Regulation of Cytokine Expression by Interferon-α in Human Bone Marrow Stromal Cells: Inhibition of Hematopoietic Growth Factors and Induction of Interleukin-1 Receptor Antagonist

By M. Javad Aman, Ulrich Keller, Günther Derigs, Mansour Mohamadzadeh, Christoph Huber, and Christian Peschel

We investigated the effects of interferon-α (IFN-α) on the expression of cytokines by human bone marrow stromal cells. Production of granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF), and interleukin-1β (IL-1β) in stromal cell layers was induced by incubation with IL-1α, tumor necrosis factor (TNF), or lipopolysaccharide (LPS). Addition of IFN-α to such stimulated cultures resulted in a strong downregulation of mRNA expression of GM-CSF and IL-1β. Similarly, the protein levels of GM-CSF and IL-1β were significantly reduced by IFN-α, whereas G-CSF production was only moderately inhibited. In contrast, IFN-α markedly stimulated the production of IL-1 receptor antagonist (IL-1RA) by stromal cells. The inhibition of cytokine expression resulted in a reduced hematopoietic activity of stromal cells, indicated by a reduced proliferation of the factor dependent cell line M07e on IFN-α-treated stromal cells. In the presence of cycloheximide (CHX), IFN-α failed to inhibit IL-1 mRNA expression, whereas the regulation of GM-CSF and IL-1RA by IFN-α was not affected. Our results indicate that the myelosuppressive effects of IFN-α, as observed in therapeutic applications or associated with viral infections, are, in part, indirectly mediated by inhibition of the paracrine production of hematopoietic growth factors. © 1994 by The American Society of Hematology.

LONG-TERM HEMATOPOIESIS in vitro is supported by nonhematopoietic connective tissue cells of marrow origin collectively referred to as stromal cells.1 For stem cell renewal and sustained hematopoiesis, a complex cellular interaction with stromal cells is required, which includes yet poorly defined signals mediated by cellular and matrix structures and the production of humoral factors by stromal cells. Hematopoietic growth factors are detected in stromal cell lines and Dexter type cultures of murine or human origin on stimulation with cytokines, such as interleukin-1 (IL-1), or lipopolysaccharide (LPS).6,11 However, granulocyte-macrophage colony-stimulating factor (GM-CSF) is also constitutively produced by stromal cells in small, but biologically relevant, amounts.12,13 Novel cytokines, including IL-7, IL-11, and stem cell factor, have been detected as products of stromal cells.14-16 Negative regulators, such as transforming growth factor-β (TGF-β) and tumor necrosis factor (TNF), are also produced by bone marrow stromal cells to maintain homeostasis of the hematopoietic system in vitro, and apparently, in vivo.17,18 In addition to stromal cells, the cellular composition of the bone marrow microenvironment comprises immune regulatory cells, including monocytes and T lymphocytes. The role of many cytokines produced by those immune cells in the regulated expression of factors with stimulatory or inhibitory effects on hematopoiesis remains to be determined.

Interferon-α (IFN-α) has a well-defined beneficial role in the treatment of myeloproliferative diseases, including chronic myelogenous leukemia (CML),19 polycythemia vera,20 and essential thrombocytopenia.21 Although the mechanism of a selective suppression of the malignant clone by IFN-α in subpopulations of CML patients is unclear, cytogenetic responses are clearly associated with the more frequent hematologic responses of CML patients,19 which can be explained by the myelosuppressive effect of IFN. Direct antiproliferative effects of IFNs on hematopoietic progenitors are well known.22 However, the regulation of cytokine cascades by type-I IFNs, as recently observed for IL-823 and IL-1 receptor antagonist (IL-1RA),24 might contribute to the role of IFN as an inhibitory agent of hematopoiesis.

In this study, we used human stromal cell cultures to evaluate the effects of IFN-α on the expression of cytokines that are involved in the regulation of hematopoiesis. We demonstrate that IFN-α substantially inhibits the production of cytokines with stimulatory activity on hematopoietic progenitors and additionally counteracts IL-1 effects by inducing IL-1RA. These results indicate that the myelosuppressive effects of IFN-α are, at least, in part mediated by modulating the expression of hematopoietic growth factors.

MATERIALS AND METHODS

Materials. rh-IFN-α 2b with a specific activity of 1.8 × 10^5 U/mg was obtained from Essex Pharma (München, Germany). rh-GM-CSF was kindly provided by Sandoz AG (Nürberg, Germany), rh-IL-1α by Hoffmann La Roche (Nutley, NJ), and rh-TNF-α (6.6 × 10^6 U/mg) by Knoll AG (Ludwigshafen, Germany). LPS was purchased from Sigma Chemicals (Deisenhofen, Germany), actinomycin-D and cycloheximide (CHX) from Merck, Inc (Darmstadt, Germany), [3H]-thymidine (20 C/mmol) from DuPont (Bud Homburg, Germany), monoclonal anti-huGM-CSF antibody from Boehhringer (Mannheim, Germany) and a[3H]-labeled nucleotides from Amersham Buchler (Braunschweig, Germany).

Stromal cell culture. Bone marrow stromal cell cultures were essentially obtained as described previously25 with some modifications.26 Briefly, bone marrow mononuclear cells, separated by centrifugation over Ficoll-Hypaque, were incubated at a cell density of 1 × 10^6/mL in 25 cm² tissue flasks at 33°C in culture medium consisting of RPMI 1640 supplemented with 10% fetal calf serum.
Northern blot analysis. Total cytoplasmic RNA was prepared using the single step method of guanidinium/phenol-chloroform extraction as described previously. Ten to 15 μg RNA (depending on the least yield obtained in each experiment) were subjected to electrophoresis on a 1% agarose-formaldehyde gel and transferred onto nylon membrane (Hybond-N, Amersham Buchler). Blots were hybridized to α32P-labeled cDNA probes using random primer DNA labeling kit (Boehringer), washed, and exposed to Cronex-4 autoradiography films (DuPont) at -70°C. hu-GM-CSF cDNA, an 800-bp EcoRI fragment cloned into P91023(B)-vector, and hu-IL-1α cDNA, a 600-bp BamHI fragment in YEpsecI-vector, were obtained from American Type Culture Collection (Rockville, MD). A cDNA probe for IL-1RA was prepared by polymerase chain reaction (PCR) amplification from RNA obtained from peripheral blood mononuclear cells (PBMC) after reverse transcription using specific primer for IL-1RA, which resulted in a 492-bp fragment. Primer sequences: sense: 5’-ATGGGAATCTGCAGAGGCCTC3’, antisense: 5’TTC-GTCAGGCATATTGGTGAGGCTGAC3’. Reverse transcription and PCR amplification were carried out as previously described. Enzyme-linked immunosorbent assay (ELISA): Concentrations of GM-CSF, G-CSF, IL-1α, and IL-1β were measured using Quantikine Human Cytokine Immunoassays (R&D Systems, Minneapolis, MN) according to manufacturer’s instructions. Sensitivity of the tests was as follows: GM-CSF 7.8 pg/mL, G-CSF 31 pg/mL, IL-1β 3.9 pg/mL, and IL-1RA 31 pg/mL.

Isolation of human foreskin fibroblasts. Foreskin (obtained from the Division of Pediatrics, University of Mainz) was kept in 10% FCS/PBS for 30 minutes and subsequently washed in PBS. Epidermis was isolated from the foreskin using fine forceps and subsequently incubated in PBS supplemented with 2.5 μL dispase type II (Boehringer) at 37°C for 3 hours. After removal of dispase solution, foreskin was rinsed three times with PBS and incubated at 37°C in PBS containing 0.2% (wt/vol) trypsin type III/0.1% EDTA (Sigma) with gentle agitation for 15 minutes. A single-cell suspen-
Confluent secondary stromal cultures were incubated in medium containing 100 U/mL IL-1α with or without 1,000 U/mL IFN-α for 24 hours, and the cytokine concentration in the supernatants was assessed using specific ELISAs. Single experiments were carried out in triplicate, and data from five experiments are summarized as mean ± SEM.

Fig 2. Effect of IFN-α on the secretion of GM-CSF and G-CSF. Confluent secondary stromal cultures were incubated in medium containing 100 U/mL IL-1α with or without 1,000 U/mL IFN-α for 24 hours, and the cytokine concentration in the supernatants was assessed using specific ELISAs. Single experiments were carried out in triplicate, and data from five experiments are summarized as mean ± SEM.

Proliferation assay. M07E cells were incubated in 96-well flat bottom microtiter plates either alone or on preformed stromal cell monolayers in supplemented RPMI 1640 + 10% FCS at 37°C/5% CO₂ with the factors indicated in the Results section. After 3 days, 1 μCi/well ³H-thymidine was added. Cells were harvested after 16 hours in a PHD-Cell Harvester (Dunn Labortechnik, Asbach, Germany) and thymidine incorporation was quantified in a β-counter (Beckmann Munich, Germany).

RESULTS

Regulation of cytokine mRNA expression by IFN-α. Expression of cytokine mRNA was examined in stromal cell cultures derived from five normal bone marrow samples. Multiple stromal cell cultures of individual donors were pooled after trypsinization and expanded in secondary cultures, to avoid heterogeneity of the cellular components and cell density. In such homogenous cultures, the mRNA expression of GM-CSF, IL-1β, and IL-1RA was examined in unstimulated stromal cells after addition of various stimuli. Low levels of constitutive expression of GM-CSF and IL-1β were observed in two samples, whereas IL-1RA expression was never detected. The addition of IL-1α, LPS, or TNF-α to stromal cell cultures induced the expression of

Fig 3. Effects of IFN-α on the production of cell bound IL-1β and secreted IL-1RA. For IL-1β measurement, confluent secondary stromal cultures were incubated in medium with the indicated factors for 24 hours. Cells were lysed in 1 mL medium by sonifying, and IL-1β concentration in the lysates was assessed using a specific ELISA. IL-1β data represent mean ± SEM from one typical experiment out of five, carried out in triplicate. IL-1RA was measured in supernatants of stromal cultures incubated for 24 hours in medium with or without IFN-α. Single experiments were carried out in triplicate and summarized data from five experiments are presented as mean ± SEM. Concentrations used were the same as indicated in the legend to Fig 1.
GM-CSF and IL-1β at different quantitative levels. The strongest signals were observed on stimulation with IL-1α, followed by LPS and TNF-α. Expression of IL-1RA mRNA was only weakly induced by LPS and IL-1α, and observed only occasionally in the presence of TNF.

The influence of IFN-α on the expression of these cytokines was studied in exogenously stimulated stromal cell cultures. In stromal cells stimulated with IL-1α, LPS, or TNF, the expression of GM-CSF and IL-1β was markedly inhibited at a concentration of IFN-α 1,000 U/mL (Fig 1). In contrast to the cytokines with stimulatory effects on hematopoiesis, IL-1RA mRNA was induced by IFN-α at a much higher level as observed with LPS, suggesting a potent suppression of IL-1 mediated biologic actions. G-CSF expression, which was induced by IL-1α and TNF, was also down-regulated by IFN-α. However, the inhibitory effect was less pronounced as compared with GM-CSF and IL-1β (data not shown).

Effect of IFN-α on cytokine secretion by bone marrow stromal cells. Protein levels of GM-CSF, G-CSF, IL-1β, and IL-1RA were evaluated in stromal cell supernatants using specific ELISAs. Unstimulated stromal cells failed to produce detectable levels of GM-CSF and G-CSF, and very low levels of IL-1RA were observed in only two of five experiments. In the presence of IL-1α or LPS, significant amounts of GM-CSF or G-CSF were detected, whereas TNF-α failed to induce G-CSF secretion and stimulated only very low levels of GM-CSF. IL-1β was never detected in stromal cell supernatants, independent of the stimulant added. The effect of IFN-α on the secretion of colony-stimulating factors was evaluated in cultures, which were stimulated with IL-1α (Fig 2). Results obtained from five individual experiments showed a significant reduction of GM-CSF secretion by IFN-α (1,000 U/mL) from 96.09 ± 10.38 pg/mL to 28.2 ± 6.91 pg/mL (mean ± SEM), representing a reduction of 72.42% ± 10.38%. The reduction of G-CSF secretion by IFN-α was less pronounced and amounted to 40% (Fig 2B).

Because IL-1β was not detected in stromal cell supernatants, despite readily detectable mRNA expression, we evaluated cell-associated IL-1β in stromal cell lysates. In fact, in cell lysates prepared by sonication of stromal cells, IL-1β production was seen on stimulation with either TNF, IL-
$\alpha$, or LPS (Fig 3A). In such induced cultures, cell-associated IL-1$\beta$ was induced at comparable levels. Addition of IFN-$\alpha$ drastically reduced cell-associated IL-1$\beta$ independent of the stimulus used, as shown in Fig 3A in one representative experiment. In four independent experiments, the mean reduction of IL-1$\beta$ production by IFN-$\alpha$ in IL-1$\alpha$ stimulated stromal cells was 68% ± 14% (mean ± SEM). The strong induction of IL-1RA by IFN-$\alpha$, as indicated by the mRNA analysis, was confirmed at the protein level (Fig 3B). IFN-$\alpha$ caused a more than 30-fold increase of IL-1RA secretion as compared with unstimulated cultures. In the presence of LPS, IL-1$\alpha$, or TNF no additive effect was observed in the combination with IFN-$\alpha$ (data not shown).

The dose dependence of the modulatory effects of cytokine secretion by IFN-$\alpha$ was evaluated at INF concentrations between 10 and 10,000 U/mL (Fig 4). Apparently, GM-CSF (Fig 4A) and IL-1RA (Fig 4D) secretion was most sensitive to the IFN-$\alpha$ action. However, significant effects of IFN-$\alpha$ were seen at concentrations of 10 to 100 U/mL for all cytokines evaluated in this study.

**Biologic significance of IFN-$\alpha$-mediated downregulation of GM-CSF.** The consequences of the downregulation of hematopoietic growth factors by IFN-$\alpha$ were evaluated using the GM-CSF dependent cell line, MO7e. As shown in Fig 5, GM-CSF-induced proliferation of MO7e cells cannot be inhibited by IFN-$\alpha$. Furthermore, IFN-$\alpha$ failed to induce expression of the Mx gene, confirming the IFN resistance of this cell line (data not shown). IL-1$\alpha$ had no direct proliferative effects on MO7e cells, neither alone, nor in combination with GM-CSF (Fig 5 and data not shown). When MO7e cells were incubated in confluent stromal cultures, a moderate proliferation was observed without stimulation with an additional growth factor, which was presumably mediated by stromal cell derived growth factors. The addition of IL-1$\alpha$ to the stromal cultures, resulting in the induction of GM-CSF, caused an increase of thymidine incorporation in MO7e cells, which was comparable to that induced by GM-CSF. IFN-$\alpha$ partially inhibited the indirect stimulation of MO7e cells by IL-1$\alpha$ in stromal cell cultures (Fig 5). To prove that GM-CSF represents the stimulatory factor for MO7e cells produced by stromal cells, a neutralizing antibody against GM-CSF was used in conditioned media from stromal cultures. MO7e cells were incubated with stromal cell supernatants stimulated and, as a control, with GM-CSF or IL-3.
Anti-GM-CSF antibody completely inhibited GM-CSF-induced but not IL-3-induced proliferation of MO7e cells (Fig 6). IL-1α-induced stroma supernatants induced proliferation comparable to GM-CSF, which was completely blocked by the antibody, clearly suggesting that GM-CSF is the stimulatory cytokine in the conditioned medium. Similar results were obtained when MO7e cells were cocultured on IL-1α-stimulated stromal layers and anti-GM-CSF antibody was added (data not shown). Based on the resistance of MO7e cells to a direct antiproliferative effect of IFN-α, it can be assumed that in stromal cell cultures the inhibition of MO7e cells by IFN-α is mediated by the downregulation of stimulatory cytokines, such as GM-CSF.

Requirement of protein synthesis for IFN-α action. In experiments using CHX as an inhibitor of protein synthesis, a different pattern of gene expression was observed for IL-1β and GM-CSF in response to IFN-α. When CHX was added simultaneously with IL-1α, upregulation of both cytokines was prevented, suggesting that de novo protein synthesis was required for IL-1α-mediated cytokine induction (data not shown). In IL-1α-stimulated cultures, the addition of IFN-α after three hours still resulted in a strong inhibition of GM-CSF expression, as was the case when IFN-α was added first, followed by IL-1α (Fig 7). Therefore, IL-1α was added first, followed by CHX with or without IFN-α added 3 hours later. As shown in Fig 7, CHX caused a superinduction of GM-CSF mRNA expression, which was clearly downregulated by IFN-α. For the upregulation of IL-1RA by IFN-α, protein synthesis was not required. By contrast, IFN effects on IL-1β expression appeared to be mediated by a different mechanism. In comparison to cultures receiving IL-1α and IFN-α simultaneously or IL-1α after IFN-α, the downregulation of IL-1β expression was less pronounced when IL-1α was added first, followed by IFN-α 3 hours later. In the presence of CHX, IFN-α failed to inhibit IL-1β expression suggesting that protein neosynthesis was necessary for this inhibitory effect of IFN-α.

Influence of actinomycin D on downregulation of cytokine gene expression. For the RNA decay analysis, foreskin fibroblasts were used, which responded to IFN-α by downregulation of GM-CSF and IL-1β expression, as observed in bone marrow stromal cells (data not shown). When the RNA synthesis was inhibited by actinomycin D, the addition of IFN-α did not result in an enhanced decay of GM-CSF or IL-1β mRNA, suggesting that a posttranscriptional mechanism was not involved in the inhibitory effect of IFN-α on the expression of these cytokines (Fig 8).

DISCUSSION

Suppression of hematopoiesis is a well-established feature of IFN-α, representing the basis for the therapeutic application in myeloproliferative diseases. In vitro IFN-α inhibits proliferation of committed hematopoietic progenitor cells, which is considered to be the explanation for its myelosuppressive action in vivo. We now demonstrate that IFN-α potently inhibits the paracrine expression of hematopoietic growth factors in bone marrow stromal cells. The potency of IFN-α as a regulator of cytokine cascades has only recently been recognized. The induction of IL-1RA in PBMNC by IFN-α and the inhibitory effect of type I IFNs on the expression of IL-8 in a human fibroblast line, bone marrow stromal cells, and hematopoietic cells suggested an antinflammatory role of IFN-α. Our present results, demonstrating antagonization of IL-1 action and inhibition of GM-CSF expression by IFN-α in bone marrow stroma, are in keeping with this biologic function. However, indirect suppression of hematopoiesis appears to be the most striking functional consequence of this novel action of IFN-α.

Hematopoietic cytokines are important mediators of stromal cell-supported hematopoiesis in vitro and probably also in vivo. Although GM-CSF is rarely detected in the supernatant of unstimulated Dexter stroma, in several reports constitutive production of biologically active GM-CSF is described within the adherent layer, which is bound to glycosaminoglycans. Thus, inhibition of stromal cell-dependent hematopoiesis appears to be a logical consequence of IFN-induced downregulation of colony stimulating factors.

IL-1 and IL-6 directly act on immature progenitor cells by a synergistic activity with colony-stimulating factors, formerly described as hemopoietin-1 effect.
IL-1 is a potent stimulant of GM-CSF and IL-6 production, leading to enhanced hematopoiesis in human long-term cultures. Therefore, the inhibition of IL-1β and the concomitant induction of IL-1RA in bone marrow stromal cells by IFN-α is expected to counteract both the direct and the GM-CSF-mediated hematopoietic activity of IL-1. Although IL-1β mRNA was readily detectable in induced stromal cells, we failed to detect IL-1β protein in stromal cell supernatants. Most studies on cytokine production in bone marrow stromal cells examined mRNA expression only or used concentrated supernatants to detect IL-1 protein. After lysis of stromal cells by sonification, significant levels of immuno-reactive IL-1β were measured in the present study, suggesting that cell-associated IL-1β is the predominant species of this protein in bone marrow stroma.

Suppression of IL-1β production, which was induced by IL-1α in monocytes, has recently been described for IFN-γ and IFN-α. However, in monocyctic cells, IFN-α failed to suppress LPS-mediated IL-1 expression. Interestingly, in bone marrow stromal cells, IFN-α significantly inhibited both cytokine- and endotoxin-stimulated IL-1β production, suggesting that the mechanism of IFN-α to regulate cytokine cascades might be different in hematopoietic and stromal cells.

The biologic significance of IFN-α mediated suppression of hematopoietic growth factors was evaluated using the factor dependent cell line, MO7e. In conventional long-term cultures IFN-α inhibits the proliferation of hematopoietic progenitors. However, it would be impossible to distinguish whether IFN-α suppresses long-term hematopoiesis predominantly by direct antiproliferative effects or by indirect anti-hematopoietic effects, due to the downregulation of hematopoietic growth factors. Therefore, a factor-dependent cell line, which is resistant to direct action of IFN-α, as shown for the MO7e cell line, represents an ideal target to clarify this issue. The inhibition of stromal cell-supported proliferation of MO7e cells by IFN-α strongly suggests that downregulation of GM-CSF in stromal cells results in the functional suppression of hematopoiesis.

Downregulation of IL-1β and GM-CSF mRNA expression by IFN-α appears to be regulated at a transcriptional level. A similar mechanism has been postulated for the inhibition of IL-8 expression by IFN-α in a human fibroblast line. However, experiments using CHX as an inhibitor of protein synthesis showed that inhibition of IL-1β expression seems to require the synthesis of an IFN-α-induced protein. The nature of IFN-α-induced intermediate proteins, which are operative in IL-1 inhibition, remains to be determined. By contrast, regulation of GM-CSF and IL-1RA expression is not affected by the presence of CHX, suggesting a direct action of IFN-α.

The biologic importance of this novel action by IFN-α for the regulation of hematopoiesis in vivo is unclear. It can be assumed, however, that myelosuppressive effects of IFN-α, as observed in therapeutic administration or during viral infection, might be at least partially based on the inhibition of the paracrine production of hematopoietic growth factors. Downregulation of hematopoietic cytokines might contribute to the beneficial effect of IFN-α in CML. The reduction of the paracrine secretion of hematopoietic growth factors, which appears to be enhanced by a constitutive overexpression of IL-1 in some patients, might result in an overbalance of negative regulatory cytokines in the bone marrow microenvironment. Thus, in combination with other biologic actions, the influence of IFN-α on cytokine synthesis might contribute to the suppression of the malignant clone by IFN-α in the early phase of the disease. Significant inhibition of GM-CSF and IL-1β production, as well as induction of IL-

![Fig 8. RNA decay analysis of GM-CSF and IL-1β. Subconfluent foreskin fibroblasts were incubated with IL-1α (100 U/mL) for 4 hours to achieve maximal induction of both cytokines. Subsequently, parallel cultures were incubated with actinomycin D (10 μg/mL) alone or with IFN-α (1,000 U/mL) for the indicated times. RNA was prepared and subjected to a Northern blot analysis. Two experiments were carried out and data of one experiment are presented.](image-url)
IRA, are observed in vitro at lower concentrations of IFN-α than required for direct antiproliferative action on hematopoietic progenitors. Therefore, endogenous IFN levels, induced by viral infections, might be sufficient for the downregulation of growth factor production resulting in myeloid suppression. However, in context with bacterial infections, the strong induction of hematopoietic growth factors by bacterial products could overcome the suppressive effect of IFN, thus still allowing the expansion of the myeloid cell compartment.

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Regulation of cytokine expression by interferon-alpha in human bone marrow stromal cells: inhibition of hematopoietic growth factors and induction of interleukin-1 receptor antagonist

MJ Aman, U Keller, G Derigs, M Mohamadzadeh, C Huber and C Peschel