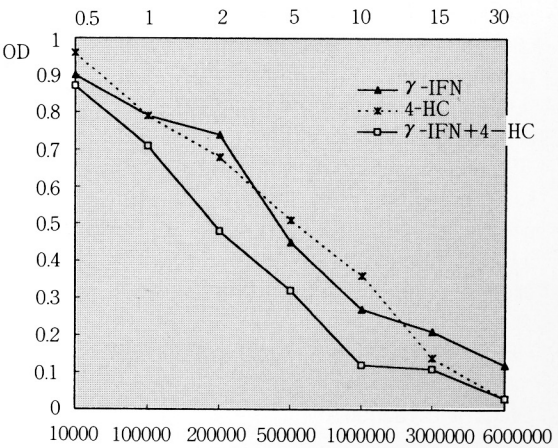

Fig. 1. Cytotoxic effects of γ-IFN, 4-HC and the combination of both drugs on K562 cells measured by MTT assay.
Upper X-axis ; 4-HC(μg/ml)
Lower X-axis ; γ-IFN(IU/ml)

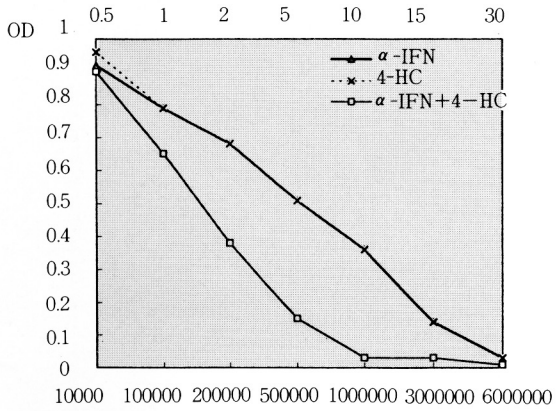

Fig. 2. Cytotoxic effects of α-IFN, 4-HC and the combination of both drugs on K562 cells measured by MTT assay.
Upper X-axis ; 4-HC(μg/ml)
Lower X-axis ; α-IFN(IU/ml)

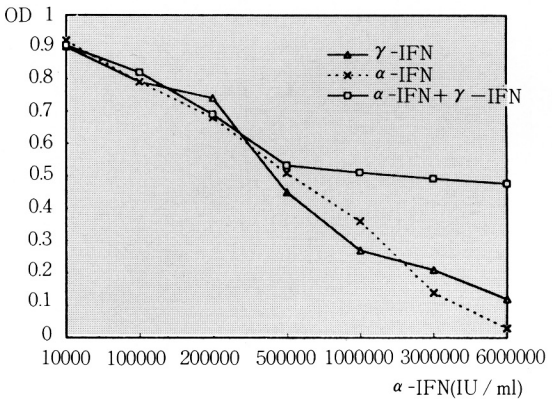

Fig. 3. Cytotoxic effects of α-IFN, γ-IFN and the combination of both drugs on K562 cells measured by MTT assay.

From these curves, the ID50(dose of 50 % growth inhibition) shown in Table 1 was calculated for each drug. Median cytotoxic doses of α-IFN, γ-IFN and 4-HC were 0.03 μM, 0.20 μM and 1.97 μM, respectively. The ID50 provided the basis for the appropriate ratio of α-IFN to 4-HC, γ-IFN to 4-HC and IFN-α to IFN-γ maintained as 1 : 80, 1 : 10 and 1 : 8, respectively, when the two drugs were studied in combination to analyze for synergism by the combination effect method of Chou and Talalay(1984). All three drugs show good concentration dependent cytotoxic effects and combination treatments of α-IFN and 4-HC, γ-IFN and 4-HC showed higher cytotoxic effects than single agent treatments. But the combination treatment of α-IFN and γ-IFN was less cytototoxic than single agent treatments.

Interaction of α-IFN, γ-IFN and 4-HC

Fig. 4 shows the combined effects for each agent. In those plots, all points with CIs above 1 indicate antagonistic effects, whereas all points with CIs equal to 1 indicate additive effects and all points with CIs less than 1 indicate synergistic effects. Combinations of α-IFN and 4-HC revealed synergistic effects at all Fa values and combinations of γ-IFN and 4-HC revealed synergistic effects at Fa values below 0.9. But α-IFN and γ-IFN showed antagonistic effects at all Fa values.

Southern blot analysis

Southern blot analysis was performed for IFN-treated K562 cells, normal hematopoietic cells and untreated K562 cells to evaluate the effects of IFN on the status of bcr gene rearrangements in K562 cells. As shown in Fig. 5, and 6, IFN therapy did not affect bcr gene rearrangements in K562 cells.

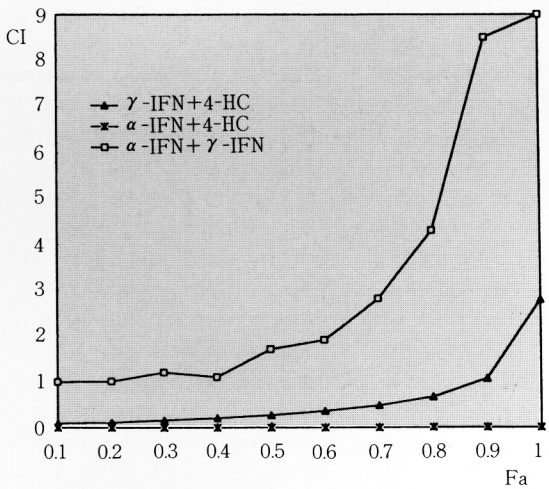

Fig. 4. Plots of the calculated combination indices using the median dose effect method of Chou and Talalay.
CI ; Combination index, Fa ; Fraction affected

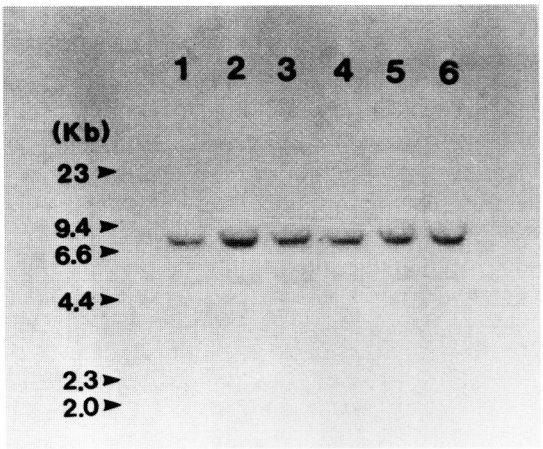

Fig. 5. Bcr gene rearrangement of K562 cells : 1 (control K562 cells), 2-6 (-IFN treated K562 cells, 2; 1.7×104, 3; 8.3 ×104, 4; 1.7×105, 5; 5×105, 6; 1×106IU/ml).

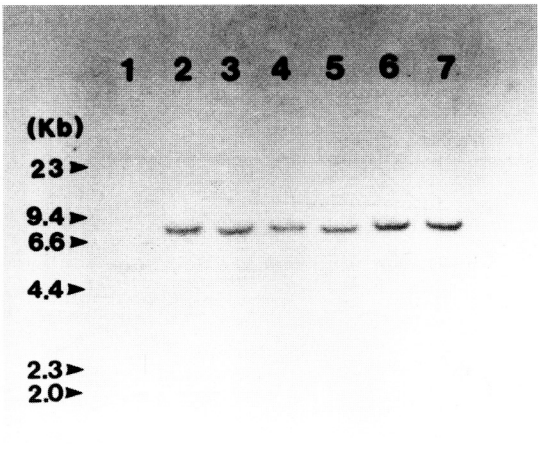

Fig. 6. Bcr gene rearrangement of K562 cells : 1 (normal cells), 2 (control K562 cells), 3-7 (-IFN treated K562 cells, 3; 1.7×104, 4; 8.3×104, 5; 1.7×105, 6; 5×105, 7; 1× 106IU/ml).

DISCUSSION

CML is a myeloproliferative disorder caused by clonal expansion of pluripotent stem cell and associated with the presence of the Philadelphia(Ph)-chromosome(Nowell and Hungerford, 1960, Rowley, 1973, Fialkow et al., 1977). Although there is now good evidence that selected patients with CML can be cured by allogeneic bone marrow transplantation (Goldman et al., 1986), many patients lack suitable marrow donors, and morbidity and mortality of the procedure is high. In the absence of an appropriate donor, autologous bone marrow transplantation may be performed. But the results of autologous bone marrow transplantation are usually worse compared with allogeneic bone marrow transplantation owing to the presence of a residual clonogenic leukemia cells within autografts and lack of the graft versus leukemia effect(Hughes et al., 1989, Meloni et al., 1989). So improvement in the treatment of CML with autologous bone marrow transplantation may depends on the establishment of a better purging method.

Our data from MTT assays indicate that γ-IFN, -IFN and 4-HC all display good dose dependent cytotoxic effects on K562 cells. These results are consistent with the previous results of other investigators(McGlave et al., 1990). The combination treatments of α-IFN and 4-HC, and γ-IFN and 4-HC, revealed significant synergistic cytotoxic effects, but interaction between α-IFN and γ-IFN was antagonistic. Interferon, the first cytokine to be produced by recombinant DNA technology, has a variety of activities such as antiviral, antiproliferative, differentiative and immunomodulating avtivities(Trinchieri and Perussia, 1985). Although both α-IFN and γ-IFN have inhibitory effects on CML derived pluripotent, erythroid and granulocyte-macrophage progenitor cells(Gutterman, 1994), α-IFN differs from γ-IFN in its biologic properties, mechanism of action, and responding cell surface receptors. Some investigators have demonstrated the synergistic antiproliferative effects of α-IFN and γ-IFN on normal human bone marrow derived progenitor cells(Broxymeyer et al., 1985, Raefsky et al., 1985). But results concerning a possible synergism between IFN-α and IFN-γ in suppressing the in vitro growth of CML-derived hematopoietic progenitor cells are few and controversial. Contradictory results of some studies may be due to different dose ratios and different scheduling regimens. Our study shows that α-IFN and γ-IFN have an antagonistic effect on K562 cells, a human CML cell line. In light of these results, the precise mechanism of the antiproliferative action of interferons and interactions between IFN-α and IFN-γ deserves further study.

Many studies, in vivo or in vitro, have shown synergistic interactions of 4-HC with various other agents in purging(Uckun et al., 1992, Zhong et al., 1994), yet there are no studies available concerning the interactions between interferons and 4-HC on chronic leukemia cells. In our studies the combined

treatments of K562 cells with α-IFN and 4-HC, and γ-IFN and 4-HC demonstrate a significant synergistic cytotoxic effect on K562 cells at almost all levels of concentrations evaluated for each drug. Since protracted engraftment and prolonged thrombocytopenic period were the major problem when marrow purged with 4-HC was employed for autotransplantation, our results are encouraging in that ex vivo combination treatment of marrow with interferons and 4-HC may reduce the dose of 4-HC without compromising the efficacy, thereby shortening the time to marrow recovery and reducing the risk of myelosuppressive complications.

We also investigated the effects of interferons on the bcr gene rearrangement using Southern blot analysis. Although in clinical trials, interferons have been shown to induce complete cytogenetic remissions in about 20 % to 30 % of CML patients, our in vitro study suggests that interferons have no effect on the suppression of the bcr gene rearrangement. In our study, however, K562 cells were exposed to interferon only for four days. To obtain cytogenetic remissions in CML patients, longterm treatment with interferons is needed. From this point of view, the exposure of K562 cells to interferons might be too short for complete eradication of leukemic cells, and long-term exposure study with CML cells or K562 cells mixed with normal cells may be needed to evaluate the efficacy of purging.

In conclusion, good dose-dependent cytotoxic effects on K562 cells were observed for α-IFN, γ-IFN and 4-HC when treated alone and combination. Treatments of K562 cells with α-IFN and 4-HC, γ-IFN and 4-HC demonstrated significant synergistic activities, while α-IFN and γ-IFN showed an antagonistic effect when combined. Short-term exposure to interferons had no effect on the bcr gene rearrangement status. Our results may provide an efficient approach to remove CML cells from the bone marrow hematopoietic stem cells.

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