Treatment of marrow stroma with interferon-alpha restores normal beta 1 integrin-dependent adhesion of chronic myelogenous leukemia hematopoietic progenitors. Role of MIP-1 alpha.

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Research Article

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Treatment of Marrow Stroma with Interferon-α Restores Normal β1 Integrin-dependent Adhesion of Chronic Myelogenous Leukemia Hematopoietic Progenitors

Role of MIP-1α

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Abstract

The mechanisms by which interferon-α (IFN-α) restores normal hematopoiesis in chronic myelogenous leukemia (CML) are not well understood. We have recently demonstrated that IFN-α acts directly on CML hematopoietic progenitors to restore their adhesion to marrow stroma by modulating β1 integrin receptor function. In the present study we examined the effect of IFN-α treatment of marrow stroma on subsequent adhesion of CML progenitors. Stromal layers were preincubated with IFN-α (10,000 μm/ml) for 48 h. Subsequent coincubation with CML progenitors for 2 h resulted in significantly increased adhesion of CML progenitors. We demonstrated that $\alpha4\beta1$ and $\alpha5\beta1$ integrin receptors were involved in the enhanced adhesion of CML progenitors, suggesting that IFN-α-treated stroma can upregulate CML integrin function. This effect is due, at least in part, to IFN-α-induced increased stromal production of the chemokine macrophage inflammatory protein-1α (MIP-1α), which upregulates β1 integrin-dependent adhesion of CML progenitors to stroma. Thus, IFN-α treatment of marrow stroma restores β1 integrin-dependent adhesion of CML progenitors, at least in part through induction of MIP-1α production. These observations provide further insights into mechanisms by which IFN-α may restore normal hematopoiesis in CML. (*J. Clin. Invest.* 1995. 96:931-939.)
Key words: chronic myelogenous leukemia • hematopoiesis • interferon-α • integrins • macrophage inflammatory protein-1α

Introduction

Chronic myelogenous leukemia (CML)[1] is characterized by a vast expansion of hematopoietic cells belonging to the malignant bcr/abl positive clone, resulting from unregulated proliferation of malignant hematopoietic progenitors (1). We, and others, have demonstrated that direct contact between normal progenitors and stroma is important for negative regulation of progenitor growth (2, 3). Normal primitive hematopoietic progenitors are usually quiescent when present in close contact with marrow stroma (2) and proliferate significantly more when cultured separated from stroma by a transwell which prevents direct progenitor-stroma contact than when cultured in contact with stroma (3). This may be the result of juxtacrine effects of stroma-bound growth inhibitory cytokines, such as TGF-β and macrophage inflammatory protein-1α (MIP-1α) (4-7). More recently, we demonstrated that inhibition of progenitor proliferation may also be the result of transduction of growth inhibitory signals subsequent to the engagement of integrin adhesion receptors on progenitors by their stromal ligands (8). Unlike normal progenitors, CML progenitors are continuously proliferating, even when in close contact with stromal layers. The abnormal proliferation of CML progenitors may be related to the abnormal adhesive interactions between CML progenitors and the marrow stromal microenvironment (9, 10).

Normal progenitors adhere to stroma through a variety of cell surface adhesion receptors, including $\alpha4\beta1$ integrin receptors, which bind to vascular cell adhesion molecule (VCAM) and the CS-1 region in the COOH-terminal heparin-binding domain of fibronectin, and $\alpha5\beta1$ integrin receptors, which bind to the RGD-containing cell-binding domain of fibronectin (11-15). Compared with normal progenitors, CML progenitors show reduced adhesion to stromal layers as well as to fibronectin and its proteolytic fragments (9, 16). However, $\alpha4\beta1$ and $\alpha5\beta1$ integrin receptors are expressed at normal levels on CML progenitors, suggesting that the function of these receptors is impaired in CML (9, 10).

IFN-α can induce hematologic remissions in up to 75% of patients with chronic myelogenous leukemia and can induce complete cytogenetic remissions in a smaller number of patients with gradual, selective suppression of the malignant clone and restoration of normal hematopoiesis (17). The mechanisms by which IFN-α restores normal hematopoiesis in CML are not clear. One possibility is that IFN-α acts by correcting the defective adhesion of CML progenitors to marrow stroma, which may in turn result in restoration of normal microenvironmental regulation of progenitor proliferation. We have recently demonstrated that treatment of CML progenitors with IFN-α results in their enhanced adhesion to marrow stroma as a result of modulation of $\alpha4\beta1$ and $\alpha5\beta1$ integrin receptor function (10). Upadhyaya et al. have demonstrated that incubation with IFN-α also restores the deficient expression of LFA-3 on CML progenitor cells (18). IFN-α may also affect adhesion of CML progenitors through effects on the marrow microenvironment

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1. *Abbreviations used in this paper:* BMMNC, bone marrow mononuclear cell; CFC, colony-forming cells; CML, chronic myelogenous leukemia; LTC-IC, long term culture-initiating cells; LTBMC, long-term bone marrow culture; MIP, macrophage inflammatory protein; VCAM, vascular cell adhesion molecule.

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besides its direct effects on CML progenitors. Dowding et al. demonstrated that culture of marrow stroma in the presence of IFN-α followed by co-culture of CML progenitors in the continued presence of IFN-α resulted in enhanced adhesion of CML progenitors to stroma (19). However, the mechanisms underlying the increased adhesion of CML progenitors under these conditions were not clear. It is possible that, aside from upregulating the expression of adhesion receptors on hematopoietic progenitors, IFN-α may act through upregulating the expression of ligands for adhesion receptors on stromal cells (20), as is well described for IFN-γ (21, 22). Alternatively, IFN-α may alter the composition of stromal extracellular matrix (23) or induce the production of other cytokines by stromal cells, which in turn may alter adhesive interactions between progenitors and stroma. In the present study, we examined the effect of IFN-α treatment of marrow stroma on the subsequent adhesion of CML progenitors. CML progenitors demonstrated significantly enhanced adhesion to IFN-α pretreated stroma as compared with untreated stroma. Increased adhesion was mediated by $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrin adhesion receptors, suggesting that IFN-α–treated stroma can restore integrin-mediated adhesion mechanisms in CML. We provide evidence that increased production of MIP-1α by IFN-α–treated stroma may play a role in the enhanced $\beta 1$ integrin–mediated adhesion of CML progenitors.

Methods

Bone marrow samples

26 patients with CML and 5 normal healthy volunteers were evaluated after informed consent was obtained using guidelines approved by the Committee on the Use of Human Subjects at the University of Minnesota. All patients were in the chronic phase of CML. 2 patients were untreated at the time of study. 24 patients were receiving treatment with Hydroxyurea alone, which was discontinued at least 4 d before the study. Of the Hydroxyurea-treated patients, 5 had been previously treated with IFN-α, which had been discontinued at least 3 mo prior to the study. Heparinized bone marrow samples were obtained by aspiration from the posterior iliac crest. Bone marrow mononuclear cells (BMMNC) were isolated using Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient separation (specific gravity 1.077) for 30 min at 37°C and 400 *g*.

Selection of purified progenitor populations

Selection of purified progenitor populations was performed using methods previously described (24, 25). Lineage-negative cells were obtained from CML or normal BMMNC by sequential counterflow centrifugation elutriation (26), sheep erythrocyte rosetting (27) and immunomagnetic bead depletion (24, 25). The resultant lineage-negative population (lin−) was labeled with anti–CD34-PE and anti–HLA-DR-FITC antibodies (Becton-Dickenson, Mountain View, CA) and sorted on a FACStar^{PLUS} laser flow cytometry system (Becton-Dickenson) equipped with a CONSORT 32 computer system. Cells were selected for low vertical and horizontal light-scatter properties and for expression of CD34 and HLA-DR antigens based on isotype control stains. In normal individuals, primitive progenitors capable of initiating long-term bone marrow cultures (LTBMC) (long term culture–initiating cells, LTC-IC) are concentrated in the Lin−CD34+HLA−DR− (DR−) fraction, and more differentiated progenitors capable of forming colonies in short-term methylcellulose progenitor cultures (colony-forming cells, CFCs) are concentrated in the Lin−CD34+HLA−DR+ fraction (DR+). In CML patients, however, CFC and LTC-IC derived from the malignant clone are both present in the DR+ fraction.

Bone marrow stromal layers

Bone marrow stromal layers were established in T-75 or T-150 flasks by plating normal BMMNC in LTBMC medium (IMDM; GIBCO BRL, Gaithersburg, MD), with 12.5% FCS (HyClone, Logan, UT), 12.5% horse serum (Terry Fox Laboratories, Vancouver, BC, Canada), 2 mM L-Glutamine, 1,000 U/ml penicillin and 100 U/ml streptomycin (Gibco Laboratories), and 10^{-6} M-hydrocortisone (A-Hydrocort; Abbott Laboratories, North Chicago, IL). Confluent stromal layers were formed after 4–5 wk of culture and were irradiated at 1,250 cGy using a cesium irradiator (Shepard and Associates, Glendale, CA), to eliminate hematopoietic cells. Adherent cells, collected after digestion with 0.1% collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN), were subcultured at a concentration of 350,000 stromal cells/well in 24-well plates (Costar, Cambridge, MA) (24).

Cell adhesion assays

Normal stromal layers in 24-well plates were incubated with IFN-α_{2b} (Intron-A; Schering Corp., Bloomfield, NJ) in LTBMC medium, at 37°C in a humidified atmosphere with 5% CO_2. The IFN-α concentrations ranged from 100 to 10,000 U/ml. Stromal layers, were exposed to IFN-α for time periods ranging from 20 min to 48 h. Control stromal layers were incubated under identical conditions but without IFN-α. After incubation, layers were washed three times with warm IMDM to remove excess unbound IFN-α. 5,000 normal DR− or DR+ or CML DR+ cells were suspended in LTBMC medium, without addition of IFN-α, and coincubated with the IFN-α treated and washed marrow stromal layers for 2 h at 37°C. Nonadherent cells were removed by three vigorous washes using warm IMDM (panning). Tightly adherent progenitor cells were harvested using trypsin and plated in a short-term methylcellulose assay to evaluate the percentage of adherent CFCs or, in LTBMC culture to evaluate the percentage of adherent LTC-ICs (24). The percentage of adherent progenitors was calculated as, [the number of progenitors adherent to stroma (panned cells) divided by the total input of progenitor cells (plated cells)] × 100. Experiments were carried out simultaneously with normal and CML progenitors.

In other experiments, adhesion assays were performed between CML DR+ cells and stromal layers exposed not to IFN-α but to MIP-1α (0.1–10 ng/ml) (R & D Systems, Inc., Minneapolis, MN), which was added during the 2-h coincubation period of the adhesion assay only.

Adhesion inhibition assays

IFN-α–treated stromal layers (10,000 U/ml; 48 h) were incubated with antibodies to VCAM or ICAM (1:50 dilution of hybridoma-supernatant) for 30 min before the adhesion assays. Alternatively, CML and normal DR+ cells were incubated with antibodies to the $\alpha 4$, $\alpha 5$, or $\beta 1$ integrins, CD44 (1:50 dilution of hybridoma-supernatant), or control mouse IgG (20 mg/ml) for 30 min before the adhesion assays. Progenitors were then plated in contact with untreated or IFN-α–treated, washed stroma in the continued presence of the indicated antibodies for 2 h. Nonadherent cells were removed, and adherent cells were plated in methylcellulose progenitor culture as described earlier. The percentage of adhesion of IFN-α–treated CML progenitors in the presence of these antibodies was calculated as [adhesion in the presence of antibody divided by adhesion in the absence of antibody] × 100%.

IFN-α–treated, washed stromal layers (10,000 U/ml; 48 h) were incubated with 10 μg/ml neutralizing antibodies to MIP-1α (capable of neutralizing 50% of the biological activity of 20 ng/ml rhMIP-1α) or 10 μg/ml control goat IgG for 30 min before the adhesion assays. CML DR+ cells were plated or panned onto the stromal layers for 2 h in the continued presence of these antibodies.

In additional experiments, CML DR+ cells were incubated with 10 ng/ml MIP-1α for 2 h, washed three times to remove excess unbound MIP-1α, and then incubated with or without anti-$\beta 1$ integrin or control mouse antibody for 30 min before the adhesion assay. Progenitors were then panned or plated onto stromal layers, not pretreated with IFN-α, in the continued presence of these antibodies.

Progenitor culture

Long-term bone marrow culture. LTBMCs were established by plating or panning 5,000 CML DR+ cells or normal DR− cells in direct contact with stromal layers subcultured in 24-well plates as described. Cultures

were maintained for 5 wk in a humidified atmosphere, at 37°C and 5% CO_2. Weekly media changes were carried out by removing half the cell-free supernatant medium and replacing it with fresh LTBMC medium. After 5 wk, cells were harvested by digesting the stromal layers with trypsin and replated in methylcellulose progenitor culture to determine the number of CFCs present in LTBMCs (24).

Short-term methylcellulose progenitor culture. DR+ cells recovered from panning assays or the progeny of cells in LTBMC were plated in methylcellulose (final concentration of 1.12%) (Fisher Scientific Co., Fairlawn, NY) supplemented with 30% FCS, 3 IU recombinant erythropoietin (Epoetin; Amgen, Thousand Oaks, CA) and 4 ng/ml recombinant interleukin-3 (a kind gift from Dr. Wong, Genetics Institute, Boston, MA) as described. Cultures were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 14–18 d. The cultures were then assessed for the presence of CFU-GM, BFU-E and CFU-MIX colonies, as previously described (27).

Measurement of MIP-1α production by IFN-α–treated stromal layers

Normal stromal layers grown in 24-well plates were incubated with IFN-α (10,000 U/ml) for varying periods of time, after which the supernatant media was collected, aliquoted, and stored at −80°C until analysis for MIP-1α was carried out by standard, commercially available ELISA assay kits (R & D Systems, Inc.). For measuring the MIP-1α content of the stromal layers themselves, layers were washed three times and then detached using non-enzymatic stroma lysis solution (Sigma). Cells were pelleted, mixed with 1 ml of cell lysis solution (25 mM Tris-HCl, 150 mM NaCl, with 1% Triton-X 100, supplemented with 1 mM PMSF) for 20 min, and centrifuged at 20,000 *g* for 10 min, after which the supernatant medium (protein extract) was collected and assayed for MIP-1α levels by ELISA.

Cytogenetic analysis of plucked colonies

Colonies were plucked from methylcellulose progenitor cultures initiated with CML CD34+HLA−DR+ cells harvested from either the adherent or nonadherent fractions from adhesion assays on IFN-α–treated stromal layers. Colonies resulting from either the adherent or nonadherent fractions were pooled and subjected to a 1.5-h colcemid incubation followed by lysis with hypotonic KCl and fixation with acetic acid/methyl alcohol as previously described. Metaphases were then analyzed by QFQ and GTG banding (28).

Antibodies

Antibodies against α4 (P4C2), α5 (P3D10), β1 (P5D2, P4C10), ICAM (P4F11), and VCAM (P8B1) were the kind gift of Dr. E. Wayner, (University of Minnesota, Minneapolis, MN). Anti-CD44 Hermes 3 was a kind gift from Dr. E. Butcher, (Stanford University, Palo Alto, CA), and antibody 50B4 was a kind gift from Dr. D. Letarte (Hospital for Sick Children, Toronto, Canada). Mouse IgG was obtained from Sigma. Anti–MIP-1α antibody and goat immunoglobulin were obtained from R & D Systems, Inc.

Statistical analysis

Results of experimental points obtained from multiple experiments were reported as mean±SEM. Significance levels were determined by two-sided Student's *t* test analysis.

Results

IFN-α treatment of stromal layers results in restored adhesion of CML progenitors to stroma. We assessed the adhesion of CML and normal progenitors to bone marrow stroma that had been pretreated with IFN-α for 48 h before the adhesion assay. Compared with normal LTC-IC, malignant LTC-IC present in the CML DR+ population showed significantly reduced adhesion to stroma not treated with IFN-α. Likewise, malignant CML DR+ CFC adhered significantly less to untreated stroma

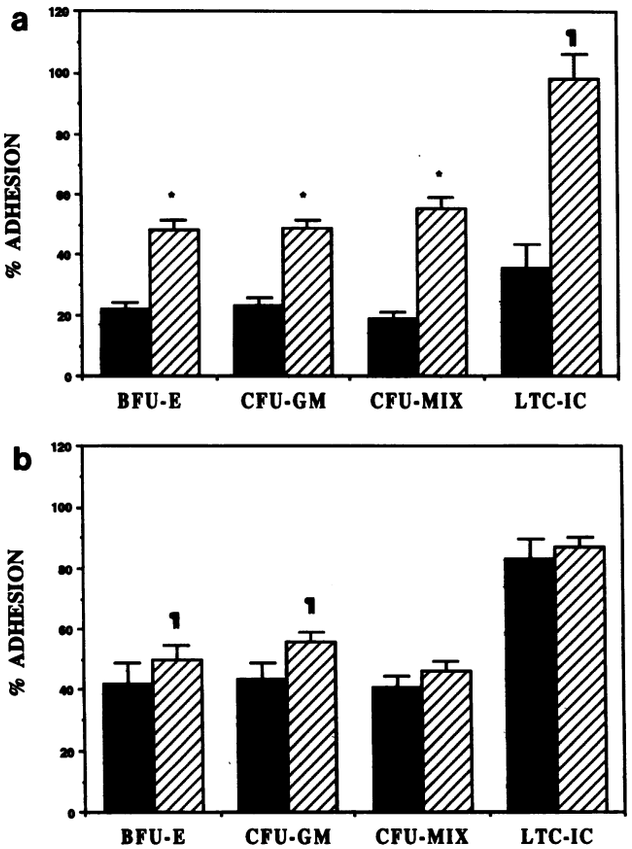

Figure 1. Increased adhesion of CML but not normal progenitors to marrow stroma treated with IFN-α. 5,000 CML DR+ cells (*a*) and normal DR− and DR+ cells (*b*) were panned or plated for 2 h on stromal layers preincubated with IFN-α (10,000 U/ml) for 48 h and then replated in either methylcellulose progenitor culture or LTBMC to assay for CFCs and LTC-ICs, respectively. The percentage of adhesion of progenitors was calculated by dividing the number of progenitors in cultures initiated with panned cells by the number of progenitors in cultures initiated with plated cells. Results are expressed as mean±SEM of results of separate experiments. Black bars represent adhesion in the absence of IFN-α; cross-hatched bars represent adhesion after IFN-α exposure. The significance levels are as follows: comparison of adhesion of progenitors to untreated and IFN-α–treated stroma, $*P < 0.001$, $¶P < 0.05$. The number of BFU-E ($n = 18$), CFU-GM ($n = 18$), CFU-MIX ($n = 14$), and LTC-IC ($n = 5$) in 1,000 CML DR+ cells was 40.4±4.6, 20.4±2.2, 3.2±0.3, and 53.9±11.5, respectively. The number of BFU-E, CFU-GM, and CFU-MIX in 1,000 normal DR+ cells ($n = 5$) was 13.7±1.8, 18.1±2.2, and 3.2±0.5, respectively. The number of LTC-IC in 1,000 normal DR− cells ($n = 5$) was 20.9±3.0.

compared with normal CFC. However, IFN-α treatment of stromal layers before the adhesion assays resulted in significantly increased adhesion of CML LTC-IC ($P < 0.001$, $n = 5$) as well as more committed CML progenitors (BFU-E, $P < 0.001$, $n = 18$; CFU-GM, $P < 0.001$, $n = 18$; and CFU-MIX, $P < 0.001$, $n = 14$). (Fig. 1*a*). In contrast, adhesion of normal bone marrow–derived LTC-IC and CFU-MIX was not significantly increased after the stroma was treated with IFN-α, while adhesion of normal BFU-E and CFU-GM was slightly, although significantly, increased (BFU-E, $P = 0.053$; CFU-GM, $P = 0.006$, $n = 5$) (Fig. 1 *b*). Altered growth of CML progenitors as a result of exposure to IFN-α–treated stroma was not respon-

Table I. CD34+HLA−DR+ CML Progenitors Adhering to IFN-α−treated Stromal Layers Are Philadelphia Chromosome Positive

Patient	Adherent colonies (Ph+ metaphases/number of metaphases analyzed)	Nonadherent colonies (Ph+ metaphases/number of metaphases analyzed)
1	20/20	20/20
2	20/20	20/20
3	20/20	20/20
4	20/20	2/2

CML DR+ cells were allowed to adhere to IFN-α−pretreated stromal layers for 2 h, after which nonadherent cells were removed by washing and the adherent cells harvested by trypsinization of stromal adherent layers. Cells from both fractions were plated in methylcellulose progenitor culture for 14−17 d, and colonies generated from these cells plucked and pooled for cytogenetic analysis as described in Methods.

sible for the results of the adhesion assays because the number of colonies obtained from DR+ cells co-cultured with stroma pretreated with or without IFN-α was equivalent with or without pretreatment of stroma with IFN-α (36.7±4.2 and 40.4±4.6 BFU-E; 18.5±1.9 and 20.4±2.2 CFU-GM; 3.2±0.3 and 3.2±0.3 CFU-MIX; and 43.3±9.0 and 53.9±11.5 LTC-IC per 1,000 CML DR+ cells, with and without IFN-α pretreatment, respectively).

CFC present within the adherent and nonadherent fractions from adhesion assays employing IFN-α pretreated stroma were of malignant origin. As shown in Table I, cytogenetic analysis of colonies plucked from methylcellulose progenitor cultures demonstrated that both adherent and nonadherent colony-forming cells were all Ph positive. This is consistent with our observations, reported previously, that CD34+HLA−DR+ cells in CML contain primarily malignant hematopoietic progenitors.

Adhesion of CML progenitors to IFN-α−treated stroma was IFN-α concentration dependent (Fig. 2). Interestingly, adhesion of CML LTC-IC to stroma treated with as low a concentration of IFN-α as 100 U/ml was no longer significantly different than the adhesion of normal LTC-IC to stroma (Fig. 2). Furthermore, increasing the length of exposure of stroma to IFN-α prior to the adhesion assays resulted in progressively increased adhesion of CML progenitors (Fig. 3).

Role of β1 integrin receptors in the adhesion of CML progenitors to IFN-α−treated marrow stroma. To characterize the receptors on CML progenitors responsible for the enhanced adhesion to IFN-α−treated stroma, we examined the ability of antibodies directed against integrin receptors, CD44, VCAM, or ICAM to block the adhesion. CML DR+ cells were allowed to adhere to IFN-α−treated, washed stromal layers for 2 h in the presence of antibodies against α4, α5, β1, CD44, VCAM, and ICAM-1. The adhesion of CML CFC to stroma that was not pretreated with IFN-α was not significantly inhibited by these antibodies (BFU-E: control adhesion, 25.9±4.8%; with anti-α4, 25.6±5.6%; anti-α5, 25.8±4.8%; anti-β1, 20.8±5.0%; anti-VCAM, 30.3±5.13%; CFU-GM: controls, 22.3±3.3%; anti-α4, 20.8±2.5%; anti-α5, 21.3±3.2%; anti-β1, 26.1±3.0%; anti-VCAM, 25.4±2.5%; $n = 4$). However, adhesion of CML BFU-E and CFU-GM to IFN-α−treated stroma was significantly inhibited by antibodies against α4 ($P < 0.05$), α5 ($P < 0.05$), and β1 ($P < 0.05$) integrin receptors as well as VCAM ($P < 0.05$, $n = 5$) (Fig. 4). Antibodies against the CD44 receptor (50B4 and Hermes-3) and ICAM-1 did not significantly affect adhesion. These results are similar to what we have shown previously for adhesion of normal CFU-GM and BFU-E to stroma (3). These results indicate, therefore, that IFN-α treatment of stroma results in enhanced adhesion of CML progenitors by restoring β1 integrin−dependent mechanisms.

Role of increased MIP-1α production in enhanced adhesion of CML progenitors to IFN-α−treated stroma. Because members of the chemokine family are capable of increasing β1 integrin−dependent adhesion (29, 30), we investigated whether MIP-1α was a secondary mediator in the enhanced adhesion of CML progenitors to IFN-α−treated stroma. We examined the effect of IFN-α on MIP-1α production by marrow stroma. IFN-α treatment (10,000 U/ml) of stroma resulted in sixfold higher levels of MIP-1α in stromal lysates (Fig. 5). Similarly, IFN-α treatment resulted in 1.5- to 2-fold higher levels of MIP-1α in stromal supernatants ($n = 4$; data not shown).

To correlate IFN-α−induced increased MIP-1α production with the increase in CML progenitor adhesion, we studied the effect of neutralizing antibodies against MIP-1α on the adhesion of CML progenitors to IFN-α−treated stroma. Anti−MIP-1α antibodies significantly reduced the adhesion of CML CFU-GM (Fig. 6) and BFU-E (not shown). These results indicate that increased adhesion of CML progenitors to IFN-α−treated stroma was at least due in part to increased stromal production of MIP-1α. This was confirmed in experiments in which increasing concentrations of MIP-1α were added during the adhesion assays between CML progenitors and stromal layers that had not been exposed to IFN-α. Addition of MIP-1α for the 2 h of the assay resulted in increased adhesion of CML CFU-GM (Fig. 7) and BFU-E (not shown) to stroma in a concentration-dependent manner.

Finally, we studied the role of β1 integrins in MIP-1α−induced adhesion of CML progenitors to stroma by examining the ability of anti−β1 integrin antibodies to block the adhesion of MIP-1α−pretreated CML progenitors to stroma (Fig. 8; BFU-E not shown). As we demonstrated earlier, anti-β1 antibodies did not inhibit the adhesion of untreated CML progenitors. However, pretreatment of progenitors with MIP-1α (10 ng/ml for 2 h) before performing the adhesion assay increased their adhesion to stroma not treated with IFN-α. The increased adhesion of CML progenitors induced by MIP-1α pretreatment could be blocked with anti-β1 antibodies, indicating that the MIP-1α−induced adhesion of CML progenitors to stroma is β1 integrin dependent.

Discussion

Treatment of CML patients with IFN-α can result in selective suppression of malignant hematopoiesis and resumption of normal hematopoiesis (17). The mechanism by which IFN-α achieves this effect is not well understood. We hypothesized that IFN-α restores important mechanisms of microenvironmental regulation of hematopoietic progenitor proliferation defective in CML. In previous studies, we have shown that IFN-α directly enhances CML progenitor adhesion to stroma through upregulation of β1 integrin receptor function (3). In this study, we show that IFN-α treatment of marrow stroma can also lead to enhanced adhesion of CML progenitors through β1 integrin−mediated mechanisms. We demonstrate that increased stromal production of MIP-1α may, at least in part, be responsible for

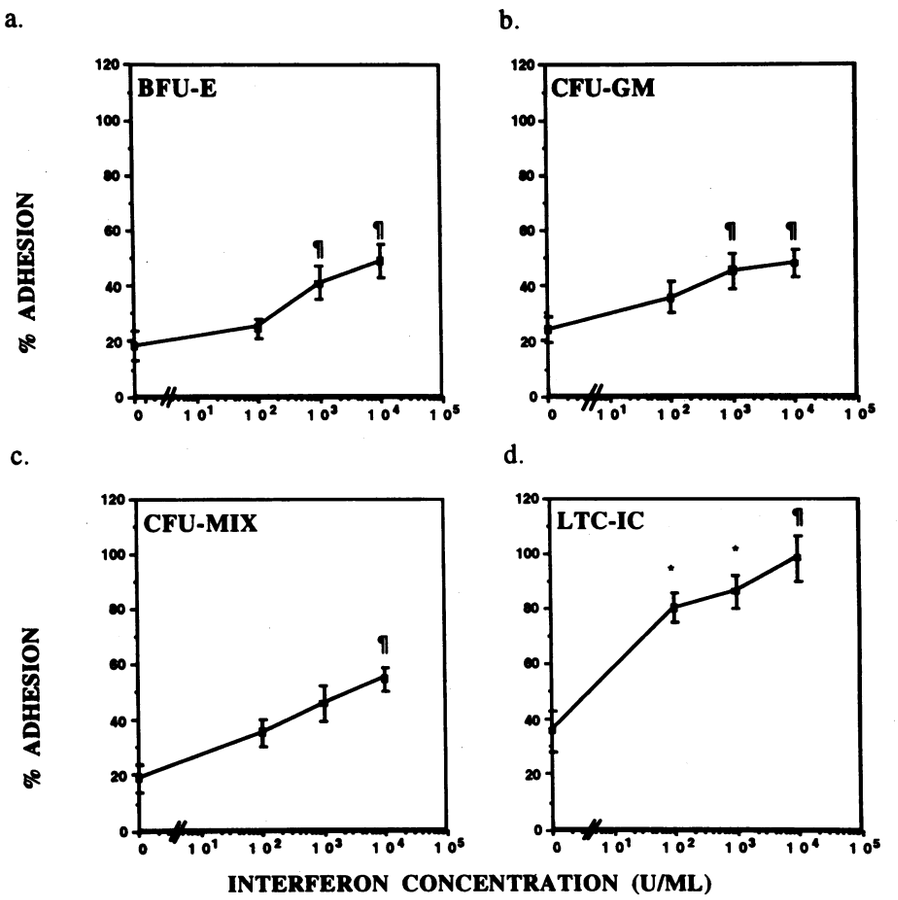

Figure 2. IFN-α treatment of marrow stroma enhances adhesion of CML progenitors in a dose-dependent manner. 5,000 CML DR+ cells (n = 5) were plated or panned for 2 h on stromal layers preincubated with increasing concentrations of IFN-α for 48 h and were then replated in either methylcellulose progenitor culture or LTBMC. The percentage of adhesion of (*a*) CFU-GM, (*b*) BFU-E, (*c*) CFU-MIX, and (*d*) LTC-IC was calculated by dividing the number of progenitors in cultures initiated with panned cells by the number of progenitors in cultures initiated with plated cells. Results are expressed as mean±SEM. Comparison of adhesion of progenitors with untreated and IFN-α–treated stroma; $*P < 0.001$, $\P P < 0.05$.

upregulation of β1 integrin function on CML progenitors exposed to IFN-α–treated stroma.

The increased adhesion of CML progenitors to IFN-α–treated stroma is not caused by residual stroma-bound IFN-α, since enhanced adhesion of CML progenitors following direct incubation with IFN-α requires at least a 12-h exposure (3). No significant increase in progenitor adhesion was seen when CML progenitors were exposed to IFN-α for only 2 h, which was the duration of the adhesion assay used here. Therefore, we hypothesized that secondary events resulting from IFN-α preincubation must be responsible for enhanced β1 integrin–mediated adhesion of CML progenitors. IFN-α is likely to have multiple effects on stromal cells, including stimulation of cytokine production and altered expression of ECM components and cell surface adhesion receptors (20, 23). Two recent studies have demonstrated that members of the chemokine family of cytokines can influence integrin-mediated adhesion. MIP-1β, a member of this family, in conjunction with proteoglycans, upregulates $\alpha 4\beta 1$-dependent T cell adhesion to VCAM (29). IL-8, another chemokine, upregulates β2 integrin–dependent neutrophil adhesion to endothelial cells (30). Another member of the chemokine family, MIP-1α, is produced by macrophages, T-lymphocytes, and bone marrow stromal cells (9, 31). We demonstrate here that IFN-α treatment of stroma results in enhanced MIP-1α production, which in turn is capable of directly enhancing β1 integrin–mediated adhesion of CML progenitors. Our results suggest that enhanced production of MIP-1α by stroma following IFN-α treatment may, at least in part, be responsible for the enhanced integrin-mediated adhesion of CML progenitors.

The mechanisms through which chemokines mediate these effects are unclear. The receptor for MIP-1α and other chemokines has recently been cloned and characterized as a member of the transmembrane G-protein–linked receptor family (32). Enhanced β2 integrin–mediated adhesion of neutrophils to stroma following IL-8 stimulation is, at least in part, the result of a direct effect of IL-8 on actin polymerization and can be blocked by pertussis toxin (33). Similar influences on the cytoskeleton may be active in restoring β1 integrin–mediated adhesion of CML progenitors following MIP-1α stimulation. Interestingly, recent studies from our laboratory indicate that MIP-1α also increases adhesion of normal committed progenitors to marrow stroma (54±1.5% without MIP-1α and 63±2% with MIP-1α, 100 ng/ml, for 2 h; $P < 0.01$) (34). The increased

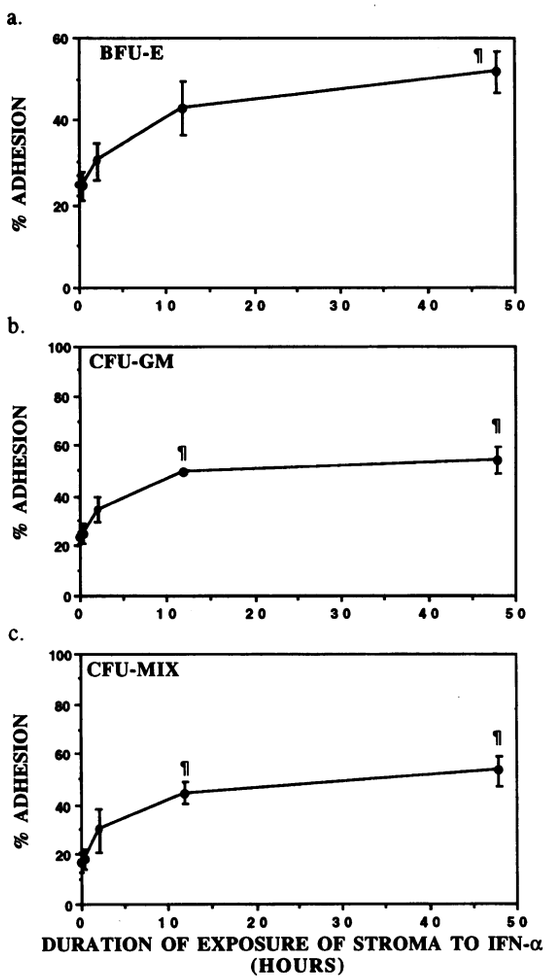

Figure 3. IFN-α treatment of marrow stroma enhances adhesion of CML progenitors in a time-dependent fashion. Stromal layers were preincubated with IFN-α (10,000 U/ml) for time periods ranging from 20 min to 48 h. 5,000 CML DR+ cells (n = 4) were panned and plated on the IFN-α-pretreated stromal layers for 2 h and then replated in methylcellulose progenitor culture. The percentage of adhesion of (a) CFU-GM, (b) BFU-E, and (c) CFU-MIX was calculated by dividing the number of progenitors in cultures initiated with panned cells by the number of progenitors in cultures initiated with plated cells. Results are expressed as mean±SEM. The significance level is as follows: comparison of adhesion of progenitors to untreated and IFN-α-treated stroma, ¶P < 0.05.

adhesion induced by MIP-1α is similar to the increased adhesion of normal committed progenitors to IFN-α-treated stroma seen in this study. This suggests that the effect of MIP-1α is universal, but more pronounced in CML because of lower baseline integrin function. The hypothesis that MIP-1α produced by stroma plays an important role in IFN-α-dependent enhanced adhesion of CML progenitors is strengthened by additional preliminary studies from our group demonstrating that stimulation of stroma with cytokines such as IL-1β and TNF-α also increased stromal production of MIP-1α (our unpublished observations) and resulted in increased adhesion of CML progenitors. Although multiple alterations in stromal adhesion ligands or cytokines following IFN-α, TNF-α, and IL-1β exposure (4, 13, 20–23) could play a part in increased CML progenitor adhesion, these studies support the hypothesis that restored adhesion may be at least partly mediated by MIP-1α.

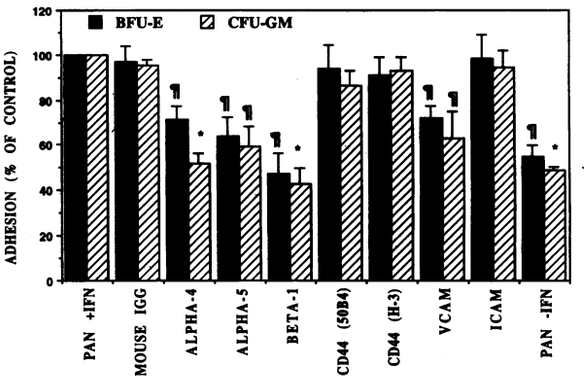

Figure 4. Increased adhesion of CML progenitors to IFN-α-treated stroma involves $\alpha4\beta1$ and $\alpha5\beta1$ integrin receptors. Stromal layers were treated with IFN-α (10,000 U/ml) for 48 h. 5,000 CML DR+ cells (n = 5) were plated and panned on IFN-α-pretreated stromal layers in the presence of blocking antibodies to the indicated receptors and control mouse IgG. Cells were replated in methylcellulose progenitor culture, and the effect of these blocking antibodies on the adhesion of BFU-E (a) and CFU-GM (b) was assessed. Results are shown as the adhesion of progenitors in the presence of antibody expressed as the percentage of adhesion in controls without antibody. Significance levels: *P < 0.001, ¶P < 0.05. The percentage of CML BFU-E and CFU-GM adherent to IFN-α-treated stroma in the absence of antibodies was 42.8±4.3% and 45.4±4.9%, respectively.

Although CML progenitors express normal levels of $\alpha4\beta1$ and $\alpha5\beta1$ integrin receptors on their cell surface, we have demonstrated that they have reduced capacity to adhere to fibronectin as compared with normal progenitors (2, 3). This suggests that these integrins, though present, are not functional. The mechanisms underlying the decreased $\beta1$ integrin-dependent adhesion of CML progenitors are unclear. Cellular adhesion

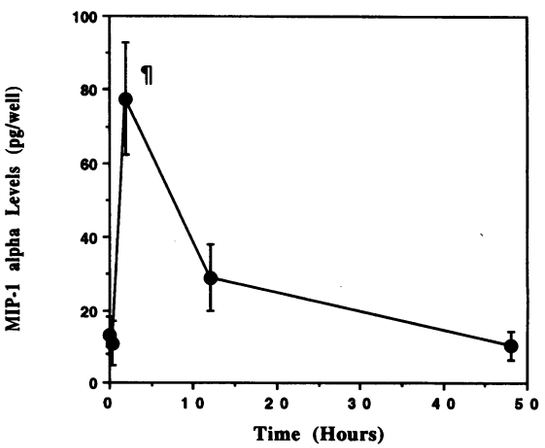

Figure 5. IFN-α treatment of stroma results in enhanced production of MIP-1α. Stromal layers subcultured in 24-well plates were incubated with IFN-α (10,000 U/ml) for varying periods of time. The MIP-1α content of the stromal layers was assayed by detaching IFN-α-treated stromal layers, preparing protein extracts, and then assaying them for MIP-1α levels by ELISA (n = 4). Significance levels: ¶P < 0.05. MIP-1α was undetectable in LTBMC media alone.

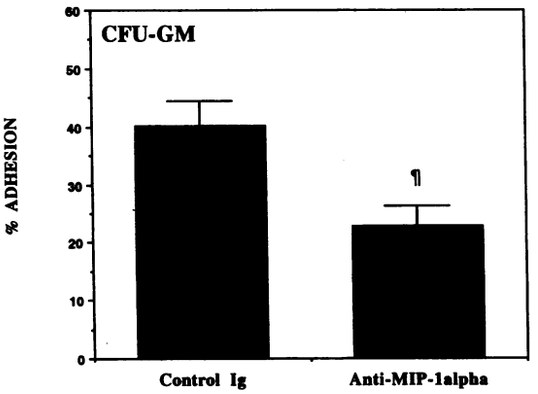

Figure 6. Neutralizing antibodies to MIP-1α reduces adhesion of CML CFU-GM to IFN-α–treated stroma. IFN-α–treated, washed stromal layers (10,000 U/ml, 48 h) were incubated with neutralizing antibodies to MIP-1α or control goat IgG for 30 min prior to the adhesion assays. 5,000 CML DR+ cells (n = 4) were plated or panned onto the stromal layers for 2 h in the continued presence of these antibodies. Cells were replated in methylcellulose progenitor culture, and the effect of these blocking antibodies on the adhesion of CFU-GM was assessed. Results represent mean±SEM of adhesion of progenitors. Significance levels; ¶$P < 0.05$. The number of CFU-GM in 1,000 CML DR+ cells was 24.0±5.6. Similar results were obtained for BFU-E (not shown).

depends not only on binding of the integrin receptor to its ligand but also on subsequent intracellular processes, including cytoskeletal rearrangement and focal adhesion formation (35, 36). Interactions of the cytoplasmic tail of the β1 subunit with cytoskeletal elements may also modulate the affinity of β1 integrins for their ligands (35–39). In CML, the p210$^{bcr/abl}$ tyrosine kinase co-localizes with the actin cytoskeleton (40, 41), and may abnormally phosphorylate several important intracellular mediators of the adhesion process, including the cytoplasmic

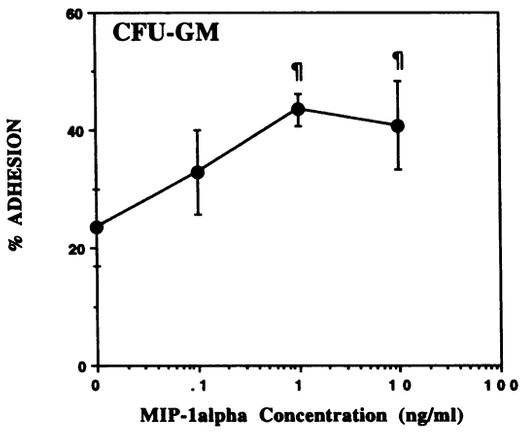

Figure 7. MIP-1α enhances the adhesion of CML CFU-GM to stroma. Adhesion assays were performed between CML DR+ cells (n = 4) and stroma that had not been exposed to IFN-α. MIP-1α (0.1–10 ng/ml) was added during the 2-h coincubation period of the adhesion assay only. Cells were replated in methylcellulose progenitor culture. Results represent the mean±SEM of percentage adhesion of progenitors in the presence of increasing concentrations of MIP-1α. Significance levels: ¶$P < 0.05$. The number of CFU-GM in 1,000 CML DR+ cells was 27.2±7.8. Similar results were obtained for BFU-E (not shown).

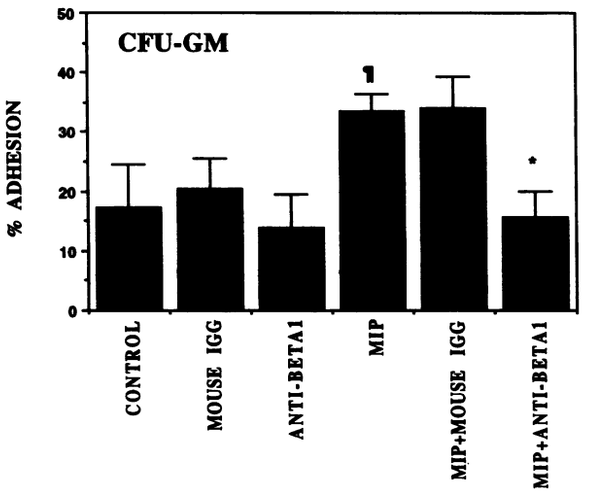

Figure 8. MIP-1α–induced adhesion of CML CFU-GM to stroma is mediated by β1 integrin receptors. CML DR+ cells were incubated with or without MIP-1α for 2 h, washed three times, and then incubated with anti–β1 integrin or control mouse antibody for 30 min prior to the adhesion assays. Progenitors were panned or plated onto stromal layers not pretreated with IFN-α, in the continued presence of these antibodies. Cells were replated in methylcellulose progenitor culture, and the effect of these antibodies on the adhesion of CFU-GM was assessed. Results represent the mean±SEM of adhesion of progenitors (n = 4). Significance levels: difference between controls and MIP-1α–treated CML progenitors: ¶$P \leq 0.05$; difference between MIP-1α–treated CML progenitors with and without anti–β1 antibody: *$P < 0.001$. The number of CFU-GM in 1,000 CML DR+ cells was 39.3±11.6. Similar results were obtained for BFU-E (not shown).

tail of the β1 integrin subunit itself (42) as well as various cytoskeletal proteins (43). Consequently, impaired adhesion of CML progenitors may result from direct effects of the p210$^{bcr/abl}$ tyrosine kinase on the cytoskeleton and/or the integrin itself. Therefore, a direct modulatory effect of chemokines such as MIP-1α on actin polymerization may explain their ability to restore β1 integrin–dependent adhesion of CML progenitors (33).

Preliminary studies demonstrate that co-culture of CML progenitors with IFN-α–treated stroma results in inhibition of proliferation of adherent progenitors (44). This supports the hypothesis that signaling through β1 integrin receptors may be important for negative regulation of progenitor proliferation and that the continuous proliferation of CML progenitors, even when in contact with normal stromal layers, may be related to defective signaling through β1 integrin receptors. Restoration of normal integrin-mediated adhesion and subsequent signal transduction in CML progenitors may then result in restoration of normal growth regulation. Of note, Eaves et al. have shown that addition of MIP-1α to long-term bone marrow culture inhibits the proliferation of normal progenitors but not CML progenitors (45). Although these results seem at odds with the results presented here, they may indicate that even though MIP-1α can restore β1 integrin–mediated adhesion of CML progenitors, this may not by itself be sufficient to restore inhibition of proliferation of CML progenitors. Indeed, although β1 integrin–dependent adhesion is associated with proliferation inhibition in normal progenitors, the two processes can occur separately. For example, we have evidence in normal hematopoiesis that

monoclonal antibody cross-linking of integrins on normal progenitors in suspension leads to proliferation inhibition in the absence of adhesion (46). Furthermore, additional stimulation through the epidermal growth factor receptor is required for integrin-directed cell motility but not adhesion of pancreatic carcinoma cells on vitronectin (47). This suggests that integrins may cooperate with cytokines and other adhesion receptors to transmit signals. It is therefore possible that, aside from increased MIP-1α production, additional IFN-α–induced microenvironmental changes, such as alteration in adhesive ligand expression (20), cytokine production (48) or extracellular matrix composition (19), are required to restore integrin-mediated signal transduction pathways, resulting in inhibition of progenitor proliferation. Studies are ongoing to explore these possibilities.

In conclusion, the present study demonstrates that IFN-α treatment of marrow stroma restores normal β1 integrin–mediated adhesive interactions between CML progenitors and the bone marrow microenvironment. Studies are currently under way to examine if and how restored progenitor adhesion may restore normal microenvironmental regulation of hematopoiesis in CML.

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