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Effect of Recombinant α-Interferon on the Expression of the *bcr-abl* Fusion Gene in Human Chronic Myelogenous Human Leukemia Cell Lines[1]

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ABSTRACT

Recent investigations have shown a therapeutic and cytogenetic response in chronic myelogenous leukemia (CML) patients treated with recombinant α_2-interferon (IFNα2). Philadelphia chromosome-positive (and many Ph[1]-negative) chronic myelogenous leukemia cells transcribe a novel *bcr-abl* fusion mRNA which may confer a growth advantage upon these cells. We investigated the effect of IFNα2 on the levels of *bcr-abl* transcript expression in three Ph[1]-positive CML cell lines, EM2, KCL22, and K562. Although IFNα2 inhibited cell proliferation in all three CML cell lines, IFNα2 had no effect on the level of *bcr-abl* mRNA expression in any of the CML cell lines. In contrast, IFNα2 increased the expression of class I HLA gene products. We conclude that while the *bcr-abl* fusion gene and its transcript undoubtedly play key roles in the pathogenesis of CML, the antiproliferative effect of IFNα2 in CML cell lines relies upon genetic mechanisms other than modulation of *bcr-abl* expression.

INTRODUCTION

Recent investigations have shown that IFNα2[4] is of clinical benefit in patients with Philadelphia chromosome-positive CML. Talpaz *et al.*(1) recently reported response in 14 of 17 CML chronic phase patients with 6 patients entering cytogenetic remission after treatment with recombinant IFNα2. This response is particularly intriguing in view of the dismal prognosis of this disease barring successful bone marrow transplantation. The present study was undertaken to determine if this selective effect on Ph[1]-positive stem cells was due to suppression of the altered *bcr-abl* transcript characteristic of CML.

The hematopoietic cells of over 90% of CML patients harbor the specific Philadelphia chromosome marker (Ph[1]), which results from a reciprocal translocation involving chromosome 9q34 and chromosome 22q11. In most cases, the c-*abl* oncogene is moved from its usual locus on chromosome 9q34 to a specific 5.8-kilobase region on the long arm of chromosome 22 known as the breakpoint cluster region (*bcr*) (2–4). This translocation generates a *bcr-abl* fusion gene which transcribes a novel 8.2-kilobase *bcr-abl* fusion mRNA (5–7). The translation of the fusion transcript forms the $P210^{bcr\text{-}abl}$ fusion protein (8). P210 exhibits enhanced tyrosine kinase activity compared with the wild-type c-*abl* P145 product (8–11). The presence of the *bcr-abl* fusion transcript in some unknown manner might somehow confer a growth advantage upon Ph[1]-positive CML cells over normal marrow precursors (12–14).

The three major types of interferon (α, β, γ) are capable of exerting a modulatory effect upon the expression of certain genes in various malignant cell lines (*i.e.*, Daudi, U937, neuroblastoma, Molt 4) including CML cell lines (15–19). The genes with expressions that are primarily affected by the interferons fall into four general categories: category 1, translation-regulatory enzymes; category 2, surface antigens; category 3, oncogenes; and category 4, unidentified proteins (20–23). More specifically, class I HLA antigens are well known to be inducible on multiple vertebrate malignant cell lines (including the K562 CML blast crisis cell line) by one or more of the three major interferon species (15–19, 21, 22, 24). Alterations in the proliferative and/or differentiative state of many *in vitro* cell lines are induced by the interferons (16, 17, 19, 25–27). Accompanying these alterations, modulation in the expression of certain "competence" protooncogenes such as c-*myc* is observed generally as cells accumulate in G_1/G_0 (16, 28). Furthermore, interferon decreases expression of the c-Ha-*ras* and the c-*src* protooncogene protein products, and c-Ha-*ras* loses its capacity for transforming 3T3 cells after addition of IFNα2 (26).

If, indeed, the *bcr-abl* fusion product confers some growth advantage, then it is possible that the antiproliferative effect of the interferons reported in the CML cell lines results from decreased expression of the *bcr-abl* fusion gene. Because of the effect of interferons on the expression of multiple genes including protooncogenes and because of the empiric effectiveness of IFNα2 in CML, we have undertaken the study of the possible effect of IFNα2 on the expression of the *bcr-abl* fusion gene in the various CML blast crisis cell lines.

MATERIALS AND METHODS

Cell Lines. The CML blast crisis cell lines K562, KCL22, and EM2 were used for studies of gene induction by IFNα2. The myeloid cell lines HL60 served as a control. Cells were cultured in RPMI-5% fetal calf serum-1% glutamine in a 4.0% CO_2 environment at 37°C. Cells were then harvested and resuspended in 50 ml fresh media at a concentration of 2.0×10^5 cells/ml in T-75 tissue culture flasks (Corning). Cells were counted using a hemocytometer counting chamber (American Optical) both before and after interferon induction.

Interferon. Commercially available recombinant α_{2b}-interferon (Schering) was diluted to a concentration of 200,000 units/ml and was then added to the flasks of each cell type in concentrations of 1,000 or 5,000 units/ml. An equal amount of RPMI without IFNα2 was added to the control flasks. For each cell line 10^8 cells were treated at each dosage level.

RNA Extraction. Following two washings in 1 × standard saline citrate, cells were pelleted, suspended in 6 ml of a solution of 6 M guanidine HCl and 0.1 M potassium acetate, and sonicated. RNA was then precipitated overnight with 3 ml of 95% ethanol, pelleted by centrifugation at 10,000 rpm for 20 min, and resuspended in 1 ml of 6 M guanidine HCL-0.1 M potassium acetate-0.02 M EDTA. This suspension was then sheared twice through a No. 22 spinal needle after intervening ethanol precipitation. After a final ethanol precipitation, the pellet was dissolved in 0.02 M Tris-0.1 M NaCl-0.2% sodium dodecyl sulfate-0.01 M EDTA. This solution was extracted twice with phenol-IAA chloroform followed by a single IAA chloroform extraction. The RNA was precipitated at −70°C in 95% ethanol, pelleted in an Eppendorf microfuge, and redissolved in an appropriate amount of water, and the concentration of RNA was estimated by determining the absorbance

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[4] The abbreviations used are: IFNα2, α_2-interferon; CML, chronic myelogenous leukemia; cDNA, complementary DNA; rIFNα2, recombinant α_2-interferon.

at 260 nm. The quality of whole cellular RNA was confirmed by running 5 μg on a denaturing 1% agarose-formaldehyde gel and then checking for the presence of 28S and 18S ribosomal fragments by ethidium bromide staining. RNA was polyadenylated, selected over an oligothydmidylic acid-cellulose column (29).

Northern Blotting. Polyadenylated RNA was run on a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized as described previously (30).

Molecular Probes. We used the following DNA fragments as probes: actin, a 2.0-kilobase chick pA 1 β-actin cDNA *Pst* fragment (31); *bcr*, a 1.0-kilobase *Pst-Sph* fragment isolated from the 5′ portion of the breakpoint cluster region (32); HLA, a 1.3-kilobase fragment of the HLA-B cDNA inserted into pBR322 (33); c-*myc*, a 1.8-kilobase fragment of human *myc* cDNA which encompases exons II and III of c-*myc*. All probes were nick translated using ^{32}P-tagged nucleotides by methods described previously (34).

RESULTS

Inhibition by IFNα2 of Cell Proliferation of CML Blast Crisis Cell Lines. The dose effect of IFNα2 on cell proliferation was determined in three cell lines, K562, KCL22, and HL60 (Fig. 1). The K562 proliferation was the most remarkably inhibited of the three. While doses of 250 units/ml or less had no effect upon proliferative rate, doses of 500, 1000, and 2000 units/ml exerted a definite antiproliferative effect on K562. The highest dose concentrations tested, 5,000 and 10,000 units/ml, resulted in minimal cell proliferation for the first 4 days in culture (Fig. 1*A*).

The antiproliferative effect was less pronounced in the KCL22 cell line although a definite decrease in rate of cell proliferation was noted at concentrations of 1000 units/ml or greater. This effect was noted within the first 2 days of IFNα2 induction and continued over the ensuing 4 days (Fig. 1*B*).

HL60 proliferation was previously found to be only slightly affected by IFNα2 (16). We confirmed this finding and in fact noted a slight stimulation to proliferation at 5000 units/ml (Fig. 1*C*).

Expression of the *bcr-abl* Gene Unaffected by rIFNα2. To study normal *bcr* as well as *bcr-abl* fusion transcript expression in the rIFNα2-treated cells, we used a 1.0-kilobase *Pst-Sph* fragment from the 5′ portion of *bcr* as a probe (32). This probe detects the normal 4.5- and 7-kilobase *bcr* transcripts as well as the aberrant *bcr-abl* fusion transcript. The K562, KCL22, and EM2 CML cell lines express the aberrant 8-kilobase *bcr-abl* fusion transcript at increased levels in the uninduced state. The normal 4.5- and 7.0-kilobase *bcr* transcripts are present in the CML cell lines as well as the HL60 and KG1 controls. We observed no effect on the steady state level of either the 8-kilobase *bcr-abl* fusion transcript or the normal *bcr* transcripts after 24 h IFNα2 induction in K562, KCL22 (Fig. 2), and EM2 (blot not shown). Similarly, we observed no effect upon the expression of the normal *bcr* transcripts in the HL60 control. Furthermore, we noted no change in *bcr-abl* transcript levels after a 12-h incubation with IFNα2 (data not shown). Hybridization of these same samples (12- and 24-h incubations) to a c-*myc* probe indicated no change in the expression of this oncogene in IFNα2-treated cells (blots not shown).

Actin Expression in rIFNα2-treated Cell Lines. A 2.0-kilobase chick pA 1 β-actin cDNA *Pst* fragment was used to quantify mRNA on Northern blots (31). While some variation in amount of mRNA per lane is present (Fig. 2, *middle*), the use of the actin probe permitted comparison of relative levels of other mRNAs alongside the actin mRNA control. The decreased *bcr-abl* expression seen in the 1000-units/ml-treated K562 cells was probably due to a slight underestimate of mRNA loaded onto the gel in this lane as the actin control also shows a comparatively reduced level in this particular lane (Fig. 2, *top*).

HLA Expression in rIFNα2-treated Cell Lines. The HL60 cells have been shown previously to express HLA class I antigens on their surfaces while K562 was known not to express such antigens unless induced by interferon (15, 21). Using a 1.3-kilobase HLA-B fragment in pBR322, designated pHLA, as a probe, we were able to demonstrate the induction of the 1.6-kilobase HLA transcript in a dose-responsive manner in the HL60, K562, KCL22, and EM2 cell lines (Fig. 2, *right*). An increased amount of the HLA class I message is seen in

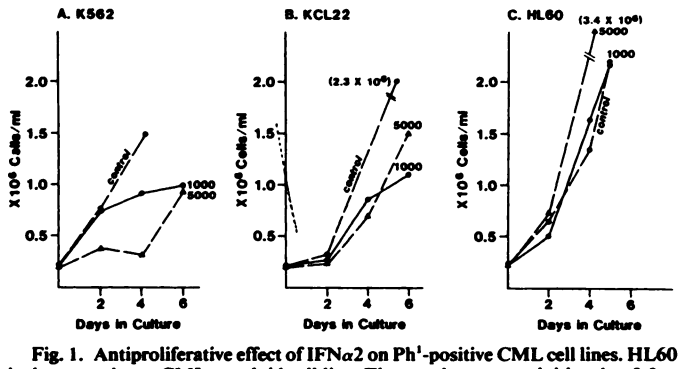

Fig. 1. Antiproliferative effect of IFNα2 on Ph¹-positive CML cell lines. HL60 is the control non-CML, myeloid cell line. Tissue cultures were initiated at 2.0 × 10^5 cells/ml. Cultures were exposed to either 1000 or 5000 units/ml IFNα2 for the time period indicated.

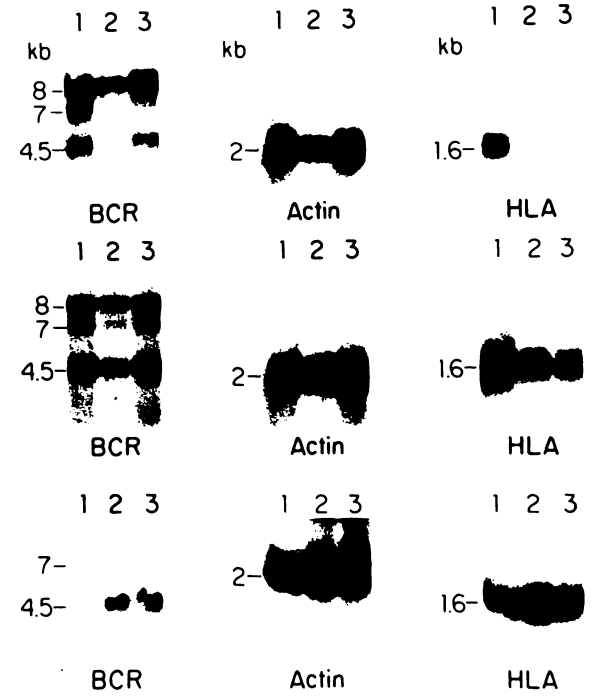

Fig. 2. Northern blot analysis of rIFNα2-treated Ph¹-positive CML cell lines K-562 (*top*), and KCL-22 (*center*), and the HL60 control cell line (*bottom*). Approximately 5 μg of polyadenylated RNA/lane were electrophoresed through a 1% denaturing agarose-formaldehyde gel. The resulting blot was transferred to nitrocellulose and successfully hybridized to the indicated probes. The three lanes under each cell type are ordered *left* to *right* according to dose levels of IFNα2: *Lane 1*, 5000 units/ml; *Lane 2*, 1000 units/ml; *Lane 3*, no IFNα2. Exposure to the interferon dose was for 24 h at which time cells were harvested for RNA extraction. The amount of polyadenylated RNA loaded into the lane for K562 cells exposed to 1000 units/ml IFNα2 probably contains less RNA than other lanes as indicated by the actin probe.

cells treated for 24 h with 1000 or 5000 units/ml IFNα2 when compared to the control cells. Increased expression of HLA mRNA was not detected after only 12 h induction in EM2 or KCL22 (data not shown).

DISCUSSION

Interferons are known to have antiproliferative effects in multiple cell lines. In particular rIFNα2 suppresses proliferation of Ph¹-positive CML cells (1). The suppression of certain genes such as *src* and c-Ha-*ras* protooncogenes by the interferons has been shown. Conversely, induction of certain genes such as class I and II HLA genes by the interferons has been described. We have studied the effect of IFNα2 on the expression of the *bcr-abl* fusion gene in Ph¹-positive CML cells with the hypothesis that if this fusion gene confers some growth advantage upon CML cells then agents inhibiting Ph¹-positive cell proliferation may act by decreasing this *bcr-abl* fusion gene expression. Our studies reveal that IFNα2 at doses which inhibit CML cell proliferation does not decrease expression of the *bcr-abl* fusion oncogene or modulate expression of the normal *bcr* gene in CML blast crisis cell lines. We therefore conclude that the antiproliferative effect exerted by rIFNα2 on these cell lines is not mediated through modulation of the *bcr-abl* transcript level. Our data also show that the induction of HLA class I mRNA can be induced in the K562, the EM2, and the KCL22 cell lines with IFNα2. This response had not been described previously in the EM2 and KCL22 CML cell line, but this result was anticipated inasmuch as increased HLA mRNA expression has been described previously in K562 cell line after treatment with γ-interferon IFN (21).

We chose doses of IFNα2 which were compatible with therapeutic levels *in vivo*. Also, others have shown that concentrations of IFNα2 in this range affect expression of other genes (24). Utilizing two high dose levels of IFNα2, 1000 and 5000 units/ml, we observed no effect on *bcr-abl* expression after 12 h in KCL22 and EM2 or after 24 h in the K562, KCL22, and EM2 cell lines. Potentially, a problem may exist with this selection of time intervals since some genes are only transiently stimulated or suppressed by the IFNα2 (20). However, the 24-h induction interval for HLA class I mRNA is compatible with previous observations of HLA mRNA induction (19, 22, 24). Also, we noted that the different cell lines vary in their responsiveness to IFNα2. Those cell lines which express HLA antigens in the uninduced state (EM2 and KCL22) express higher levels of class I HLA mRNA than K562, which does not express HLA mRNA until induced by IFNα2. Therefore, while given degrees of induction can be compared among the three dose levels within a given cell line, these responses cannot be compared among the various cell lines.

The lack of effect of antiproliferative concentrations of rIFNα2 on the *bcr-abl* fusion transcript expression does not necessarily indicate that the *bcr-abl* gene product is uninvolved in the proliferation of CML cells. It is possible that rIFNα2 exerts its antiproliferative effect on CML cells by inhibiting the enzymatic activity of the *bcr-abl* fusion protein (P210) or by inhibiting the substrate for this protein. If the P210 substrate plays an active role in cell proliferation, perhaps involving the transduction of growth-stimulatory signals from cytoplasmic membrane to nucleus, then inhibition of this substrate might have an antiproliferative effect. Presently this question of whether rIFNα2 might inhibit the activation or expression of the P210 substrate is difficult to approach experimentally because the major substrate(s) for P210 has not been clearly identified.

Finally, it is still possible that genetic mechanisms other than alterations in *bcr-abl* expression underlie the observed effect of IFNα2 on CML blast crisis cell lines. Such mechanisms might include the decreased expression of other genes critical to proliferation. Another potential mechanism might be enhanced immunogenicity of CML cells possibly resulting from increased HLA expression or simply an increased immune response resulting from IFNα2 stimulation of the immune system. Similar studies using fresh cells are required to assess the effect of IFNα2 on *bcr-abl* expression in circulating CML cells. While the *bcr-abl* fusion gene, its mRNA transcript, and its P210 protein product apparently figure centrally in the malignant characteristics of CML cells, our studies indicate that the antiproliferative effect of IFNα2 on CML blast crisis cell lines cannot be attributed to modulation of *bcr-abl* expression.

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