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Eur J Cancer, Vol. 27, Suppl. 4, pp. S21-S25, 1991
Printed in Great Britain

0277-5379/91 $3.00 + 0.00

Several New Approaches to Improvement of Alpha Interferon Therapy in Chronic Myelogenous Leukaemia

Shigetaka Asano, Hiromi Ogura, Kenzaburo Tani, Tokiko Inoue, Arinobu Tojo and Keiya Ozawa

Several basic experimental and clinical studies were carried out in an attempt to improve the efficacy of alpha interferon therapy for chronic myelogenous leukaemia (CML). First, the combined use of hydroxyurea (HU) and interferon (500-1000 mg daily) in interferon-resistant cases facilitated maintenance of reduced leucocyte production, or a reduction in the dose of interferon, although suppression of Philadelphia chromosome (Ph^1)-positive clones was not observed in most cases. In order to try and decrease the rate of lymphoblastic crisis during the course of interferon therapy, we recently added methotrexate (MTX) (10-15 mg, weekly) to the treatment protocol. Since then, no lymphoblastic crisis has been observed. Second, the *in vitro* expression of alpha interferon-stimulated gene (ISG) mRNA was shown to be markedly decreased in granulocytes of one representative interferon-resistant case, compared to that in granulocytes of the three interferon-sensitive cases. Interestingly, it was found that the transcriptional activity in this case became almost normal when the blood granulocytes were controlled by the addition of HU. These findings suggest that the *in vitro* transcriptional assay of ISG mRNA may be clinically useful for predicting alpha interferon efficacy. Third, when genetically manipulated, alpha interferon-producing NIH/3T3 cells were co-transplanted using diffusion chambers into nude mice bearing a CML cell line, KU812, the CML tumour growth was shown to be markedly suppressed. This experimental model for alpha interferon replacement gene therapy suggests some directions for future studies on interferon therapy.
Eur J Cancer Vol. 27, No. 4, pp. S21-S25, 1991.

INTRODUCTION

CHRONIC MYELOGENOUS LEUKAEMIA (CML) is one of the myeloproliferative disorders caused by the appearance of abnormal haemato-lymphopoietic stem cell clones with a Philadelphia chromosome (Ph^1) marker as a result of 9;22 reciprocal translocation. These clones generally have two biological characteristics: (1) the extensive ability to differentiate into a neutrophilic cell lineage with some growth advantages (chronic phase), and (2) the occasional transformation into more malignant clones (blastic phase) [1]. Recent molecular biology studies have revealed that the 9;22 translocation is responsible for the abnormal hybrid bcr-abl gene products which may determine the characteristics of CML cell clones [2, 3]. However, actual molecular mechanisms for blastic transformation have not yet been elucidated. Because blastic transformation is the major cause of death in most CML patients, it is of particular importance to address this issue.

For CML, a major advance was the introduction of alpha

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interferon therapy, originally reported by Talpaz *et al.* [4], and subsequently by many other investigators [5-11]. It is well known that the long-term administration of alpha interferon frequently induces haematological remission with suppression of Ph¹-positive clones, especially when the treatment is started in the early chronic phase and also when prolonged leukopenia is induced by the treatment. However, therapy with alpha interferon raises a number of problems. Recently, we have embarked on some basic experimental and clinical studies to address these problems. In this paper, we report some of the results of these ongoing studies.

METHOTREXATE AND/OR HYDROXYUREA IN COMBINATION WITH INTERFERON

In an attempt to improve further the survival of CML patients treated with alpha interferon, we are currently investigating the efficacy of methotrexate (MTX) and/or hydroxyurea (HU) in combination with alpha interferon in the stable chronic phase. Twenty-seven patients with CML have been enrolled in this study. Alpha interferon therapy was initiated within 1 year from diagnosis at a dose of 3-10 million units (MU)/day, once a day or twice a week, in order to maintain blood leucocyte counts between 3 x 10^9 and 5 x 10^9/L and platelet counts of more than 50 x 10^9/L. In 18 of the 27 patients, treatment with alpha interferon was well tolerated; the other nine patients experienced severe persistent anorexia or lassitude, or poorly controlled blood leucocyte counts. HU was given to the latter patients at doses of 500 to 1,000 mg/day in order to allow a reduction in the dose of alpha interferon. In *all* patients, oral MTX at doses of 10 to 15 mg once a week was added to the interferon therapy at a median of 22.4 months (range 5 to 56), as a result of sudden onset of lymphoblastic crisis in two patients.

When analyzed irrespective of MTX and/or HU administration, life expectancy was significantly prolonged in the alpha interferon-treated group in a historical comparison with a conventional busulphan-treated group of patients (Fig. 1). However, when the alpha interferon-treated groups were compared, there was no difference in survival between the

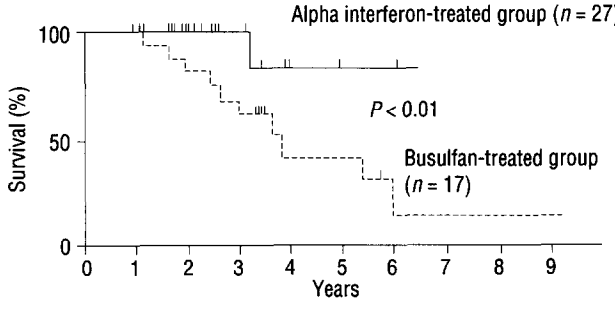
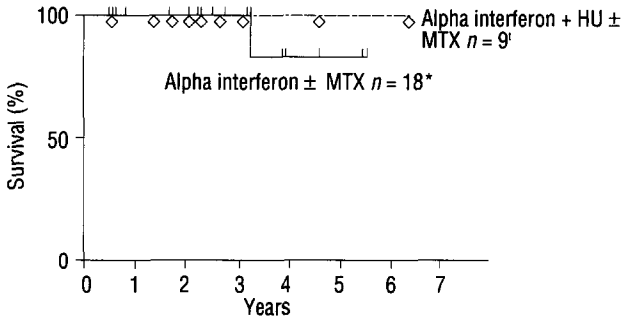


Fig. 1. Survival curves of the alpha interferon-treated patients and busulphan-treated patients (Kaplan-Meier). The interferon treated group comprised patients receiving a combined therapy of MTX and/or HU with alpha interferon. A statistically significant difference was noted between the two groups ($P < 0.01$).

HU/interferon ± MTX group and the interferon ± MTX group (Fig. 2), despite the fact that there was much greater suppression of Ph¹-positive clones in the former group than in



' Additional chromosomal abnormality 4/9
* Additional chromosomal abnormality 4/18

Fig. 2. Survival curve of the alpha interferon ± MTX-treated patients, with and without HU (Kaplan-Meier).

the latter patients (Fig. 3). The comparative data for interferon therapy with and without MTX are summarized in Table 1.

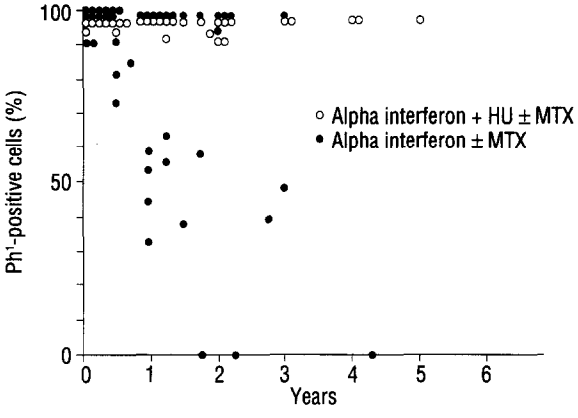


Fig. 3. Proportion of Ph¹-positive metaphases in alpha interferon ± MTX-treated patients, with and without HU.

Although the duration of follow up is too short and patient numbers are too small to allow conclusions to be drawn at this stage, development of lymphoblastic crisis has not been observed since the introduction of MTX to the treatment protocol.

IN VITRO EXPRESSION OF INTERFERON-STIMULATED GENE mRNA

We have examined the *in vitro* transcriptional activity of

Table 1. Incidence of blastic crisis in patients treated with alpha interferon with or without MTX

MTX	Median observation period months (range)	Lymphoblastic crisis *n*
Before	22.4 (5-56)	2/19
After	13.3 (4-20)	0/17

interferon-stimulated genes (ISG) [12-15] in mature granulocytes from normal volunteers and four patients with CML as well as in various cell-lines, using Northern blot analysis. Both of the interferon-sensitive Daudi and KU812 cells [16] tested showed marked induction of ISG mRNA dose-dependently under stimulation with alpha interferon, while the interferon-resistant HL60 cells did not (Fig. 4). Interestingly,

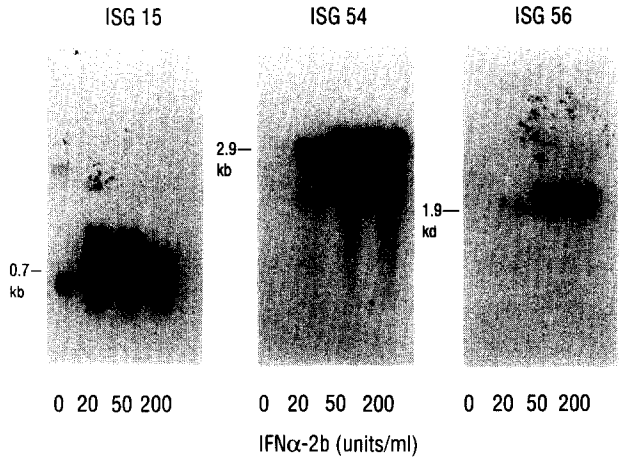


Fig. 4. Northern blot analysis for *in vitro* expression of ISG mRNA in human leukaemia cell lines. Fifty million human leukaemia cells (Daudi, HL60, or KU812 obtained from Japanese Cancer Research Resource Bank, Japan) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum in the absence and presence of interferon alfa-2b (final concentrations: 20, 50 and 200 U/ml) for 90 min. RNA was extracted from cultured cells using guanidine hydrothiocyanate; it was electrophoresed (40 μg/lane) on a 1.0% agarose gel containing formaldehyde, and was then transferred to a nitrocellulose filter. The cDNAs of ISG15, 54, and 56 (a generous gift from Dr E. Reich, The Rockefeller University, U.S.A.) were labelled with ^{32}P dCTP15 using a multiprime DNA labelling system (Amersham, Buckinghamshire, U.K.), and were used as probes.

similar findings were also obtained in investigations with mature granulocytes (Fig. 5). Thus, the transcript was clearly demonstrated in granulocytes from the three representative alpha interferon-sensitive CML patients (M.T., S.K., K.T.) as well as from normal volunteers (data not shown), while markedly reduced in granulocytes from one representative alpha interferon-resistant patient (M.I.). More interestingly, the transcript became almost normal in this patient (M.I.) when re-tested after improved control of the neutrophil count by combined use of HU/interferon.

AN EXPERIMENTAL MODEL OF INTERFERON-SUPPLEMENTATION GENE THERAPY USING DIFFUSION CHAMBERS

A model system for fibroblast-mediated alpha interferon supplementation gene therapy has been developed [17-19]. In this system, diffusion chambers containing a constant number of genetically-manipulated NIH/3T3 fibroblasts (3T3-interferon), which now secrete a large amount of human alpha interferon, or control fibroblasts (3T3) were implanted subcutaneously into nude mice bearing an alpha interferon-sensitive CML cell-line, KU812 (Fig. 6); [20, 21]. The alpha interferon levels in sera of nude mice with 3T3-interferon cells

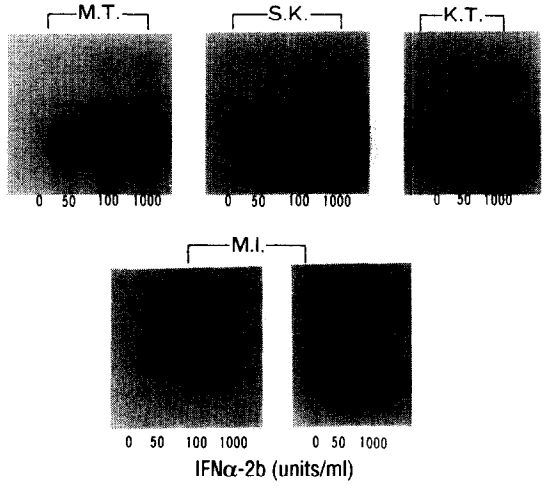


Fig. 5. Northern blot analysis for *in vitro* expression of ISG56 mRNA in CML granulocytes. Granulocytes were isolated from heparinized bone marrow and peripheral blood of each of the four representative CML patients in the stable chronic phase (M.T., S.K., K.T., M.I.) by Ficoll-metrizoate density centrifugation at 400G and by ammonium chloride-induced haemolysis. M.T., S.K., and K.T. responded well to the treatment with alpha interferon alone, while M.I. was resistant to this treatment but was successfully treated with the combined use of alpha interferon and HU. The methods used here were as described in Figure 4, except for the concentration of interferon alfa-2b. Only the data using ^{32}P ISG56 cDNA as a probe are shown. In M.I., the data obtained both in the resistant phase (right) and in the sensitive phase (left) are shown.

became much higher than those with 3T3 cells (222.0 ± 77.5 versus 6.0 ± 0.3 U/ml 2 weeks after implantation), without any physical or haematological changes. Consistent with these findings, the KU812 tumour growth in nude mice with 3T3-interferon cells was markedly suppressed compared to the controls (Fig. 7). In two out of the six mice with 3T3-interferon, the tumour had completely disappeared 2 weeks later.

DISCUSSION

A prolonged life expectancy has been achieved in alpha interferon-treated CML patients, even without suppression of Ph^1-positive clones, as shown in the alpha interferon/HU-treated patients. Other studies using polymerase chain reaction amplification have shown a persistent bcr-abl rearrangement, even in eight patients treated with alpha interferon who had become both Ph^1-negative and bcr-abl rearrangement negative on Southern blot testing [22]. It is therefore likely that the most prominent feature of alpha interferon efficacy is a suppression, not a complete elimination, of Ph^1 clone proliferation, presumably associated with an inhibition of the development of blastic transformation, particularly of the myeloblastic type. In order to establish the possible benefits of combined use of MTX with alpha interferon, it will be necessary to conduct a randomized controlled study involving a larger number of patients and with a long follow-up period.

Several possible mechanisms of alpha interferon resistance have so far been investigated. They include decreased binding to specific surface receptors, loss of inhibitory activity on the bcr-abl product, failure to induce 2',5'-oligoadenylate synthetase, and loss of down-regulation of c-*myc* gene product.

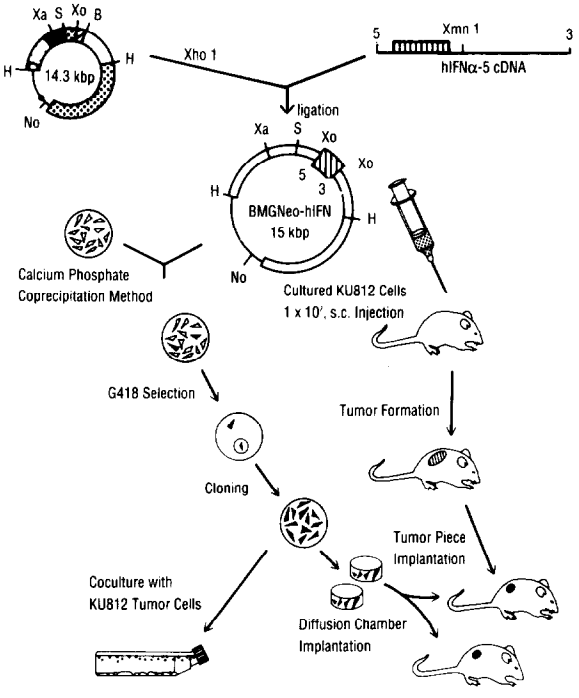


Fig. 6. Fibroblast-mediated interferon supplement gene therapy. The 682-base pair *Bgl*II-*Xmn*I fragment of human alpha interferon 5 cDNA (a generous gift from Dr Nagata, Osaka Bioscience Institute, Japan) [20] was blunted, ligated with *Xho*I linker, and inserted into the *Xho*I site of the BMGNeo vector (a generous gift from Dr Karasyama, University of Tokyo, Japan) [21]. The BMGNeo-interferon DNA and control BMGNeo vector DNA were introduced into the NIH/3T3 cells (clone no. 5611) (Japanese Cancer Research Resource Bank, Japan) cultured in DMEM supplemented with 10% bovine serum, using the calcium phosphate co-precipitation method. The transformed cell clones were selected by culturing in the medium with G418, and were assessed for the episomal amplification of the BMGNeo plasmid and alpha interferon 5 mRNA, by Southern and Northern blot analysis, respectively. One clone (3T3-interferon) secreting a large amount of human alpha interferon 5 and one interferon-non-secreting control clone (3T3) were selected by estimating the levels in the culture-conditioned medium using radioimmunoassay employing anti-human interferon monoclonal antibody (Dynabott, U.S.A.). It was also demonstrated in the *in vitro* co-culture studies that KU812 cells became pyknotic within 7 days on the confluent 3T3-interferon cell layers but not on the control 3T3 cell layers (data not shown). Ten million 3T3-interferon cells or 3T3 cells in the culture medium were enclosed in a diffusion chamber, each of which was implanted subcutaneously in a BALB/c nu/nu mouse bearing a growing KU812 tumour.

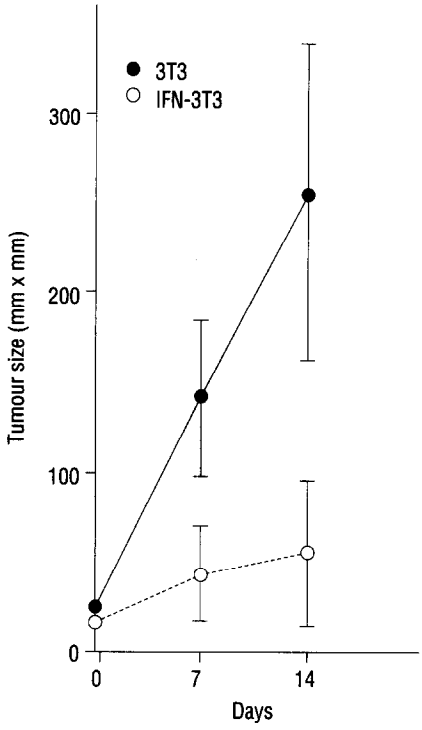


Fig. 7. Growth curves of KU812 tumours in nude mice implanted with the diffusion chambers containing 3T3-interferon and 3T3. The methods are described in Fig. 6. The KU812 tumour size was measured weekly. Each value is mean ± standard deviation from 10 mice.

However, the former two possibilities have almost been excluded, while the latter two have not yet been confirmed. To address this interesting issue, we have been investigating the *in vitro* transcriptional activation of ISG. Our preliminary data demonstrated that the *in vitro* expression of ISG mRNA was markedly suppressed in the alpha interferon-resistant patient. The fact that normal granulocytes, like CML granulocytes, respond to alpha interferon does not explain the selective inhibition of CML clones often observed *in vivo*. However, these findings strongly suggest that the product of ISG may be acting as an important intracellular negative regulator for cell growth and also that the *in vitro* transcriptional activity in granulocytes may provide a useful tool for prediction of alpha interferon efficacy in CML.

We have also shown an experimental model system of gene therapy. This model not only provides clear evidence for the direct suppressive action of alpha interferon on CML cell growth, but also shows that the diffusion chamber system makes it possible to utilize the malignant cells as a vehicle, and to regulate the number of these cells and, in turn, the interferon levels. Furthermore, this system, although not practical at the moment, addresses one of the problems of alpha interferon therapy; that is, the distress caused by continuous injections.

CONCLUSIONS

In summary, these studies indicate that:

1) the combined use of MTX and/or HU with alpha interferon seems to be efficient in alpha interferon-resistant patients.
2) the *in vitro* transcriptional ISG activity of CML granulocytes might be used to predict the clinical efficacy of alpha interferon therapy.
3) fibroblast-mediated gene therapy using the diffusion chamber method may be useful for the purpose of interferon supplementation in CML.

We realize that the present data are not sufficiently mature, but the approaches described here may contribute toward improving alpha interferon therapy in the future.

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Acknowledgements—We thank Dr Kiyoshi Kiamura, the 3rd Department of Internal Medicine, Faculty of Medicine, and many attending physicians, Department of Hematology-Oncology, Institute of Medical Science, University of Tokyo, for kindly supplying the clinical data of the patients.

Eur J Cancer, Vol. 27, Suppl. 4, p. S25, 1991
Printed in Great Britain

0277-5379/91 $3.00 + 0.00

Interferon Alfa-2b in the Treatment of Chronic Myelogenous Leukaemia

Masami Bessho, Nobutaka Kawai and Kunitake Hirashima

ABSTRACT

Recent reports have indicated that alpha interferon can be an effective treatment for patients with chronic myelogenous leukaemia (CML) [1]. In order to evaluate the clinical usefulness of interferon alfa-2b, we treated six patients with chronic phase CML and observed their clinical course.

The patients consisted of four males and two females, aged between 39 and 58 years, who had previously received either treatment with busulfan (four patients) or no therapy (two patients). Interferon alfa-2b was administered intramuscularly at a dose of 3-10 million units (MU)/body, either daily or three times per week, for more than 8 weeks.

All six patients showed a fall in white blood cell count from a mean of 101.8 x 10^9/L (range 15.6-330) before treatment to a mean of 25.7 x 10^9/L (range 4.0-117) after treatment with interferon alfa-2b. Haemoglobin remained largely unchanged, and platelet counts fluctuated. Two of the six patients also showed a slight reduction in the percentage of Ph^1-positive clones (to 98% and 96%, respectively).

Complete haematological response was achieved in four patients, partial haematological response in one and no response in one. All six patients are alive at a mean of 69 months (range 38-108 months) from diagnosis and are either in chronic phase (five patients) or post bone marrow transplant (one patient).

Major side effects of alpha interferon included fever, general fatigue, and nausea, but all were tolerable.

In conclusion, alpha interferon was useful for controlling blood cell counts in chronic phase CML patients, with tolerable side effects. Five of six patients achieved long-term haematological remission, and alpha interferon slightly reduced the fraction of Ph^1-positive clones in two patients.

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