Interferon-α Overrides the Deficient Adhesion of Chronic Myeloid Leukemia Primitive Progenitor Cells to Bone Marrow Stromal Cells

By Charles Dowding, Ai-Pu Guo, Jürgen Osterholz, Martin Siczkowski, John Goldman, and Myrtle Gordon

Primitive blast colony-forming cells (Bi-CFC) from chronic myeloid leukemia (CML) patients are defective in their attachment to bone marrow-derived stromal cells compared with normal Bi-CFC. We investigated the effect of recombinant interferon-α (IFN-α) on this interaction between hematopoietic progenitor cells and bone marrow-derived stromal cells by culturing normal stromal cells with IFN-α (50 to 5,000 U/mL). At 50 U/mL we found that: (1) the capacity of stromal cells to bind two types of CML primitive progenitor cells (Bi-CFC and long-term culture-initiating cells) was increased; and (2) the amount of sulfated glycosaminoglycans (GAGs) in the stromal layer was increased. However, sulfated GAGs were not directly involved in binding CML Bi-CFC, unlike binding by normal Bi-CFC, which is sulfated GAG-dependent.

Recombinant interferon-α (IFN-α) has proven to be useful in the treatment of Philadelphia chromosome-positive chronic myeloid leukemia (CML). About 70% of patients show long-lasting hematologic control. In some responding patients, IFN-α permits the re-emergence of a significant degree of Ph-negative hematopoiesis, but the mechanism of its apparently selective action in CML is unknown. IFN-α has a direct antiproliferative effect on both normal and CML progenitor cells in vitro but no selective anti-leukemic activity on progenitor cell proliferation has been demonstrated. In contrast to IFN-α, recombinant IFN-γ has proved to be ineffective in treating patients with CML either as a single agent or in combination with IFN-α.

The bone marrow (BM) microenvironment is a possible alternative target for IFN-α. The binding of CML progenitor cells to stromal cells in vitro is deficient, in vivo this deficiency could lead to release of progenitor cells into the circulation and hematopoiesis in extramedullary sites. We therefore speculated that IFN-α might act by modifying the interaction between CML and stromal cells in a way that allows normal microenvironmental control to be restored. Accordingly, we investigated the binding of CML progenitor cells to stromal cells cultured with IFN-α or IFN-γ and the effect of IFN-α on the proliferation of Ph-positive and Ph-negative long-term culture-initiating cells.

We also investigated the influence of IFN-α and IFN-γ on extracellular matrix (ECM) synthesis by stromal cells. Heparan sulfate and chondroitin sulfate, both sulfated glycosaminoglycan (GAG) molecules, are highly negatively charged ECM components identified in cultures of BM-derived stromal cells. Heparan sulfate has been implicated in normal hematopoietic cell-stromal cell interactions and as a growth factor sequestraotr within the ECM of stromal cells. Another negatively charged molecule, neuraminic acid, is also involved in cell-to-cell interactions. Therefore, we investigated the effects of IFN-α and IFN-γ on (1) the synthesis of sulfated GAG by stromal cells, and (2) the role of sulfated GAG and neuraminic acid molecules in the attachment of primitive myeloid progenitor cells to BM-derived stromal cells.

Neuraminidase-treated control stromal cells bound an increased number of CML Bi-CFC, reproducing the effect of IFN-α, whereas the binding to IFN-α-treated stromal cells was unaffected by neuraminidase treatment. Thus, the enhanced attachment by primitive CML progenitor cells to IFN-α-treated stromal cells might be due to changes in the neuraminic acid composition in the stromal cell layer. Our in vitro evidence may provide insights into the mechanism of action of IFN-α in vivo. Prolonged administration may alter the marrow microenvironment in some patients such that it can restrain the aberrant proliferation of Philadelphia chromosome-positive (Ph)-positive stem cells while permitting Ph-negative stem cells to function normally.

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MATERIALS AND METHODS

Samples. Peripheral blood cells from 13 patients with Ph chromosome-positive CML in chronic phase were collected before initiation of therapy with cytotoxic drugs. The median white blood cell (WBC) count for the patients investigated was 198 × 10⁹/L and ranged from 124 to 287 × 10⁹/L. Normal BM cells collected from iliac crests of donors of marrow for allogeneic transplantation were used as controls. Informed consent was given to use samples in experimental procedures. For most experiments mononuclear cell fractions (MNCs) from blood or marrow were prepared by centrifugation over Lymphoprep (density 1.077 g/mL; Nycomed, Oslo, Norway) according to the method of Boyum. For the long-term culture experiments, MNCs were prepared by centrifugation (400g, 30 minutes) over iso-osmotic Percoll (density 1.065 g/mL; Pharmacia, Uppsala, Sweden). MNCs prepared by both techniques were washed twice in Hank's balanced salt solution (HBSS; Gibco, Paisley, UK) before use.

Stromal cell cultures with IFN-α or IFN-γ. Stromal layers were grown by plating 5 × 10⁶ normal BM MNCs per milliliter in α medium (GIBCO) containing fetal calf serum (FCS; 15%, GIBCO), methylprednisolone (1.6 × 10⁻⁷ mol/L; Upjohn, Crawley, UK), with (50 to 5,000 U/mL) or without recombinant IFN-α, (2 × 10³ U/mg protein; Roferon, Hoffman La Roche, Basel, Switzerland) or with (25 to 100 U/mL) or without recombinant IFN-γ (2 to 4 × 10³ U/mg protein; Biogen S.A., Basel, Switzerland) in 35-mm petri dishes or 25-cm² flasks (GIBCO). Cultures were fed weekly by total replacement of medium and additives. Stromal layers achieved confluence after 3 to 5 weeks and were used in the procedures described below. The effect of IFN-α (50 to 5,000 U/mL) on attachment of primitive myeloid progenitor cells to BM-derived stromal cells was unaffected by neuraminidase treatment. However, IFN-α treatment increased the capacity of stromal cells to bind primitive myeloid progenitor cells (Bi-CFC and long-term culture-initiating cells) compared with control stromal cells cultured without IFN-α.

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stromal cell colony formation was assayed using a modification of a standard CFU-F assay. Stromal cultures were initiated and colonies composed of $\geq 50$ cells were scored on day 10. Colonies of cells with fibroblastoid morphology were identified by staining with hematoxylin for 2 minutes. In one representative experiment all colonies stained positively with alkaline phosphatase, confirming a fibroblastoid phenotype.

Attachment of progenitor cells to stromal layers. Adherent cells were removed from normal BM or CML peripheral blood MNCs by incubating $4 \times 10^5$ cells in $\alpha$ medium (8 mL) containing PCS (15%) for 2 hours in 25-cm$^2$ tissue culture flasks. The nonadherent MNCs were assayed for blast colony-forming cells (BI-CFC) using the standard assay previously described. In brief, the nonadherent equivalent of $5 \times 10^3$ MNCs was added to each petri dish culture of stromal cells (three replicate dishes per experimental point) and were incubated for 2 hours. Cells not attached to the stromal cells were decanted and the stromal layers were washed vigorously three times with HBSS, overlaid with $\alpha$ medium (1 mL) containing PCS (15%) and agar (0.3%; Difco Laboratories, Detroit, MI), and incubated at 37°C in 4% CO$_2$ in humidified air. Colonies were counted 5 or 6 days later and dishes were desiccated and stained with May-Grünwald/Giemsa to identify colonies composed of blast cells. In different experiments, stromal cells grown $\pm$ IFN-\(\alpha\), $\pm$ IFN-\(\gamma\), or $\pm$ enzyme treatment (see below) were used.

The binding efficiency of CML BI-CFC was determined by measuring the recovery of BI-CFC following incubation of cell suspensions with stromal layers. Adherent cell-depleted CML MNCs were incubated ('panned') on stromal cells (primary) for 2 hours. Unattached cells were removed by sucking the medium up and down 10 times with a Pasteur pipette and were then transferred to a second dish of control stromal cells (secondary) and incubated for a further 2 hours. Blast cell colonies were scored in both primary and secondary cultures and the attachment of CML BI-CFC to primary stromal cells as a proportion of the primary $+$ secondary total was calculated.

To determine the recovery of normal or CML burst-forming unit-erythroid (BFU-E) and granulocyte-macrophage colony-forming cells (GM-CFC) from stromal cells cultured $\pm$ IFN-\(\alpha\) (50 U/mL), the nonadherent equivalent of $5 \times 10^3$ normal or CML MNCs were assayed directly using standard clonogenic techniques or 'panned' for 2 hours on stromal cells before assay. Unattached cells were decanted from the stromal layers and the number of BFU-E and GM-CFC was calculated.

Attachment to and culture of long-term culture (LTC)-initiating cells with stromal cells grown $\pm$ IFN-\(\alpha\). In replicate experiments, MNCs (4 x 10$^5$, of density $\leq 1.065$ g/mL) from four newly diagnosed CML patients were suspended in $\alpha$ medium (8 mL) containing PCS (15%) and were then added to flasks (25 cm$^2$) of irradiated (30 Gy; 13Cs source) normal allogeneic stromal cells grown $\pm$ 50 U/mL IFN-\(\alpha\) from culture initiation. After 2 hours the flask was rocked ten times and the nonadherent colonies (2h non-adh) were removed, and the remaining cells attached to stromal cells were cultured in LTC medium (8 mL) consisting of $\alpha$ medium containing horse serum (12.5%), FCS (12.5%), methylenepinsolone (2 x 10$^{-7}$ mol/L) and $\pm$ IFN-\(\alpha\) (50 U/mL). The '2h non-adh' cells were pelleted, resuspended in LTC medium, and added to identical (secondary) flasks of irradiated stromal cells matched with the first according to exposure to IFN-\(\alpha\). LTGs set up using IFN-\(\alpha\)-treated stromal cells were maintained in the presence of IFN-\(\alpha\) (50 U/mL), whereas LTGs set up with control stromal cells were maintained without IFN-\(\alpha\). Cultures were incubated at 33°C in 4% CO$_2$ in humidified air. On days 7, 17, and 27 the cultures were semi-depopulated and re-fed with fresh medium plus additives. In addition, cultures were harvested by trypsinization (1:20 in EDTA, 15 to 30 minutes at 37°C; GIBCO) of the adherent layer and the numbers of GM-CFC per flask calculated. The numbers of GM-CFC on day 27 were presumed to reflect the numbers of LTC-initiating cells seeded because at this time few of the original GM-CFC seeded remain.

Generation of Ph-positive/negative GM-CFC. Individual granulocyte-macrophage colonies derived from the 27-day-old LTGs from the same four CML patients studied above were analyzed cytogenetically using a standard technique with minor modifications. In brief, on days 7 to 9 of culture a mixture of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; 1,000 U/mL; Genetics Institute, Cambridge, MA) and recombinant interleukin-3 (IL-3; 1,000 U/mL; Genetics Institute) in a 50 $\mu$L volume was added to a 35-mm dish containing the colonies. After 24 hours incubation at 37°C, coclemid (50 $\mu$L, 5 $\mu$g/mL; GIBCO) was added dropwise to the culture and was incubated for 2 to 4 hours at 37°C. Individual colonies were then plated from the culture and resuspended in a droplet of KCl (0.075 mol/L) placed on a poly-L-lysine-treated slide. After 30 minutes the colony was fixed in methanol-acetic acid (3:1)\(^{12}\) and stained with Giemsa stain and the chromosomes were analyzed. For 'direct' cytogenetic analysis of cells from LTC, cocciid (40 $\mu$L, 50 $\mu$g/mL) was added to the culture, and after 4 hours the cells were pelleted and resuspended in KCl (0.085 mol/L). After 30 minutes of incubation at 37°C, the cells were pelleted, fixed in methanol-acetic acid (3:1), and stained with Giemsa stain.

Sulfated GAG in stromal layers. To estimate the amount of sulfated GAG in stromal layers grown $\pm$ IFN-\(\alpha\) or $\pm$ IFN-\(\gamma\), confluent cultures (in 25-cm$^2$ flasks or 35-mm dishes) were incubated for 72 hours in fresh culture medium $\pm$ IFN-\(\alpha\) (50 to 500 U/mL) or IFN-\(\gamma\) (25 to 100 U/mL) containing $[^{35}S]$ sulfate (50 $\mu$Ci/mL, specific activity 20 to 40 Ci/mmol; Amersham, Aylesbury, UK). The medium was then decanted and the cell layer was rinsed three times in $\alpha$ medium and incubated in fresh medium for a further 4 hours at 37°C to chase free $[^{35}S]$. The stromal cells were washed three times, air dried, and the sides were cut off the culture vessel. The surface to which the stromal cells were attached was then placed against an X-ray film (X-Omat; Kodak, Rochester, NY) within a cassette. The autoradiographs were developed after 4 to 12 hours. The intensity of the film blackening gave an indication of the $[^{35}S]$ sulfate incorporation by GAGs in the stromal layer because 90% of sulfate is used in sulfation of GAG molecules with only a small proportion incorporated into glycoprotein molecules. Autoradiographs were scanned using a light densitometer (Chromscan 3; Joyce-Loebl, Gateshead, UK). Quantitation of $[^{35}S]$ sulfate incorporation into the stromal cell layer was also measured using G25 gel filtration. Fractions were counted on an LKB 1217 Rackbeta liquid scintillation counter (Pharmacia, Uppsala, Sweden). The amount of incorporated isotope was determined from the isotope in the first of the two peaks.

Role of GAGs or neuraminic acid in binding CML BI-CFC. Stromal layers grown $\pm$ 50 U/mL IFN-\(\alpha\) were incubated with neuraminidase (isolated from Clostridium perfringens, 0.1 U/mL; Sigma), heparanase (5 U/mL; Sigma), or chondroitinase ABC (0.1 U/mL; Sigma, St Louis, MO) dissolved in $\alpha$ medium, for 30 minutes at 37°C. Control cultures were incubated with $\alpha$ medium alone. The stromal cells were rinsed three times in $\alpha$ medium and used in the BI-CFC assay (above).

Statistics. Dr R. Szydlo (MRC/LRF Leukaemia Unit) performed the statistical analyses. A Student's $t$-test was used to compare differences between colony numbers under various culture conditions. A paired $t$-test was used to evaluate the effect of IFN-\(\alpha\) on the binding of LTC-initiating cells to stromal cells. The IFN-\(\alpha\) dose-response curve was evaluated using an analysis of variance with repeated measures. A $\chi^2$-test was used to compare the effect of IFN-\(\alpha\) on generation of Ph-negative GM-CFC in LTC.
RESULTS

Effect of IFN-α and IFN-γ on stromal cell capacity to bind progenitor cells. The formation of blast cell colonies on IFN-α-treated (50 U/mL) stromal cells by CML (169.5% ± 21.5% of the control, mean ± SEM, P < .05; Fig 1A), but not by normal, Bl-CFC (104.7% ± 10.2%) was increased. At higher IFN-α concentrations, there was a non-significant increase in colony formation by both normal and CML Bl-CFC (data not shown). We sought to confirm that the peak in CML blast colony numbers detected on IFN-α (50 U/mL)-treated stromal cells resulted from increased numbers of Bl-CFC attaching rather than an increased ability of stromal cells to support colony formation. In 'panning' experiments comparing binding to IFN-α-treated and -untreated stromal cells, the proportion of CML Bl-CFC attaching to IFN-α-treated stromal cells in 2 hours was 81% compared with 40% attachment to untreated stromal cells (Fig 1B). In contrast, blast colony formation by CML Bl-CFC on IFN-γ-treated (25 to 100 U/mL) stromal cells showed a dose-dependent decrease (Fig 1C). At 100 U/mL of IFN-γ, blast colony numbers were reduced to 37.1% of the control (P < .05, n = 3) compared with the twofold increase found in the presence of IFN-α.

The effects of IFN-α on progenitor cell binding were further tested in the LTC system. The presence of IFN-α (50 U/mL) during the period of LTC had no significant effect on the relative numbers of LTC-initiating cells detected for any of the four patients studied (Fig 2 and see below). Using cells from these four CML patients, the mean proportion of LTC-initiating cells (d ≤ 1.065 g/mL) that bound to IFN-α-treated stromal cells (50 U/mL) during 2 hours of incubation was 0.59 compared with 0.38 on control stromal cells (P < .05, n = 4; Fig 1D). Stromal cells cultured with IFN-α (50 U/mL) slightly increased the attachment, though not significantly, of CML GM-CFC or BFU-E during a 2-hour 'panning' procedure (89.0% ± 12.1% and 85.8% ± 10.3% of numbers recovered from control stromal cells, respectively; mean ± SEM, n = 5 for both).

Effect of IFN-α (50 U/mL) on proliferation of CML cells in LTC. The total numbers of GM-CFC, in the 2h-adherent and -nonadherent fractions combined, after 27 days of LTC had no significant effect on the total numbers of GM-CFC detected after 27 days of LTC despite the altered distribution of LTC-initiating cells between the 2h-adherent and -nonadherent cultures. The generation of Ph-negative GM-CFC colonies in 27-day-old LTCs grown ± IFN-α (50 U/mL) is shown in Table 1. In three of four patients, only Ph-positive GM-CFC were detected before LTC. In the fourth patient a small proportion of Ph-negative GM-CFC were detected.

![Fig 1. Binding of primitive progenitor cells to stromal cells grown ± IFN-α or ± IFN-γ during 2 hours of incubation.](image)

![Fig 2. Numbers of GM-CFC/flask in four LTCs of CML peripheral blood cells of density less than 1.065 g/mL seeded onto irradiated normal BM stromal cells. See Materials and Methods for culture details.](image)

<table>
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<th>No.</th>
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<th>Post-Culture</th>
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<td>4.2 (24)</td>
<td>6.3 (206)†</td>
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<tr>
<td>4</td>
<td>0 (46)</td>
<td>0 (52)</td>
<td>5.3 (75)</td>
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See Materials and Methods for culture details. 
*Day 27 of LTC.
†Only direct metaphases analyzed.
before LTC. After LTC without IFN-α, a small proportion of Ph-negative GM-CFC (one patient) and Ph-negative metaphases in an experiment where colony analysis failed (one patient) were identified. In two patients, no Ph-negative GM-CFC were found. In cultures seeded on IFN-α-treated stromal cells and grown in the presence of IFN-α, the proportion of Ph-negative GM-CFC or metaphases detected was increased over that in control cultures. In patient 4, whose preculture cells and post-control culture cells contained no Ph-negative GM-CFC, there were 5.3% Ph-negative GM-CFC following LTC with IFN-α. Overall, the differences did not achieve statistical significance, but in no case was the number of Ph-negative GM-CFC lower in cultures grown on IFN-α-treated stromal cells.

Effect of IFN-α and IFN-γ on stromal cell proliferation and GAG synthesis. Normal BM-derived stromal cell colony formation was unaffected by culture with IFN-α at concentrations ≤100 U/mL (Fig 3A). At concentrations of ≥100 U/mL there was a dose-dependent reduction in stromal cell colony numbers (P < .02).

Representative autoradiographs showing [35S] sulfate incorporation by stromal layers cultured with IFN-α or IFN-γ are shown in Fig 3B and C. The intensity of film blackening corresponded to the amount of sulfate incorporated into GAG. Figure 3B shows that at 50 to 100 U/mL, IFN-α increased the amount of sulfated GAG in the stromal layer compared with controls, whereas at higher concentrations the amount of sulfated GAG was reduced in parallel with inhibition of proliferation (Fig 3C). Densitometric scanning of the autoradiograph at 50 U/mL IFN-α showed a 39% increase in intensity compared with the control. Quantitation using G25 chromatography indicated a mean 25% increase in sulfated GAG incorporation into the stromal layer (Table 2). Incubation of stromal cells with IFN-γ (25 to 100 U/mL) did not appear to alter the amount of sulfated GAG in the stromal layer (Fig 3C).

Involvement of stromal cell-derived GAGs and neuraminic acid on attachment by CML Bl-CFC. Stromal layers grown ± IFN-α (50 U/mL) were stripped of sulfated GAGs and neuraminic acid to see if there was any relationship between the effects of IFN-α on these matrix components and its effect on CML Bl-CFC binding. The results of these experiments are shown in Fig 4. Neither heparanase nor chondroitinase ABC treatment of stromal cells had a significant effect on attachment of CML Bl-CFC to stromal cells grown ± IFN-α, but neuraminidase treatment of control stromal cells (ie, without IFN-α) increased blast colony formation by CML cells (P < .05; Fig 4). Thus, the enhancing effect of IFN-α on CML binding to stromal cells was reproduced by treating control stromal cells with neuraminidase. Consequently, the results are consistent with IFN-α acting via modulation of the neuraminic acid content in the hematopoietic microenvironment.

<table>
<thead>
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<th>Experiment No.</th>
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<td>18,079 (130.0%)*</td>
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| Background counts less than 30. See Materials and Methods for labeling details. |
| Percentage of control (no IFN-α) counts. |
to stromal cells. The increased efficiency in binding by CML BM-CFC approached the efficiency reported for normal BM-CFC of which greater than 90% bind to stromal cells. In this previous study designed to determine the rate of attachment of BM-CFC to stromal cells using serial 2-hour panning steps, approximately 90% of normal BM-derived BM-CFC attached to stromal cells during the first 2-hour incubation step. However, apparently only about 40% of the total CML BM-CFC adhered to stromal cells during the first 2-hour panning step because progressively decreasing numbers of BM-CFC could be detected by re-panning the nonadherent cell fraction on stromal cells for further 2-hour incubations. Blast colony-forming cells from CML patients did not have a different binding rate to stromal cells because maximal binding occurred after 2 hours. 

In two of four patients, between 4% to 8% of the circulating LTC-initiating cells were Ph-negative as previously reported and in three of four patients IFN-α increased, though not significantly, the proportion of Ph-negative GM-CFC detected on day 27. These IFN-α-mediated increases in Ph-negative GM-CFC detected could reflect differences in the responses of Ph-positive and Ph-negative LTC-initiating cells to the direct effect of IFN-α or could reflect an effect through modification of the microenvironmental cells,29 suggesting that under different conditions the attachment or release of lineage-committed progenitor cells might also be enhanced by IFN-α.

In this study we showed that IFN-α, but not IFN-γ, increased the attachment of two different types of CML primitive progenitor cells (BM-CFC and LTC-initiating cells)
also, we have recently demonstrated that CML BI-CFC lack a phosphatidylinositole-anchored CAM that is an essential requisite for efficient and selective binding by normal BI-CFC. It is possible that expression of CAMs by hematopoietic progenitor cells controls their precise location in the marrow microenvironment so that they are properly regulated. The operation of a different cell adhesion mechanism by CML BI-CFC, in the context of stromal cells exposed to IFN-α, raises the possibility that the immobilized leukemic cells could be placed at a proliferative disadvantage because they are subjected to inappropriate rather than normal control mechanisms.

In summary, we have presented evidence for a functional difference in the effect of IFN-α on normal and CML progenitor cells that may prove to be associated with the finding of IFN-α-induced normalization of nuclear protein-DNA complexes observed in IFN-α-sensitive leukemic cells. To a great extent IFN-α overcame the deficient attachment of CML BI-CFC to stromal cells and increased the proportion of LTC-initiating cells that attached to stromal cells. This effect was associated with changes in the neuraminic acid content of the stromal layer. It is possible that the mechanism of action of IFN-α in CML patients might include modification of BM microenvironmental cells such that the interaction between hematopoietic cells and stromal cells is enhanced; this could lead to suppression of the leukemic clone and thus allow re-emergence of normal hematopoiesis.

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