

Thesis for the Ph.D. degree

Interferon-alpha in pediatric oncology

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INTRODUCTION

The Department of Pediatrics of the University of Debrecen is a regional center of the Hungarian Pediatric Oncology Group. The number of new cases of children with cancer in Hungary is about 300 among which there are newly diagnosed tumoric and 70-80 leukemic cases a year and 35-40 new children suffering in hematological malignancies or in other malignant disease are cared in our clinic. Similarly to international mortality data malignant diseases of childhood are the second main cause of mortality in Hungary and treatment of one third of all cases representing malignant hematological patients is the supreme therapeutic challenge nowadays.

In the last decades, due to the advances of medical biology and in the diagnostic tools and therapeutic possibilities, the previously fatal malignant diseases of childhood became curable in 70% of cases. This favorable change was due to the great progress in the field of intensive combined chemotherapy and supportive treatment that improved the results of treatment in ALL dramatically. Presently, children suffering acute lymphoblastic leukemia (ALL) can be cured in 80% however, 20% of patients will succumb therapy resistance, relapse, toxic and infective side effects of the therapy. Unfortunately, improvement in the survival rates is slow and there was no further major progress advance in the last decade. Any progress in percentage of surviving children required powerful forces. It is a special contradiction that the use of anti-leukemic, anti-neoplastic protocols is limited by its toxicity - especially myelotoxic and immunosuppressive effects-, but intensity of the therapeutic scheme affects the efficiency of cure, as clinical studies certified. Beside the survival results, quality of life has a growing importance.

The results of treatment of pediatric acute lymphoblastic leukemia had reached were so pioneer in character that it had a fruitful effect on „adult” hematology. We can declare with serendipity that pediatric hematologists were the „flagships” of the development of combined chemotherapeutic protocols. However, there are certain diseases presenting frequently in adulthood, like myeloproliferative and myelodysplastic syndromes that existence was debated - except for chronic myeloid leukemia - in childhood. There was no review study about the pediatric myeloproliferative disorders recently in the Hungarian literature. With respect of these two groups of diseases the pediatricians required the help from adult hematologists. My interest was raised by the possibility and the need for further improving of the results of pediatric acute leukemias and chronic hematological malignancies, respectively.

It is an advance that patients are stratified according to risk factors and therapy is individually planned for the type of disease. Less toxic compounds and methods to increase defense mechanisms of the body or to modify the biologic character of the leukemia/tumor cells are new therapeutic elements. These modalities begin to find their place in the therapeutic armamentarium just now, but they seldom can be used in childhood. Enhancing immune-response of a patient contributes to fight infections and to destroy pathological clones. Extracellular regulation and manipulation of the genetic structure of leukemia cells are able to control the balance between the self renewal and terminal differentiation in the pathological clone. It is hoped that intelligent combination of monoclonal antibodies, cytokines, differentiation induction or angiogenesis inhibitory agents and other biomodulant compounds will improve the results achieved by cytoablative therapy and hematopoietic stem cell transplantation. The experimental data of recent years, including our experiences, support the notion that the proliferation and maturation of normal bone marrow hematopoietic progenitors or leukemia cells can influence in different ways: certain growth factors and their combinations promote in vitro proliferation of leukemia cells, while others can cause mainly death of these cells by increasing apoptosis or promoting terminal differentiation. Out of well-known cytokines the granulocyte-stimulating factor (G-CSF), the granulocyte-macrophage (GM)-CSF and the interleukin (IL)-3 have been introduced into therapeutic schemes of patients suffering from leukemia or other lympho-hematopoietic diseases to stimulate normal bone marrow cells. The potential stimulatory action of these hematopoietic growth factors on leukemic blast cells may interfere negatively with the therapeutic effects of antileukemic regimens. As stem cell factor stimulates the earliest stem cells and the G-CSF and GM-CSF react on myeloid lineage-restricted progenitor cells, especially of the granulocyte-macrophage lineage, their stimulatory effect on myeloid leukemic cells is not surprising. SCF receptors have been confirmed on blast cells of patients with T-ALL, B-cell lineage ALL or My+ ALL, and SCF stimulates proliferation of leukemic cell lines of various origin including T-lymphoblastic leukemia cell lines. Beside unfavorable proliferation promoting action of cytokines their inhibitory effect on proliferation in combination or alone is an interesting possibility and it can be utilized in the treatment of neoplasms. Anti-neoplastic effect of IL-2 and tumor necrosis factor (TNF) are mostly investigated. The former may act by activation of tumor-specific T-cells and lymphokine-activated-killer cells (LAK), and the latter by enhancing monocyte cytotoxicity and increased production of peroxid products. There are encouraging studies about products regulating T-cell operation, angiogenesis inhibition and proinflammatory cytokines having anti-tumor effect, as IL-12, IL-18 and IL-27.

The various cytokines and their combinations change the balance between self renewal and terminal differentiation of leukemia/lymphoma cells in different ways. This differential regulatory effect can be utilized to kill quickly and totally the leukemic cells, as monotherapy or in combination with cytostatic drugs and of course relatively sparing normal elements of hematopoiesis.

The interferons, beside their antiviral effects, promote differentiation in mammalian cells. They are widely used in adults with malignant hematopoietic disorders or solid tumors on the bases their antiproliferative effect and because of enhancing the anti-tumor protective function of the body, inducing powerful anti-neoplastic action. The favorable cytogenetic response and survival advantage observed during treatment of Philadelphia chromosome positive (Ph+) chronic myeloid leukemia opened a new era in the use of these anti-neoplastic glycoproteins. Beside Ph+ CML, IFN α is registered in Hungary for treatment of follicular non-Hodgkin lymphoma (NHL), cutaneous T-cell lymphoma, Kaposi sarcoma and malignant melanoma, respectively. Therapeutic effectivity is of proven merit in myeloproliferative disorders frequently appearing in adults, but rarely in childhood. The 50 cases of essential thrombocythemia (ET) in childhood reviewed so far in the literature reported only few experiences with IFN α treatment however, in adults reported favorable action in 78% of cases. There are few clinical studies including small groups of patients that demonstrated clinical activity of IFN α therapy in malignant pediatric disorders. The Ph+ CML, in which the IFN α treatment has a proven merit, is only a small fragment of pediatric neoplasia. There is a need to perform careful in vitro experiments and clinical studies to ascertain the safe application of these cytokines -introduced in therapeutic arsenal - in pediatric-oncohematology.

Aims of the study

1. To survey the literature for the effect and contemporary use of type-I interferons in childhood cancer, especially in rare childhood myeloproliferative disorders.
2. To characterize in vitro activity of IFN α -2b on proliferation and differentiation of normal umbilical cord blood B-lymphocytes and B-cell leukemia/lymphoma cell lines, respectively.
3. To investigate the effect of IFN α -2b on bone marrow progenitor cells in a patient with essential thrombocythemia (ET), by colony assay.

4. To demonstrate the therapeutic potential of IFN α in children with therapy-resistant cancer and neoplastic disorders with unfavorable prognosis. A special emphasis was given to the patient with ET since colony assay demonstrated in vitro inhibition of the proliferation of myeloid progenitors by IFN α -2b. Side effects of IFN α used alone and in combination with isotretinoin were to be closely observed.

MATERIALS AND METHODS

Cells

Cell lines

The EBV genome positive JY, the EBV genome negative BL-41 and the HHV-8 positive, EBV and HIV-1 negative BCBL-1 human B-lymphoma/leukemia cell lines were maintained at 37°C in CO₂ thermostat (5% CO₂), in RPMI 1640 (GIBCO, Grand Island, NY) medium supplemented with 10% fetal calf serum (FCS; GIBCO, Grand Island, NY), L-glutamine and antibiotics. Cells were splitted 2 or 4 daily depending on the cell line and for further experiments cells in the exponential phase of growth were used.

Bone marrow and umbilical cord blood mononuclear cells

Bone marrow sample of patient with ET was obtained with a sterile biopsy needle, by puncture of the iliac crest. Umbilical cord blood samples were collected from dissected umbilical cords of healthy newborns after written informed consent obtained from the parents. Samples were heparinized to prevent clotting. Mononuclear cells were separated by Ficoll-Iodamide (Pharmacia, Uppsala, Sweden) gradient centrifugation at 1000g for 15min and washed from the interphase twice with McCoy's 5A medium (GIBCO, Grand Island, NY) containing 5% FCS.

Cell culture methods

Colony assay

Semi-solid cultures contained 1x10⁴ BL-41, JY and BCBL-1 cells. Samples at every examined time, types and doses of exogenous stimuli were seeded in triplicates. The McCoy's 5A modified medium was supplemented according to Pike and Robinson with amino acids, vitamins, Na-pyruvate, NaHCO₃, penicillin and streptomycin as well as with 2-5x10⁻⁵ M mercaptoethanol (LOBA Feinchemie, Fischamend, Germany) and 20% FCS. Methylcellulose (Methocel, 3000-5000 centipoises, FLUKA, Neu-Ulm, Germany) was used at 1.2 % as the support matrix for semisolid culture. Using 35 mm plastic petri dishes (Greiner, Nürtingen, Germany), cells were plated in 1 mL medium and were incubated for 7 days at 37°C in a humidified atmosphere containing 5% (v/v) CO₂.

Recombinant human (rh) IFN α -2b (Shering-Plough, Brinny, Ireland) was added to the soft gel cultures just before plating in final concentrations of 10, 100, 500, 1000 and 10,000 U/mL. Colonies containing more than 50 cells were scored at the end of incubation period under a dissecting microscope (SZ6045; Olympus, Hamburg, Germany).

For the secondary cultures, cells from primary cultures were resuspended in McCoy's medium, washed, replated and incubated for further 7 days under identical conditions as were primary cultures. Primary and the secondary colony formation were expressed as relative plating efficiency, i.e. as percentages of control cultures not containing IFN α -2b.

The bone marrow mononuclear cells of the patient with ET were plated and incubated under identical conditions as were cell lines. No stimulation agent was used to evaluate spontaneous colony formation. A combined stimulation with 5 U/mL rh erythropoietin (EPO) and 10 ng/mL of rh granulocyte (G) colony-stimulating factor (CSF) was used to study erythroid bursts (BFU-E) and colonies (CFU-E) after 14 and 7 days of cultures, respectively. Myeloid colonies were observed after applying optimal concentrations of rh G-CSF (300 ng/mL), rh granulocyte-macrophage (GM)-CSF, (100 ng/mL), rh stem cell factor (SCF, 100 ng/mL) or 10% (v/v) of phytohemagglutinin A-stimulated leukocyte conditioned medium (PHA-LCM). Cultures were added 100 and 1000 U/mL rh IFN α -2a to investigate inhibition of PHA-LCM-induced colony formation. The controls were subjected to bone marrow aspiration because of suspected hematological disorders, but they proved to have a healthy hematopoiesis.

Phytohemagglutinin-stimulated human leukocyte medium

Blood for human leukocyte cultures was drawn from a healthy adult volunteer (I.SZ.) according to the Helsinki Declaration. Mononuclear cells at 4×10^6 /mL were cultured according to Dresch et al. in McCoy's 5A medium containing 15% autologous human serum, 17 mg/mL phytohemagglutinin A (DIFCO Laboratories, Detroit, Mich) and 10 mg/mL Levamisole (Decaris, Richter Gedeon, Budapest, Hungary) at 37°C, in a 5% CO₂ atmosphere. Supernatant was decanted on day 5 of culture by centrifugation and stored at -20°C in 1 mL aliquots.

Suspension cultures

To analyze cell surface marker expression patterns and apoptosis, JY, BL-41 and BCBL-1 cells in the exponential phase of growth and cord blood-derived mononuclear cells were cultured in 5×10^5 /mL concentration in 5 mL RPMI 1640 medium in 10 mL plastic culture flasks (Greiner), at 37 °C in a 5% humidified CO₂ atmosphere for 72 hours (cell lines) or 24 hours

(umbilical cord blood mononuclear cells), respectively. Suspension cultures were supplemented with a final concentration of 100 U/mL and 1000 U/mL rh IFN α -2b, and 10,000 U/mL, 10 ng/mL and 50 ng/mL of rh IFN- γ , rh GM-CSF and rh SCF, respectively. Control suspensions did not contain exogenous cytokines.

Immunofluorescence labeling and flow cytometry

Cell suspensions were simultaneously incubated with fluorescein-isothiocyanate (FITC)-conjugated anti-CD32 (anti-Fc γ RII; IV.3, Medarex, West Lebanon, NH) and phycoerythrin (PE)-conjugated anti-CD19 (Becton Dickinson, San Jose, CA) monoclonal antibodies. Other subpopulations of the mononuclear cell suspensions were also determined. Anti-CD4 and anti-CD8 monoclonal antibodies were used to label T-lymphocytes, anti-CD14 monoclonal antibody was used to label monocytes and anti-CD34 monoclonal antibody was used to label primitive hematopoietic progenitor cells (each monoclonal antibody was purchased from Becton Dickinson). Isotype-identical, irrelevant mouse monoclonal antibodies were used as control. After lysing red blood cells by FacsLysing (Becton Dickinson) suspension cells were washed twice by phosphate buffered saline (pH 7.4; PBS, Sigma, St Louis, Mo) and fixed in 1% (vol/vol) paraformaldehyde (Kat-Chem, Budapest, Hungary).

Samples were analyzed on a FacScan flow cytometer equipped with 15 mW argon laser using Lysis II software (Becton Dickinson). The characteristic of leukemia/lymphoma cells and the umbilical cord blood cells were determined by investigating 10.000 cells in every event. The sample evaluated as positive for an antigen if 20% of cells showed considerable higher fluorescent intensity as compared to cells labeled with irrelevant isotype control murine monoclonal antibodies. Ratio of positive cells and mean fluorescence intensity (MFI) were assessed as percentage of control.

In the course of detection of the CD19 and CD32 coexpression the suspension cultures contains 100 U/mL and 1000 U/mL final concentration of rh IFN α -2b, and 10.000 U/mL, 10 ng/mL, 50 ng/mL final concentration of rh IFN- γ , rh GM-CSF and rh SCF, respectively. The control suspensions did not contain exogenous cytokines.

For apoptosis analysis, cells were incubated in suspension cultures. 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. The

suspension cultures contain IFN α -2b, IFN γ , GM-CSF and SCF in concentration using above. The control suspensions did not contain exogenous cytokines.

To detect platelet P-selectin (CD62) expression level in peripheral blood samples of patient with ET, were incubated with PE-labeled anti-CD62 monoclonal antibody together with FITC-labeled anti-CD42a monoclonal antibody referring to GPIX antigen (CD42a) of the thrombocyte. Isotype-identical, irrelevant mouse monoclonal antibodies were used as control. The samples were washed and fixed as reviewed formerly in the case of mononuclear cells. The analysis occurred on FacScan flow cytometer equipped with 15mW argon laser (Becton Dickinson) using the CellQuest software (Becton Dickinson).

Clinical Study

Between January 1994 and May 2000, 24 pediatric patients with specifically unfavorable, therapy-refractory or relapsing malignant disease were subjected IFN α therapy. In the course of clinical study the data collection and evaluation happened retrospectively from 1994 to 1997, and then prospectively, rely upon the documentation of patients.

Of the 24 patients 15 were male and 9 were female (age at diagnosis ranged between 1 month and 18 years, mean 5,3 year, median 3 year). Six patients suffered from hematopoietic malignancies. Three cases of acute leukemia; 1 acute mixed leukemia (AmixL: CD7-, CD10-, CD19-, CD33-positive) and 2 acute myeloid leukemia (AML: one M7: CD33-, CD41-, CD42a-, CD7-positive, and one M2: CD33-, CD13-, CD7-positive); 2 cases were MDS (1 JMML and 1 RAEB). One patient had essential thrombocythemia (ET). Two patients had disseminated forms of LCH, four patients were diagnosed with gastrointestinal malignancies: 1 hepatocellular carcinoma, 2 hepatoblastoma, 1 colon adenocarcinoma. Nine patients had tumors of neural crest origin: 6 neuroblastoma, 1 ganglioneuroblastoma, 2 primitive neuroectodermal tumors (PNET). Two patients had central nervous tumors: one medulloblastoma and one astrocytoma (A") of the cervical and thoracic spinal cord. One patient had rhabdomyosarcoma.

IFN α therapy was initiated at time of diagnosis in three cases: in the JMML patient with hyperleukocytosis and organomegalia, introduction of IFN α therapy was based on excellent in vitro antiproliferative properties of IFN α as determined by colony assay; in ET IFN α therapy is of proven merit in adult patients and the in vitro efficiency of IFN α therapy I confirmed by colony assay in the case of ET including the clinical study; in the patient with colon adenocarcinoma IFN α therapy in combination with 5-Fluorouracil was started after radical surgery (hemicolectomy) in order to suppress minimal residual disease. All the other patients were treated previously according to standard antineoplastic protocols. Twelve patients were

included at relapse: 8 local (including bone marrow relapses in AML), 3 local and metastatic, and 1 metastatic relapses. Three patients were refractory. Six patients exhibited partial responses to previous therapy and had macroscopic residual tumors. The IFN α (IFN α -2b; Intron A, Schering-Plough, Brinny, Ireland; IFN α -2a; Roferon A, Hoffman-LaRoche, Basel, Switzerland) was applied three times a week subcutaneously (s.c.) as monotherapy in seven cases, and with other antineoplastic drugs and protocols or biologic response modifier agents, such as retinoic acid (RA) derivatives and low dose cytarabine (LD-ARA-C) in the other cases. Daily dose of IFN α was 3 MU/m² of body surface area in the majority of cases. The initial dose was escalated to 6 MU/m² in 2 cases. In two cases the initial dose was 10 MU/m² (later escalated to 20 MU/m²) and 5 MU/m², respectively.

Before the planned treatment, written informed consent was obtained from the parents of the patients. The parents of JMML patient refused any further therapy after 51/2 months of treatment before completing the consolidation phase of AML-BFM 93 protocol being applied together with IFN α therapy.

Responses to IFN α therapy were assessed by performing physical examination, complete blood count, bone marrow aspiration in patients with hematopoietic malignancies, image analysis including conventional X-ray pictures, ultrasound, CT, MRI investigation, ⁹⁹Tc bone scan and in neuroblastoma patients ¹²⁵I metaiodobenzylguanidine (MIBG) scan, blood chemistry tests (including lactate dehydrogenase, neuron specific enolase and ferritin), repeatedly. Complete and partial remission (CR and PR), stable and progressive disease (SD and PD) were defined according to conventional criteria referring to therapeutic effect. Survival of patients was registered. Besides the therapeutic effect of IFN α treatment the side effects of IFN α was investigated.

Out of 24 patients the case of patient with ET has been reviewed in detail, her bone marrow mononuclear cells were characterized by colony assay at the time of diagnosis. Cases of 2 patients have been described too, in whom Sweet syndrome developed during 13-cis-retinoic acid (isotretinoin; Roaccutan, Roche, Basle, Switzerland) treatment applied parallel with IFN α .

Statistical analysis

The statistical significance of differences between the effects of various doses of IFN α -2b on the rate of apoptosis, inhibition of colony formation, 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 (p<0,05) were considered significant.

RESULTS

Effect of IFN α -2b on primary and secondary colony formation by leukemia/lymphoma B-cell lines

IFN α -2b caused a dose-dependent decrease both in primary and in secondary colony formation of all three B-cell leukemia/lymphoma cell lines. Maximal inhibition on primary colony formation was observed at dose of 1000 U/mL IFN α -2b in the case of BL-41 and JY cells and near maximal inhibition at dose of 100 U/mL IFN α -2b in the case of BCBL-1 cells. Inhibition of colony formation was not complete 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 of IFN α -2b and 100 U/mL of IFN α -2b resulted in 100% abolishment of clonal proliferation in primary and secondary methylcellulose cultures, respectively.

Effect of IFN α -2b on apoptosis and cell surface Fc γ RII manifestation of normal umbilical cord blood B-cells and leukemia/lymphoma B-cell lines

In the investigated cell lines in the exponential phase of growth there were 2.3-4.1% and 3.6-6.7% of apoptotic BL-41 and JY cells, respectively. This spontaneous apoptosis rate started to elevate after 72 hours (JY cells) and 96 hours (BL-41 cells) of culture without splitting. IFN α -2b 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. In IFN α -2b-treated BCBL-1 cell line cell necrosis was abundant. In contrast to leukemia/lymphoma cell lines 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 α -2b 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. In contrast to IFN α -2b, IFN γ , GM-CSF and SCF were not able to prevent spontaneous apoptosis in umbilical cord blood samples.

The IFN α -2b treatment affected the cell surface manifestation of Fc γ RII (CD32). Over 99% of BL-41 and JY cells were CD32-positive, i.e. all cells within the cell population manifested the receptor constitutively. Incubation of BL-41 and JY cells with 1000 U/mL IFN α -2b for 24 hours resulted in a 13 ± 9 % and a 7 ± 3 % increase in the mean fluorescence intensity as compared to non-treated cells. 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 α -2b, the proportion of CD19-CD32-coexpressing cells did not decrease (10.0 ± 5.5 %) from the initial level. IFN α treatment did not exhibit similar change in other subpopulations (T-lymphocytes, monocytes, CD34-positive primitive hematopoietic progenitor cells) of cord blood mononuclear cells. The effects of IFN γ , GM-CSF and SCF did not prove significant in preventing a decrease in the proportion of Fc γ RII-expressing cord blood B-lymphocytes.

The change in the mean CD32 fluorescence intensity rate 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) as compared to the CD32 mean fluorescence intensity rate of the IFN α -2b 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. IFN α -2b did not change significantly the proportion of T-lymphocytes ($70 \pm 5\%$), monocytes ($9 \pm 5\%$) and primitive CD34-positive hematopoietic progenitor cells ($1.5 \pm 1\%$) 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.

In vitro and in vivo effect of IFN α in a patient with essential thrombocythemia (ET)

A 3-year-old Caucasian girl was investigated in our clinic for severe headache, dizziness and mild splenomegaly. Complete blood count (CBC) exhibited an isolated, marked increase in platelet count ($3000 \times 10^3/\mu\text{L}$). Mean platelet volume was 10.5 fL. Bone marrow biopsy revealed a moderately hypercellular hematopoiesis, with a normal ratio and localization of erythropoietic and myelopoietic precursors. The frequency of megakaryocytes was markedly increased. The megakaryocytes were larger than normal, their nuclei were strongly hyperlobulated. Reticular elements were mildly increased (Bauermeister grade 1-2). Prussian' blue reaction did not reveal ringed sideroblasts. Cytogenetic analysis showed a normal female karyotype (46, XX).

Mononuclear cells, obtained from bone marrow aspirate were subjected to colony assay. No spontaneous colony formation was observed. A combined stimulation with 5 U/mL recombinant human (rh) erythropoietin (EPO) and 10 ng/mL of rh granulocyte (G) colony-stimulating factor (CSF) resulted in normally appearing erythroid bursts (BFU-E) and colonies (CFU-E) after 14 and 7 days of cultures, respectively. Myeloid colonies, consisting of relatively few number (50-200) of large cells, that were negative for factor VIII (FVIII)

antigen, were observed after applying optimal concentrations of rh G-CSF (300 ng/mL), rh granulocyte-macrophage (GM) -CSF, (100 ng/mL), rh stem cell factor (SCF, 100 ng/mL) and 10% (v/v) of phytohemagglutinin A-stimulated leukocyte conditioned medium (PHA-LCM). 1000 U/mL rh IFN α -2a resulted in complete-dose dependent inhibition of PHA- LCM-induced colony formation.

Based on its excellent in vitro inhibitory effect and on the favorable therapeutic action in adults IFN α therapy was started with 3 MU s.c. thrice a week. The dose of IFN α was escalated to 6 MU. The patient was given 5 mg/kg aspirin to inhibit platelet aggregation until platelet counts decreased below $1000 \times 10^3/\mu\text{L}$. Platelet counts decreased continuously and reached a near-normal level ($440 \times 10^3/\mu\text{L}$) 65 months after initiation of IFN treatment. The patient's symptoms ceased within two months of treatment, she has casual way of life, attends school, and she is free of complaints and clinical signs. She experienced only mild flu-like side effects with a low grade fever. After IFN α treatment have been stopped the platelet counts of the patient vary between $400 \times 10^3/\mu\text{L}$ and $600 \times 10^3/\mu\text{L}$ on checkup examinations.

Three months after having stopped IFN treatment, bone marrow flow cytometric analysis showed normal myeloid/erythroid, T cell/B cell and CD4/CD8 ratios. There was a segregated population (6%) with an intensive GPIIb and GPIX expression and a CD45dim/CD34/cyFXIII-A characteristics. The flow cytometric analysis of the peripheral blood sample showed an extremely increased (30%) P-selectin expression on platelets as related to normal control (0-3%) representing platelet activation. Fifteen months after having stopped IFN treatment, these markers were expressed within the normal ranges.

Clinical experiences on IFN α treatment of children with cancer

IFN α was applied as first line therapy in the case of ET because of its effectivity reviewed in literature and in the case of JMML on the grounds of favorable effect of IFN α . The patient suffering colon adenocarcinoma received IFN α as a maintenance treatment with Fluoro-uracil in combination, based on literature data. Out of these cases the two patients with LCH, and the two patients with residual neural crest tumor and the patient with astrocytoma received the IFN α – all through or partially – in monotherapy. All the other patients were treated with IFN α in combination with chemotherapy match to diagnosis.

Fourteen of 24 patients (58,3%) responded with complete responses (CR) and partial responses (PR) to IFN α therapy. Survival of this subgroup of patient was 9-167 months (mean 79,4 months, median 79 months). All the surviving patients were clustered among the responsive cases. Beside the 7 surviving patients favorable response was detected in four

patients with hematological malignancies, ad interim in patient with colon adenocarcinoma, in a patient with neuroblastoma and in two patients with LCH, who later all succumbed. 3 patient with stable disease and 8 patients with progressive disease were lost without exception (11/24, 45,8%). In this subgroup the mean survival time was 35 months (8-67 months, median 32 months). Poor responses were noted among patients with gastrointestinal lesions, MDS, PNET and neuroblastoma, medulloblastoma and rhabdomyosarcoma, respectively.

Majority of patients received IFN α in a dose of 3 MU/m². In five patients was divergence from this dosage method. In patient with AmixL showed no response to standard therapy the initial dose of IFN α was 10 MU/m² escalated later to 20 MU/m² without increasing in side effects. In the case of hepatocellular carcinoma the initial 3 MU/m² dose was escalated to 5 MU/m² while in the patient with ET, a patient with neuroblastoma and the patient with astocytoma to 6 MU/m². The favorable responses and the individual tolerance were considered to escalate dose. In the case of patients receiving IFN α in raise dose the frequency and intensity of the side effects was the same than in group receiving standard dose.

All the patients tolerated well IFN α therapy except for mild flu-like symptoms and the inconvenience caused by s.c. administration. Only in one case developed great fatigue, mild deficiency in attention, and poor appetite with weight loss.

Severe side effects were noted in two cases receiving 13-cis-retinoic-acid in conjunction with IFN α treatment. An 18 years-old Caucasian girl treated with craniospinal irradiation and VEP (Vincristine/Elobromol/Procarbazine) because of cerebellar medulloblastoma. Three years later, she developed a secondary myelodysplastic syndrome (RAEB: refractory anemia with excess blasts) and underwent allogenic bone marrow transplantation. The original dysplastic clone re-emerged 5 months after transplantation. Donor buffy coat transfusions were given twice followed by IFN α (3MU/m² trice weekly), isotretinoin (120mg/m²) and repeated courses of low-dose ARA-C. Eight days after having started isotretinoin treatment, the patient developed painful erythematous nodules, fever and leukocytosis (mature neutrophils) lasting for 2 months, with elevated ESR and negative blood cultures, repeatedly. Four weeks later gross proteinuria and severe fluid retention developed that persisted throughout the remaining life of patient, in contrast to other clinical signs that improved with isotretinoin withdrawal and using of 6-methylprednisolone. The clinical picture was the same in a patient with neuroblastoma accompanied by local relapse and hepatic metastases treated with CEV (carboplatin/etoposid/vincristine) cycles, IFN α and isotretinoin in

combination. The patient's signs of side effects (erythematous subcutaneous nodules, fever) ceased after isotretinoin withdrawal and 6-methylprednisolon treatment. The symptoms observed in connection with treatment of these two patients fit for Sweet syndrome.

DISCUSSION

The effect of IFN α -2b was investigated on clonal growth, apoptosis and expression of certain cell surface molecules, causing phenotypic changes in three B-cell leukemia/lymphoma cell line, the EBV-infected, immortalized JY, the EBV-genome free BL-41 and the HHV-8 genome positive BCBL-1 leukemia/lymphoma cell lines. In the investigated 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 leukemia stem cells as well as on their progeny undergoing terminal cell divisions. The suppression of colony formation was accompanied by an elevation in the proportion of apoptotic JY and BL-41 cells. IFN α alone did not result in a complete inhibition of colony formation, nor in 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 escaping programmed cell death induced by IFN α .

There are no data in the literature about the effect of IFN α to induce inhibition of apoptosis in human B-cell leukemia/lymphoma cell lines. Similar to our results, Trubiani et al observed induction of apoptosis elicited by IFN γ -treatment in differentiated human B-cell lines.

Complete suppression of both primary and secondary colony formation by IFN α was observed in HHV-8-infected BCBL-1 cells. Here, an excess 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)-1 β , IL-10, IL-12, two macrophage inflammatory proteins belonging to the β -chemokine family, transforming growth factor- β 1 and viral IL-6. 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. 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. 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. In contrast to experienced in case of three leukemia/lymphoma cell lines IFN α -2b treatment prevented the in vitro spontaneous apoptosis of cord blood mononuclear cells. This new observation support that IFN α is a potent survival signal for mature B-cells.

In parallel with its anti-apoptotic effect on umbilical cord blood B-lymphocytes, IFN α -2b prevented the decrease in the proportion of CD32-positive, Fc γ RII-expressing cord blood B-lymphocytes maintained in culture. Parallel with these observations, Ruuth et al confirmed the apoptosis inhibition effect of IFN α on peripheral B-cells that could be blocked with a specific phosphatidil-inositol-3 (PI3)-kinase inhibitor, suggesting that the second messenger PI3 plays a key role in this process. Since the proportion of other subpopulations (T-lymphocytes, monocytes, CD34-positive primitive hematopoietic 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 α -2b also increased the manifestation of Fc γ RII in the leukemia cell lines, as proved by the elevation of mean CD32 fluorescence intensity in IFN α -2b treated cultures. The effect of IFN α proved to be unique since neither IFN γ , nor GM-CSF, nor SCF was able to elicit similar changes in the proportion of apoptotic cells and the level of Fc γ RII manifestation. Although an indirect action of inducing other B-cell surviving factors secreted by monocytes or T-lymphocytes, present in the mononuclear cell fraction by IFN α 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 Fc γ receptors on monocytes/macrophages and on megakaryocytic cells. IFN γ induces the rapid induction of specific protein complexes, called IFN γ response region (GRR) resulting in a robust increase in the transcription rate for the Fc γ RI gene. IFN α also was shown to induce the formation of similar complexes assembling on the GRR, yet the IFN α -induced complex failed to activate the transcription of Fc γ RI in monocytic cells. The increased expression level of Fc γ receptors observed myself was suggested to correlate with differentiation events and increased responsiveness to IFN α in B-cells.

Inhibition of clonal proliferation of leukemia cell and differential regulation of apoptosis in neoplastic and normal B-cells seem 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 leukemia 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.

Essential thrombocythemia (ET) is a clonal myeloproliferative disorder of unknown origin characterized by overproduction of platelet precursors. In contrast to acute and chronic myeloid leukemia, there are no cytogenetic or molecular genetic aberrations. The Philadelphia translocation [t(9;22) / bcr-abl translocation] is also negative. A recent study investigating 7 children with non-familial ET and one child with secondary thrombocytosis did not reveal alteration in either coding region, including the flanking intronic sequences of *TPO* and *c-mpl* genes suggesting that the primary defect might affect a different step in the process of response of megakaryocytes to thrombopoietin (TPO), possible at the level of signal transduction. The studying the clonal proliferation of hematopoietic precursors in adult patients helped to understand the pathophysiology of this disorder. Formerly Ash et al. verified spontaneous CFU-MK colony formation, later Juvonen et al. showed significantly elevated CFU-MK colony formation in adult patients by the use of suboptimal dose of PHA-LCM, similar to that was observed in other myeloproliferative disorders. Randi et al. investigating 5 children with ET reported on the lack of spontaneous BFU-E formation. Florensa et al. were the first to study CFU-MK and CFU-GM formation in children with ET. In contrast to Randi et al, they have identified 4/5 of their cases with spontaneous BFU-E. Spontaneous CFU-MK was noted in 4/5 cases and spontaneous CFU-GM in 0/5 cases. After proper stimulation with exogenous cytokines, mean BFU-E, CFU-MK and CFU-GM were 78.4, 6.6 and 36.4, respectively. Investigating adult patients with ET, Gugliotta et al. reported that very low concentration (1 and 10 U/mL) of IFN alpha-2a inhibited the CFU-MK growth.

In pediatric patients, the indications and types of treatment are less certain than in adults. In some asymptomatic cases, a watch-and-wait approach may be justified. In symptomatic patients, introduction of anti-platelet or cytoreductive therapy may be necessary. Alkylating agents such as busulfan or hydroxyurea may increase the risk of leukemic transformation. Long term use of low-dose aspirin has been shown effective in relieving symptoms, although the use of acetylsalicylic acid in the management of ET with high platelet count is controversial. More recently, anagrelide has been advocated for correction of elevated platelet counts both in adult and children with ET. The use of IFN has been

suggested as an alternative therapeutic option. Interferon-alpha was shown to directly inhibit TPO-induced megakaryocyte growth by suppressing TPO-induced signaling through induction of SOCS-1. As the proliferation of human megakaryocyte progenitors can be stimulated via TPO-independent pathways, an indirect inhibitory effect of type-I interferons on colony forming unit-megakaryocyte (CFU-Mk) growth cannot be ruled out. IFN is nonmutagenic and nonleukemogenic although flu-like side effects and the subcutaneous administration may limit its use. Major side effects, such as irreversible neurological damage, have been described only infrequently.

A symptomatic 3 year-old Caucasian girl who fitted the diagnostic criteria of the Polycythemia Vera Study Group is one of the youngest patients with ET published. In addition to the clinical presentation, erythroid and myeloid colonies of bone marrow-derived mononuclear cells were studied. The patient did not exhibit spontaneous colony formation. The number of induced BFU-E, CFU-E and CFU-GM formation was similar to the samples reported by Florensa et al. At the time of investigation there was no opportunity to characterize the effect of TPO on in vitro colony formation of the patient, however colonies grown in response to G-CSF, GM-CSF, SCF, PHA-LCM stimulation were negative for intracellular FVIII antigen. Similar to the adult samples, IFN α proved very efficient in inhibiting PHA-LCM-induced myeloid colony formation.

Based on the excellent in vitro effect and on the favorable in vivo clinical studies with adult patients, IFN α was introduced to treat our patient. There was a significant decrease in the platelet count and a resolution of clinical symptoms. The effect of IFN treatment seems to be long lasting, as the patient's platelet counts subsequently varied between 400 to 600 X 10³/ μ L even after having stopped the IFN treatment for 26 months.

Elevated expression of GPIIb, GPIX and P selectin, indicating in vivo platelet activation, were noted three months after having stopped IFN treatment. A repeated investigation after 12 months indicated normalization of in vivo platelet activation. Abnormal platelet activation has been demonstrated both in inflammatory conditions in vivo and after an exposure of purified megakaryocytes to exogenous stimuli in vitro. Type I interferon treatment was accompanied by decreases in P-selectin expression on platelets in patients with chronic hepatitis C suggesting that IFN stabilizes activated platelets. This observation suggests that an inhibition on platelet activation by IFN treatment may contribute to the beneficial effect in ET in addition to inhibition on platelet production.

We applied IFN α first to treat a greater group of patients containing 24 children in a cohort. Out of ET reviewed in detail, 23 children with recurrent or poorly responding neoplasms, i.e. a group of patients who had very poor outlook with respect to cure or even improvement. Therapy was applied as up-front treatment in a patient with JMML where excellent in vitro activity indicated the clinical application, as in ET, and based on the literature refer to adult cases in the child with colon adenocarcinoma after radical surgery resection, respectively. All the other patients were treated by IFN α because of macroscopic residual tumor after chemo- irradiation therapy, tumor progression or recurrence.

Despite the advanced nature of the underlying malignancies, 14 of 24 patients exhibited responses to IFN α therapy. The response of CD7-positive leukemic patients is of particular interest, since the CD7 antigen is expressed on immature hematopoietic stem cells that are not irreversibly committed to T-cell lineage. The leukemic transformation of this cell may cause a previously unrecognized leukemia syndrome that seems to be responsive to IFN α therapy. Complete or partial responses were also seen in children with ET, JMML, disseminated LCH, astrocytoma and neuroblastoma. In the latter cases favorable responses were noted when IFN α was applied after standard multimodality treatment, in partial remission, as maintenance therapy alone or in conjunction with 13-cis-retinoic acid. Similar to data from recent meta-analysis, we did not see merit of IFN α therapy in gastrointestinal cancer. Seven of the 14 responding patients survived. Six of the surviving patients show no evidence of disease activity, one of them is in very good partial remission.

IFN α therapy was well tolerated in the patients with only minimal to moderate side effects.

During the IFN α treatment all the patients had flu-like symptoms with fever varies in intensity and duration. Two children (8%) had pain in connection with s.c. injection. In one case had to stop IFN α treatment for a while because of fatigue, poor appetite and excessive weight loss.

In two children who received isotretinoin parallel with IFN α therapy, development of Sweet syndrome was observed, described originally by Sweet et al. as a rare disease in children characterized by neutrophil dermatosis. The exact pathomechanism of the syndrome is not known. Dysregulation of IL-1, IL-3, IL-6, IL-8, G-CSF and IFN γ may play a causative role. Characteristic histopathological changes are the nodular perivascular neutrophil infiltration, neutrophil karyorrhexis without evident vasculitis. Cutaneous eruptions, erythematous plaques and papules, high grade intermittent fever, flu-like symptoms localized

to the upper respiratory tract, conjunctivitis, iridocyclitis, arthritis and sterile organ inflammation (alveolitis, osteomyelitis, acute renal failure, hepatitis, pancreatitis, affecting of central nervous system) may occur. The syndrome often associated with hematologic neoplasia, solid tumors, immunologic and inflammatory diseases and the use of certain drugs including colony stimulating factors and all-trans retinoic acid therapy. The two cases are the first described Sweet syndrome in association with 13-cis-retinoic acid treatment in children depending on the induction of specific cytokine background through neutrophyl activation.

Beside its direct and immune-mediated anti-tumor activity, IFN α inhibits angiogenesis. The use of IFN α in hemangioendothelioma, metastatic hemangiopericytoma and Kasabach-Merritt syndrome is based on this latter effect. Between ages 1-5 70% regression and 30% stable disease can be achieved in cases of life-threatening hemangiomas. In infancy, where this disease is characterized by rare spontaneous regression, there was a favorable effect in 68% of cases. Anti-angiogenic effects of IFN α may contribute to an effective response in neuroblastoma, as showed by an animal model, although phase II human trials were not demonstrative. There are favorable results with IFN α treatment in cases of CD7-positive, undifferentiated acute leukemia, in case of JMML relapsing after allogenic bone marrow transplantation and advanced, therapy refractory or relapsing cases of Hodgkin's lymphoma, respectively.

In adults with ET there are a lot of clinical experiences with the use of IFN α , but not in children. There are a few reports about the IFN α treatment of PV both in the international and in the Hungarian literature. There are favorable clinical experiences with IFN α treatment of aggressive mastocytosis and disseminated LCH, similarly to our results.

A randomization of patients with osteosarcoma is on under the EURAMOS (European and American Osteosarcoma Study Group) study. Primary objective is to estimate the survival advantage and the efficiency of IFN α therapy as maintenance treatment modality in resectable, cisplatin-doxorubicin-methotrexate responsive cases. Several studies investigate the effectivity of IFN α therapy in pediatric brain tumors. Rajkamur et al. observed good responses in 5/9 cases of high grade (HG) gliomas, while other authors observed increasing in toxicity when used IFN α in combination with chemotherapy. The use of fibroblast-IFN was result in 36% remission rate in LG (low grade) astrocytomas. IFN α treatment was successful in the treatment of a T-cell lymphoblastic leukemic patient suffering hepatitis-B infection and liver dysfunction, not allowing continue consolidation therapy. So, IFN α treatment maybe an

alternative therapeutic modality -especially as maintenance therapy- in cases of pediatric ALL with hepatitis B or C infection, not allowing further chemotherapeutic treatment.

The therapeutic activity of IFN α can be potentiated by cytostatic drugs and biologic response modifiers, such as platinum compounds, nucleotide analog and antagonists, epipodophillotoxin derivatives, anthracyclin antibiotics, alkylating agents, and retinoic acid derivatives. Exploiting these positive interactions and carefully choosing the phase of cytostatic drugs to be applied in conjunction with IFN α may extend its use in childhood cancer where the reported experience is limited.

SUMMARY

Cancer is one of the most important factor of childhood mortality. Despite of recent advances, 20-30% of children with cancer still succumb to death due to their disease even in the 21st century. Promising results were obtained with biologic response modifiers which enhance anti-cancer defense mechanisms of the body and may help to reverse disturbed differentiation/maturation processes. Interferon-alpha (IFN α) is a well studied representative of biologic response modifiers however, its role in the pediatric oncology remains to be established.

I have performed a literature survey on anti-tumor effects and application of type-I interferons with a particular emphasis on rare childhood myeloproliferative disorders. Effects on proliferation and differentiation of IFN α were studied in vitro using leukemia/lymphoma cell lines of the B-cell lineage, umbilical cord blood-derived B-lymphocytes and bone marrow-derived mononuclear cells of a pediatric patient with essential thrombocythemia (ET). I have assessed the therapeutic application of IFN α in children with advanced cancer, associated with particularly unfavorable outcome.

IFN α has exerted a significant, dose-dependent inhibition both in primary and secondary colony formation of three leukemia/lymphoma cell lines of the B-cell lineage. The drug has enhanced, in parallel, programmed cell death. In contrast to leukemia/lymphoma cell lines however, IFN α has prevented spontaneous in vitro apoptosis in cord blood derived healthy B-lymphocytes. In parallel with the robust inhibition of clonal proliferation of myeloid progenitor cells, IFN α treatment resulted in a long-lasting partial remission in a child with ET. In addition, 14/24 children with advanced cancer exhibited a favorable therapeutic response (CR or PR) upon IFN α treatment. The mild side effects, observed in most cases,

allow the safe application of IFN α in pediatric patients. Severe complication, in form of Sweet syndrome, was observed only in two patients receiving IFN α therapy in combination with isotretinoin.

These results, together with the observations of other groups suggest that IFN α is a promising agent in certain forms of childhood cancer.

LIST OF OWN PUBLICATIONS

This thesis is based on the following publications

1. Kiss C, Kiss M, **Szegedi I**, Árvai K, Tóth J, Oláh É. Interferon-alpha therapy in children with malignant diseases: clinical experience in twenty-four patients treated in a single pediatric oncology unit. *Med Ped Oncol* 2002;39:115-119. **IF: 1.216**
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3. **Szegedi I**, Kiss C, Karászi É, Vámosi G, Szöllösi J, Kovács P, Benkő I. Differential regulation of umbilical cord blood and leukemic B cells by interferon-alpha (IFN- α): observations in cultured cells. *Pathol Oncol Res* 2006;12:159-163. **IF: 1.16**
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Additional publications

1. Karászi É, Kiss C, Jakab Z, **Szegedi I**, Hevessy Z, Kappelmayer J. Gyermekkori akut leukémiák vizsgálata sejtfelszíni markerek és ploiditás alapján. *Gyermekgyógy* 1998; 49:319-329.
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MAIN ORIGINAL OBSERVATIONS

- I was the first in Hungary to review the clinical picture, recent classification and therapy of the pediatric myeloproliferative syndromes, with a particular emphasis on the therapeutic use of IFN α .
- Together with my co-authors, I was the first to observe that IFN α -2b caused a dose-dependent inhibition of clonal proliferation of B-cell leukemia/lymphoma cell lines, in parallel with its pro-apoptotic effects, while in case of normal umbilical cord blood B-cells it caused a differential effect, inhibiting spontaneous in vitro apoptosis.
- Together with my co-authors, I was the first to observe that IFN α increased the expression of CD32 (FC γ RII) both on B-lymphocytes and lymphoblasts.

- Together with my co-authors, I was the first in Hungary to describe the case of a 3 year-old patient with essential thrombocythemia, I have characterized colony formation of the patient's bone marrow mononuclear cells and described in vitro and in vivo favorable effects of IFN α treatment.
- Together with my co-authors, I was among the first to observe favorable effect of IFN α treatment in a larger cohort of pediatric patients with severe, advanced forms of cancer.
- We were the first to observe development of Sweet syndrome in conjunction with IFN α and isotretinoin treatment.

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The peaceful circumstances at home and my children's patient assured this work.