Cytokines can reduce clonal, CD34-positive cells in acute myeloid leukemia in vitro

Abstract We studied the influence of cytokine mixes on the survival of acute myeloid leukemia (AML) bone-marrow (BM) cells in a 14-day culture assay in vitro. Southern-blot analysis using a panel of different probes in combination with densitometry and flow cytometry were used to detect and compare the amount of clonal or CD34-positive BM cells before and after the culturing procedure. A significant reduction of CD34-positive cells after incubation with a cytokine mix [interleukin (IL)-1β, IL-3, IL-6, stem cell factor (SCF), erythropoietin (EP) with granulocyte macrophage/colony-stimulating factor (GM-CSF, Cytok1) could be achieved in all 16 cases with a CD34-positive blast phenotype studied at diagnosis (P<0.001), in 3 of 10 cases at relapse, and in 8 of 18 cases in complete remission. In healthy donors, an increase of CD34-positive cells was demonstrated in 5 of 5 samples. A reduction of clonal DNA through incubation with Cytok1 was achieved in 5 of 5 (100%) cases studied at diagnosis, in 1 of 4 (25%) cases at relapse, and in 7 of 9 cases (78%) in complete remission. Cytokine cocktails with GM-CSF (Cytok1) were more efficient in reducing (clonal) CD34-positive cells than cocktails without GM-CSF (Cytok2). AML patients at diagnosis and in complete remission had a better survival probability if their CD34-positive or clonal cells could be reduced in vitro by cytokine cultivation (P<0.05). Vitality of BM cells was not influenced by 14-day cytokine treatment; however, the total cell count could be increased by Cytok1 and Cytok2 by 55–174%, but not by the control medium. Our data show that: (1) clonal cell populations can be regularly detected at diagnosis, during complete remission, and at relapse; (2) CD34-positive cells in AML can be demonstrated to be clonal, gene-rearranged cells; (3) incubation of AML BM-cells with Cytok1 leads to a reduction of the CD34-positive, clonal cell load in all cases at diagnosis and in 78% of the cases in complete remission of AML, but in only 25% of the cases at relapse; (4) in all healthy BM samples, proportions of ‘healthy’ CD34-positive cells were increased. Moreover, absolute cell counts were increased by cytokine incubation of cells obtained at diagnosis, relapse, or complete remission of AML and from healthy donors indicating a selective stimulation of healthy, but not of leukemic CD34-positive cells; (5) cytokine cocktails containing GM-CSF are more efficient in reducing leukemic cells than cocktails without GM-CSF; and (6) in vitro reactivity of clonal or CD34-positive BM cells against Cytok1 has clinical relevance. We conclude, that Southern-blot analysis and flow cytometry are suitable methods to detect and quantify leukemic disease and to distinguish between clonal or non-clonal CD34-positive cells. The ex vivo or clinical application of specific combinations of cytokines might be a feasible and successful application of immunotherapy in AML that merits further investigations.

Key words Southern blot · Clonality · AML · Cytokines · Residual disease · Flow cytometry

Introduction

Acute myeloid leukemia (AML) results in accumulation of leukemic blasts through clonal proliferation of an abnormal progenitor cell. These blasts are morphologically and biologically homogeneous [1]. Leukemic
cell populations can be identified by flow cytometry using a panel of different antibodies [2]. Most of the AML cases show a CD34-positive blast phenotype. Clonal cell populations can be identified by cytogenetics, polymerase chain reaction, or Southern-blot analysis. Clonal bone-marrow (BM) cells, rearranged in the T-cell receptor, in immunoglobulin genes, in growth factor genes [granulocyte macrophage/colony-stimulating factor (GM-CSF), G-CSF, interleukin (IL)-3], or in M-bcr genes can be detected in about 50% of the AML cases [3–9]. Gene rearrangements of the retinoic acid receptor alpha (RARα) gene are typical of promyelocytic leukemia (AML-M3). Therefore, gene rearrangements can be used to study the presence and amount of clonal, gene-rearranged cells in the course of the disease [10].

About 70% of patients with AML in complete remission relapse in the following 2 years. Therefore, residual leukemic cells must have survived [11]. In patients who do not relapse, mechanisms may exist that inhibit or even eradicate those leukemic cells. Several cytotoxic mechanisms, mediated for example by natural killer cells, are known to suppress leukemic growth in vivo [12]. In addition to cellular mechanisms, several soluble factors seem to play a role in the suppression of leukemic cells. Until now, animals have been treated in some trials using several cytokines such as IL-2 or interferon-alpha (IFNα) in order to find tumor-toxic factors or to test the efficiency of factors in restoring normal BM cells [13, 14]. Moreover, IL-3, GM-CSF, or G-CSF regulate the proliferation of leukemic cells [15].

Based on preclinical data showing synergistic effects of stem-cell factor (SCF), GM-CSF, IL-3, IL-6, and erythropoietin (EPO) on the proliferation of healthy hematopoietic progenitor cells, we investigated the influence of combinations of growth factors including SCF on the survival of CD34-positive AML cells in vitro [16]. Moreover, the influence of cytokines on the proliferation and differentiation of granulocytes (CD15-positive cells), on CD19-positive B cells, and on CD3-positive T cells was studied. Presence of persisting clonal cells was proven by Southern-blot analysis in cases with a gene rearrangement at diagnosis. The clinical relevance of our in vitro studies is demonstrated. This data may be important with regard to the biological features of AML cells and for the clinical application of cytokines to restore or keep stable remissions in AML.

**Patients and methods**

**Patients**

Cultured and uncultured BM samples from 16 patients at diagnosis of AML, 17 patients in complete remission, and 10 patients at relapse according to cytological and cytochemical criteria were examined. All patients were previously untreated and entered at the time of initial therapy. Patients were treated according to approved therapy standards of the European Organization of Research on Treatment of Cancer (EORTC). Complete remission was determined to be achieved when the BM was normocellular, containing less than 5% blasts, and when neutrophil granulocytes in peripheral blood (PB) had recovered to 1500/μl and platelets to 100,000/μl according to Cancer and Leukemia Groups (CALGB) criteria [17]. Relapse was diagnosed when the BM contained 25% leukemic blasts or when leukemic cell infiltration occurred at any other site. As a control, BM cells obtained from five healthy BM donors were studied.

**Cell preparation**

BM cells were obtained after informed consent by aspiration from the posterior iliac crest of the patients and were collected in preservative-free heparin. Mononuclear cells (MNCs) were obtained from BM cells by Ficoll density gradient (density 1.077, Seromed) centrifugation and were then washed in Hank’s balanced salt solution with NaHCO3 (Seromed).

**Culture of BM cells with cytokine cocktails**

BM-MNCs of AML patients obtained at different times in the course of their disease and BM-MNCs from healthy BM donors were cultured in cytokine-containing media for 14 days at 37 °C and 5% CO2 in a humidified atmosphere. Medium (± GM-CSF (Cytok1, Cytok2), IS/CFS) was changed once to twice per week. Cell counts adjusted to 0.5–1.0 × 10⁶ cells per ml medium. We used two different cytokine cocktails and a control [16]:

1. IL-1β (10 ng/ml, Boehringer-Ingelheim) + IL-3 (20 U/ml, Sandoz) + IL-6 (100 U/ml, Sandoz) + EPO (IU/ml, Boehringer-Mannheim) + SCF (100 ng/ml, Amgen) + GM-CSF (100 ng/ml, Sandoz) in Iscove’s modified Dulbecco medium (GibcoBRL) and 20% fetal calf serum (FCS, HyClone)

2. Had the same composition as Cytok1 without GM-CSF

3. ISC/FCS. Control medium without added cytokines; Iscove’s modified Dulbecco medium containing 20% FCS

**Surface marker analysis**

Flow cytometric analysis was performed on uncultured and cultured BM-MNCs in order to estimate the percentage of blast cells (positive for CD34), granulocytes (positive for CD15 and CD65), T (positive for CD3) and B cells (positive for CD19 and CD20) [2]. Antibodies conjugated with fluorescent dyes [phycocerythrin (PE) or fluorescein isothiocyanate (FITC)] were used: CD34 (class III clone 381, PE-labeled; Immunotech), CD15 (FITC or PE-labeled; Sigma), CD65 (FITC-labeled; Ortho), CD19 (PE-labeled; Ortho), CD20 (PE-labeled, Dako), or CD3 (FITC-labeled; Ortho). Blast phenotypes at diagnosis were regularly evaluated using a panel of different leukocyte antibodies in combination according to consensus protocols [2]. In the further course of AML, the patient’s typical antibody combinations – including CD34 antibodies in combination with pan-myeloid (e.g., CD13, CD33) and pan-leukocyte antibodies (CD45) – were used to quantify CD34-positive cells. To avoid unspecific or epitope class-specific variations of antibody binding, always the same CD34 antibodies from the same company were used. Analyses were performed using a flow cytometer (Cytoron Absolute, Ortho Diagnostic Systems). Data were evaluated using special software (ImmunoCount 2) from Ortho Diagnostic Systems. The percentage increase or decrease of cells being positive for the antibodies mentioned above before and after the cytokine treatment was evaluated.