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ARTICLE

Differential Regulation of Umbilical Cord Blood and Leukemic B Cells by Interferon-Alpha (IFN-α): Observations in Cultured Cells

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The exact mechanism of the beneficial therapeutic action of interferon-a (IFN-α) in B-cell-lineage malignancies has not been adequately explained. Here we report on the differential effect of IFN-α2b on non-malignant B cells of umbilical cord blood and leukemic B-cell lines JY, BL-41 and BCBL-1. Leukemic cell proliferation was characterized by colony assay, whereas apoptosis was investigated by flow cytometry of propidium iodide-stained cells. The degree of differentiation was evaluated by measuring the expression level of Fcγ receptor-II (FcγRII) labeled with anti-CD32-FITC monoclonal antibody using flow cytometry. IFN-α protected umbilical cord blood CD19-positive B lymphocytes from apoptotic cell death in vitro. IFN-α significantly decreased colony formation of all three cell lines, and in contrast to normal cells, induced apoptosis in JY and BL-41 and excessive necrosis in HHV-8 infected BCBL-1 cells. FcγRII was upregulated both in normal and in leukemic B cells as indicated by an increase both in the proportion of CD32-positive cells and the mean fluorescence intensity. From our results it seems that antiproliferative, apoptotic and differentiative effects of IFN-α are interrelated but distinct cellular events, which are differentially regulated in normal, leukemic and virus-infected cells of the B-cell lineage. (Pathology Oncology Research Vol 12, No 3, 159–163)

Key words: apoptosis, B cells, colony formation, interferon-alpha, type II Fcγ receptor

Introduction

Interferon-alpha (IFN-α) proved effective in treating patients with neoplastic diseases of the B-cell lineage.[4,9] Antiproliferative effects of IFN-α are exerted by a number of ways such as interrupting autocrine and paracrine growth loops, stimulating terminal differentiation and apoptosis, inducing phenotypic changes that influence the manifestation of regulatory cell surface molecules, downregulating certain genes that mediate proliferation signals and enhancing the cytolytic activity of effector cells involved in tumor cell lysis.[5,6] Direct antineoplastic effects of IFN-α on target cells are mediated by IFN-alpha/beta receptor (IFNAR-2) polypeptides, which have been shown to be highly expressed on B cells.[14] Signal transduction of IFNAR-2 involves the JAK/STAT pathway, an attractive therapeutic target in B-cell malignancies.[8]

We have investigated the effect of IFN-α2b on apoptosis and differentiation of B cells from the umbilical cord blood mononuclear cell (MNC) fraction of healthy newborns. The effects of IFN-α on non-malignant cells were compared to that of permanent cell lines of the B-cell lineage, the EBV-positive human lymphoma-derived JY cell line, the human Burkitt's lymphoma-derived BL-41 cell line, and the human herpes virus 8 (HHV 8)-infected

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Abbreviations

FcγRII: type II receptor for the constant fragment of immunoglobulin G, FITC: fluorescein-isothiocyanate, GM-CSF: granulocyte-macrophage colony-stimulating factor, HHV-8: Human Herpesvirus 8, IFN-α: interferon-alpha, IFN-γ: interferon-gamma, IRF: interferon regulatory factor, MFI: mean fluorescence intensity, MNC: mononuclear cell, PE: phycoerythrin, SCF: stem cell factor (c-kit ligand)

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BCBL-1 cell line.[16,18,19] Terminal differentiation and self-renewal of the leukemic cell lines were assessed by primary and secondary colony formation in semi-solid culture.

Materials and Methods

Cells

Umbilical cord blood samples were collected from dissected umbilical cords of healthy newborns after obtaining written informed consent from the parents. Mononuclear cells were separated by Ficoll-Iodamide (Pharmacia, Uppsala, Sweden) gradient centrifugation as described.[2] BL-41, BCBL-1 and JY human B-lymphoma/leukemia cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), L-glutamine and antibiotics. For further experiments, cells in the exponential phase of growth were used.

Immunofluorescence labeling and flow cytometry

Immunofluorescence labeling of cells and flow cytometric measurements were performed as described.[2] Briefly, cell suspensions were simultaneously incubated with fluorescein-isothiocyanate (FITC)-conjugated anti-CD32 (anti-FcγRII; IV.3, Medarex, West Lebanon, NH) and phycoerythrin (PE)-conjugated anti-CD19 (Becton Dickinson, San Jose, CA) monoclonal antibodies at a concentration suggested by the manufacturers' instructions, at 0ºC for 30 min. Other subpopulations of the mononuclear cell suspension were also determined. We used anti-CD4 and anti-CD8, anti-CD14 and anti-CD34 monoclonal antibodies to label T lymphocytes, monocytes and primitive hematopoetic progenitor cells, respectively (all purchased from Becton Dickinson). Cells were washed and fixed in 1% (vol/vol) paraformaldehyde. Samples were analyzed on a FACScan flow cytometer equipped with argon laser using Lysis II software (Becton Dickinson). Percentage of positive cells and mean fluorescence intensity (MFI) were assessed, as compared to cells labeled with isotype control murine monoclonal antibodies. In umbilical cord blood samples, coexpression of CD32 was detected on CD19-positive B lymphocytes. MFI rate was calculated so as to provide information on the level of marker expression. Effect of in vitro treatment with recombinant human (rh) IFN-α2b (Shering-Plough, Brinny, Ireland) for 24 hours at various concentrations on cell surface FcγRII (CD32) manifestation was also measured. Effects of three additional rh cytokines, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF) were also investigated at 10,000 U/mL, 20 ng/mL and 50 ng/mL concentrations, respectively. These three cytokines were purchased from Genzyme (Cambridge, England).

For apoptosis analysis, cells were incubated in suspension cultures in the presence or absence of varying concentrations of IFN-α2b as described above for periods up to 72 hours (cell lines) or for 24 hours (umbilical cord blood samples). After washing, fixing in 70% ethanol and staining with propidium iodide (Sigma, San Louis, MO), samples were analyzed on a FACScan flow cytometer using the CellFIT program. After doublet discrimination, DNA histograms were obtained. Cells containing less DNA than cells in the G0/G1 peak were considered as apoptotic cells with DNA fragmentation. Necrotic cells were distinguished from apoptotic ones by fluorescence microscopy after acridin orange/ethidium bromide staining when apoptotic cells appear as shrunken cells with green fluorescence signal, whereas necrotic cells appear with orange fluorescence signal. In vitro effects of IFN-α2b and, in case of cord blood samples, IFN-γ, GM-CSF and SCF were also studied at concentrations similar to those used for CD32 expression experiments.

Colony assay

Methylcellulose (Methocel, 3000-5000 centipoise, FLUKA, Neu-Ulm, Germany) at 1.2% was used as the support matrix for semisolid culture. McCoy's 5A modified medium was supplemented with amino acids, vitamins, Na-pyruvate, $NaHCO_3$, penicillin and streptomycin as well as with $2\text{-}5x10^{-5}$ M mercaptoethanol (LOBA Feinchemie, Fischamend, Germany) and 20% FCS. Using 35 mm plastic petri dishes (Greiner, Nürtingen, Germany), $1x10^4$ B-lymphoma/leukemia cells were plated in 1 mL volume of this medium and were incubated for 7 days at 37ºC in a humidified atmosphere containing 5% (v/v) CO_2 supplemented with 20% fetal bovine serum and methylcellulose. Recombinant human IFN-α2b was added to the soft gel cultures just before plating, at final concentrations of 10, 100, 500, 1000 and 10,000 U/mL. Colonies containing more than 50 cells were scored under a dissecting microscope (SZ6045; Olympus, Hamburg, Germany) at the end of the incubation period. Cells from primary colonies were resuspended, washed, replated and incubated under identical conditions as primary methylcellulose cultures. Colonies were seeded in triplicates or quadruplicates. Data are given as percentage of control cultures not containing IFN-α2b (relative plating efficiency).

Statistical analysis

Data are given as mean $\pm$ standard deviation (SD) of three to four parallel experiments. The statistical significance of differences between the effects of various doses of IFN-α2b on the rate of apoptotic as well as of CD19- and CD32-expressing cells was determined using Student's t-test after checking the normality of the distribution. Differences with a p value less than 0.05 were considered significant.

Results

Effect of IFN-α2b on apoptosis and cell surface FcγRII expression of normal umbilical cord blood and leukemic B cells

The proportion of apoptotic mononuclear umbilical cord blood cells showed a tenfold increase after 24 hours in suspension culture without any exogenous growth factor added. Supplementing the medium with IFN-α2b resulted in a significant ($p<0.05$) decrease in the proportion of apoptotic cells at 100 U/mL and a further decrease at 1000 U/mL (*Table 1*). IFN-γ, GM-CSF and SCF were not able to prevent spontaneous apoptosis in umbilical cord blood samples (data not shown). In contrast to the normal umbilical cord blood B cells without IFN-α2b treatment, during the first 72 hours there was a constant low proportion of apoptotic leukemic cells that made up 2.3-4.1% and 3.6-6.7% of BL-41 and JY cells, respectively. The spontaneous apoptosis rate started to increase after 72 hours (JY cells) and 96 hours (BL-41 cells) of culture without splitting. IFN-α2b was observed to increase significantly ($p<0.05$) the proportion of apoptotic cells after 48 hours and 24 hours of culture in BL-41 and JY cells, respectively (*Table 1*). In IFN-α2b-treated BCBL-1 cell line cell necrosis was abundant.

Table 1. **Effect of IFN-α2b on apoptosis of normal umbilical cord blood mononuclear cells, JY and BL-41 human B-cell leukemia cell lines in suspension cultures.**

Incubation time time(h)	*Proportion of apoptotic cells (%)*		
	Without IFN-α2b	*100 U/mL IFN-α2b*	*1000 U/mL IFN-α2b*
		Cord blood MNC	
0	1.0±0.2		
24	10.0±6.4*§	6.5±4.8*	5.1±3.6§
		JY cells	
4	3.6±0.8	6.6±0.7	12.0±2.3
24	6.0±0.8*§	15.3±3.1*¶	22.6±2.0§¶
48	3.8±1.1*§	11.9±2.0*	16.3±1.8§
72	6.7±2.8*§	22.4±3.2*	27.4±1.9§
96	11.0±1.4§	20.7±10.0	30.5±3.8§
120	23.4±7.6	30.6±18.2	43.7±11.5
		BL-41 cells	
4	3.5±0.3	6.6±0.7	12.0±2.3
24	2.3±1.2	4.3±3.0	5.6±1.9
48	1.8±0.33*§	8.7±0.3*	12.0±1.2*§
72	4.1±0.4*§	13.3±1.3*	26.7±11.2§
96	3.7±0.4	23.7±16.3	27.6±17.9
120	25.7±1.2*§	33.3±1.9*	57.8±1.9§

Numbers show mean ± SD values (n=4)

*§¶ denote significant differences ($p<0.05$) between the corresponding groups of BL-41, JY and cord blood MNC, incubated for the same period of time in the absence or presence of different concentrations of IFN-α2b

In parallel with these changes, we measured the cell surface manifestation of FcγRII (CD32) on CD19-positive umbilical cord blood B lymphocytes and on two B-cell leukemia/lymphoma permanent cell lines as related to in vitro treatment with IFN-α2b. The proportion of CD19-CD32-coexpressing B lymphocytes was 10.8±5.3% among umbilical cord blood mononuclear cells. This proportion fell significantly ($p<0.05$) to 5.8±3.5% after 24 hours of culture in the absence of any added growth factors. In the presence of 1000 U/mL IFN-α2b, the proportion of CD19-CD32-coexpressing cells did not decrease (10.0±5.5%) from the initial level. The effects of IFN-γ, GM-CSF and SCF did not prove significant in preventing a decrease in the proportion of FcγRII-expressing cord blood B lymphocytes (data not shown). Changes in the mean CD32 fluorescence intensity showed a similar pattern: a significant decrease ($p<0.05$) from 4.8±2.8 to 2.2±0.7 in the absence of exogenous growth factors, and no significant change (4.3±1.9) in the presence of IFN-α2b, as compared to the pretreated sample. IFN-γ, GM-CSF and SCF did not exert a significant effect on CD32 mean fluorescence intensity of CD19-positive cord blood B lymphocytes in culture (data not shown). IFN-α2b did not change significantly the proportion of T lymphocytes (70±5%), monocytes (9±5%) and primitive CD34-positive hematopoetic progenitor cells (1.5±1%) in the mononuclear cell fraction obtained from umbilical cord blood samples during the same period of incubation as in the case of CD19-positive B lymphocytes. Over 99% of BL-41 and JY cells were CD32-positive, i.e. all cells within the cell population constitutively expressed the receptor. Incubation of BL-41 and JY cells with 1000 U/mL IFN-α2b for 24 hours resulted in 13±9% and 7±3% increase, respectively, in the mean fluorescence intensity as compared to non-treated cells.

Effect of IFN-α2b on primary and secondary colony formation by leukemic B cells

IFN-α2b caused a dose-dependent decrease both in primary (*Figure 1a*) and in secondary (*Figure 1b)* colony formation of all three B-cell leukemia/lymphoma cell lines. Maximal or near-maximal inhibition was observed at 1000 U/mL (BL-41 and JY cells) and at 100 U/mL in the case of BCBL-1 cells. Inhibition of colony formation was not com-

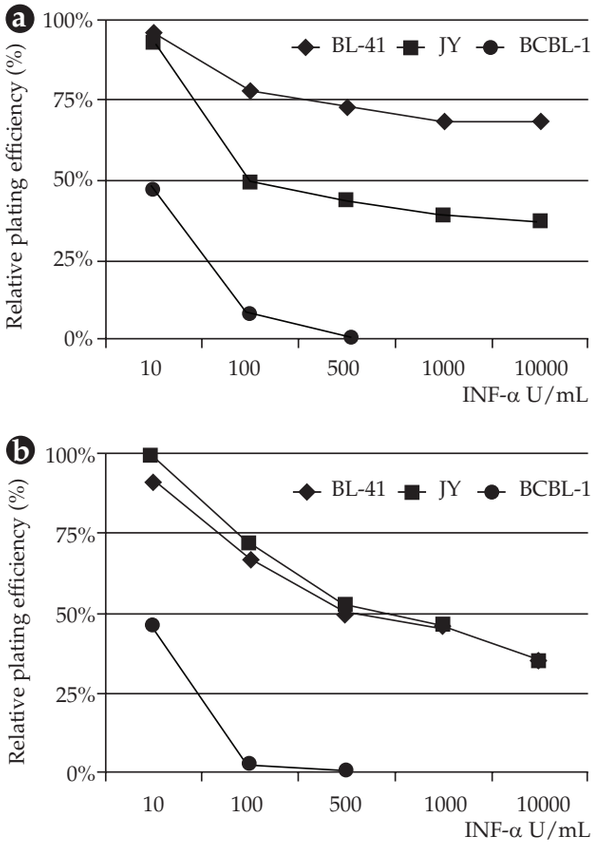

Figure 1. *Effect of interferon-alpha (IFN-α) on primary* ***(a)*** *and secondary* ***(b)*** *colony formation of BL-41, JY and BCBL-1 human B-cell-lineage leukemic cell lines in soft gel cultures*

plete in the case of BL-41 and JY cells, 70% and 35% of these cells survived in primary soft gel cultures, while 45% and 35% survived in secondary cultures, respectively. In the case of BCBL-1 cells, 500 U/mL or 100 U/mL of IFN-α2b resulted in 100% abolishment of clonal proliferation in primary and secondary methylcellulose cultures, respectively.

Discussion

IFN-α2b-induced changes in clonal growth, apoptosis and phenotype of umbilical cord blood B lymphocytes were compared with those of three permanent B leukemic cell lines. Similar to the effect of myeloproliferative cytokines on granulocyte and granulocyte/macrophage progenitor cells, IFN-α2b mediated a potent survival signal to normal umbilical cord blood B lymphocytes in vitro. Spontaneous apoptosis rate of these cells in suspension cultures without any exogenous growth factors decreased significantly after supplementing the culture with IFN-α2b but not with IFN-γ, GM-CSF and stem cell factor. In contrast, IFN-α2b treatment increased significantly the rate of apoptosis (JY and BL-41) and cell necrosis (BCBL-1) in the investigated leukemic B-cell lines.

In parallel with its anti-apoptotic effect on umbilical cord blood B lymphocytes, IFN-α2b prevented the decrease in the proportion of CD32-positive, FcγRII-expressing cord blood B lymphocytes maintained in culture. Since the proportion of other subpopulations (T lymphocytes, monocytes, CD34-positive primitive hematopoetic progenitor cells) of cord blood mononuclear cells did not exhibit a similar change, there are reasons to believe that antiapoptotic action of IFN-α was exerted primarily on B lymphocytes. IFN-α2b also increased the manifestation of FcγRII in the leukemic cell lines, as proved by the elevation of the mean CD32 fluorescence intensity in IFN-α2b-treated cultures. The effect of IFN-α proved to be unique since IFN-γ, GM-CSF or SCF were not able to elicit similar changes in the proportion of apoptotic cells and the level of FcγRII expression. Although an indirect action of other B-cell survival factors secreted by monocytes or T lymphocytes, induced in the mononuclear cell fraction by IFN-γ, is not likely, this possibility cannot completely be ruled out. This observation is a new, however, not unexpected finding. IFN-γ but not IFN-α was known to upregulate the expression of class I and II Fcγ receptors on monocytes/macrophages and on megakaryocytic cells.[7] IFN-γ caused a rapid induction of specific protein complexes that assemble on a 39-nt sequence, called IFN-γ response region (GRR) resulting in a robust increase in the transcription rate of the FcγRI gene.[12] IFN-α was also shown to induce the formation of similar complexes assembling on the GRR, yet the IFN-γ-induced complex failed to activate the transcription of FcγRI in monocytic cells. The increased expression level of Fcγ receptors was suggested to correlate with differentiation events and increased responsiveness to IFN-α in B cells.[1,17]

In the investigated leukemic cell lines IFN-α exerted a potent, dose-dependent decrease in the plating efficiencies of both primary and secondary colonies. Since primary colony formation is thought to define terminal differentiation whereas secondary colony formation represents self-renewal, our results indicate a direct antiproliferative effect of IFN-α exerted at the level of the leukemic stem cells as well as on their progeny undergoing terminal cell divisions.[11] The suppression of colony formation was accompanied by an elevation in the proportion of apoptotic JY and BL-41 cells maintained in IFN-α-supplemented suspension cultures, which was a differential effect of IFN-α2b in leukemic vs. normal B cells. IFN-α alone did not result in a complete inhibition of colony formation or the induction of 100% apoptosis in JY and BL-41 cell lines. The surviving fractions may represent either subpopulations naturally resistant to IFN-

α or the existence of bypassing mechanisms that allow to escape programmed cell death induced by IFN-α.

Complete suppression of both primary and secondary colony formation by IFN-α was observed in HHV-8-infected BCBL-1 cells. Here, an elevated number of necrotic cells were found in IFN-α-treated suspension cultures. These cells were shown to express detectable levels of mRNA for human interleukin (IL)-1b, IL-10, IL-12, two macrophage inflammatory proteins belonging to the b-chemokine family, transforming growth factor-b1 and viral IL-6.[10,13] IFN-α may inhibit proliferation by interrupting autocrine loops of growth by interfering with these cytokines. Recently, inhibition of infectious HHV-8 production by IFN-α was demonstrated in BCBL-1 cells.[15] On the other hand, the long unique region of HHV-8 contains ORF-K9, encoding for a protein with high homology to other interferon regulatory factors (IRF). IFN-α may exert its growth suppressing activity by inducing IRF-1, since viral IRF does not compete with IRF-1 binding to DNA.[20] Recently, type I consensus IFN gene transfer into BCBL-1 cells was shown to induce apoptosis and abrogate tumorigenicity in SCID mice by inhibiting activation of the viral lytic cycle.[3]

Inhibition of leukemic cell clonal proliferation and differential regulation of apoptosis of neoplastic and normal B cells seems to be a promising effect of IFN-α, which can potentially be exploited in anti-tumor regimens aimed at childhood B-cell malignancies. Further studies employing de novo leukemic cells of the B-cell lineage and investigating possible interactions of cytostatic drugs with IFN-α are required to define its role in the therapeutic arsenal of pediatric oncology.

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