Growth inhibitory effect of the antibody to hematopoietic stem cell antigen CD34 in leukemic cell lines*

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Growth-inhibitory and proapoptotic effects of the monoclonal antibody to CD34 molecule, clone 4H11, were tested in CD34+ leukemic cell lines (MOLM-9, JURL-MK1, HEL) and CD34- cell lines (PS-1, ML-2 and CTV-1). We have found that the monoclonal antibody to CD34 inhibited the proliferation and induced apoptosis of all CD34+ cell lines. We did not observe induction of differentiation by anti-CD34 antibody, but a growth arrest of cells in the G0/G1 phase of the cell cycle was detected in all the cell lines studied. Combinations of anti-CD34 antibody with both type I (α, β) or type II (γ) interferons did not enhance the effects on the cell growth or inhibition of cellular proliferation of the antibody alone. Our data suggest that the monoclonal antibody to CD34 molecule prepared from clone 4H11, after sufficient experimental and preclinical testing on laboratory animals, may provide a new basis for targeted antibody therapy of acute or chronic myeloid leukemia.

Key words: monoclonal antibody therapy, CD34 antigen, leukemia cell lines, proliferation, apoptosis, cellular senescence

The precondition for antibody therapy of tumors is the ability of antibody to react with the specific target molecules expressed on the surface of tumor or leukemic cells and the ability to induce either direct or immune system mediated cell death or inhibit the proliferation by activation of cellular differentiation program or by induction of apoptosis or senescence. Attempts to treat tumors by antiidiotypic antibodies to membrane immunoglobulins in chronic lymphocytic leukemia (CLL) or to T-cell antigens in T-lymphoma were performed already in early eighties of the last century [1, 2, 3]. Unfortunately, there were problems with the immune response of the host to the therapeutic antibody and with the unability of mouse antibodies to activate human complement and the effectors of the immune response. A further problem was the antigenic modulation. Recently, the progress in construction of both chimeric and recombinant antibodies using the phage liberaries first enabled the initiation of relevant clinical studies aimed at the targeted therapy of human tumors including leukemias and malignant lymphomas using of monoclonal antibodies. One of the first antibodies introduced to the clinic of CLL and non-Hodgkin's lymphomas was the chimeric monoclonal antibody to B-cell antigen CD20 rituximab (Mabthera). This antibody consists of mouse Fab part and human Fc part of immunoglobulin IgG, which is able to activate human complement. It selectively kills cells expressing the membrane antigen CD20 by induction of apoptosis, probably by a mechanism of complement mediated cytotoxicity (CDC) or antibody mediated cytotoxicity (ADCC). This antibody is recently used not only in the treatment of malignant lymphomas and CLL of B-cell type, but also for the treatment of autoimmune diseases, e.g. rheumatoid arthritis. Another monoclonal antibody used in the clinic of acute myeloid leukemia (AML) or promyelocytic leukemia (APL) is targeted to the myeloid surface membrane antigen CD33. This antibody is conjugated to anthracyclin cytostatic agent calicheamicin. Treatment of AML by this antibody is however limited. Therefore we concentrated our effort on the possibilities for the development of new therapeutic antibodies targeting leukemic stem cells. One of the candidates of antibody reactive with the leukemic stem cells of some patients with AML and CML is the antibody to the surface

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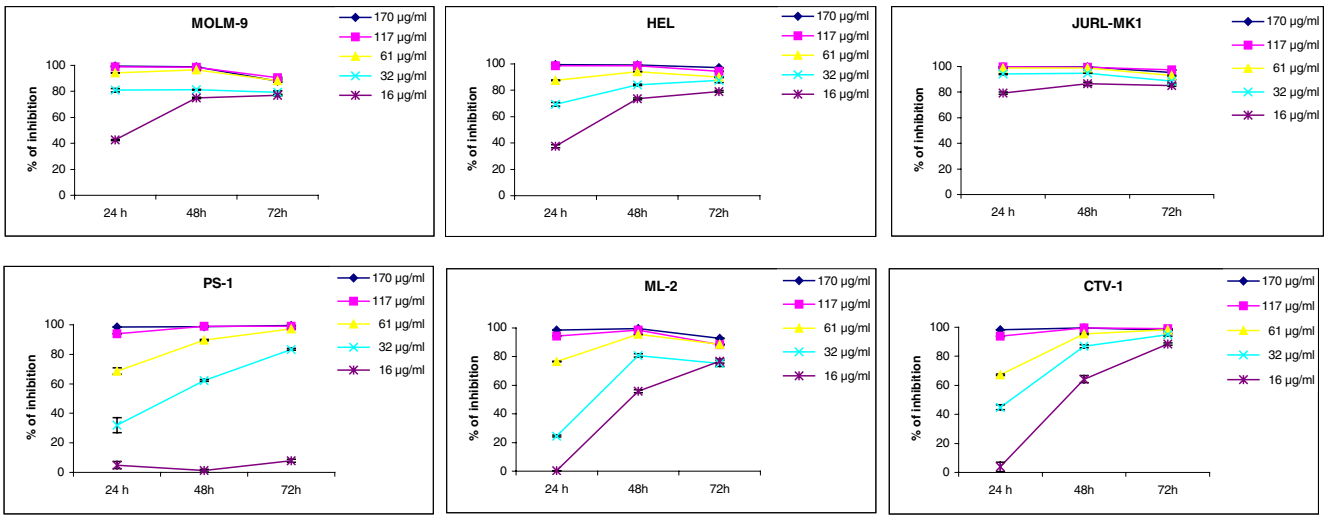


Figure 1. Inhibition of proliferation of CD34+ cell lines (MOLM-9, HEL, JURL-MK1) and CD34- cell lines (PS-1, ML-2, CTV-1) after treatment with 4H11 antibody. Cells were incubated in the presence of 16 –170 µg/ml of purified immunoglobulin from supernatant of mouse monoclonal antibody to CD34 molecule (clone 4H11) for up to 72 h, and the cell aliquots in quadruplicates were assayed on proliferation with ^{3}H-thymidine for 4 h. The cells were harvested and the ^{3}H-thymidine radioactivity was measured in counts/min (cpm). Results are expressed as the mean of three experiments.

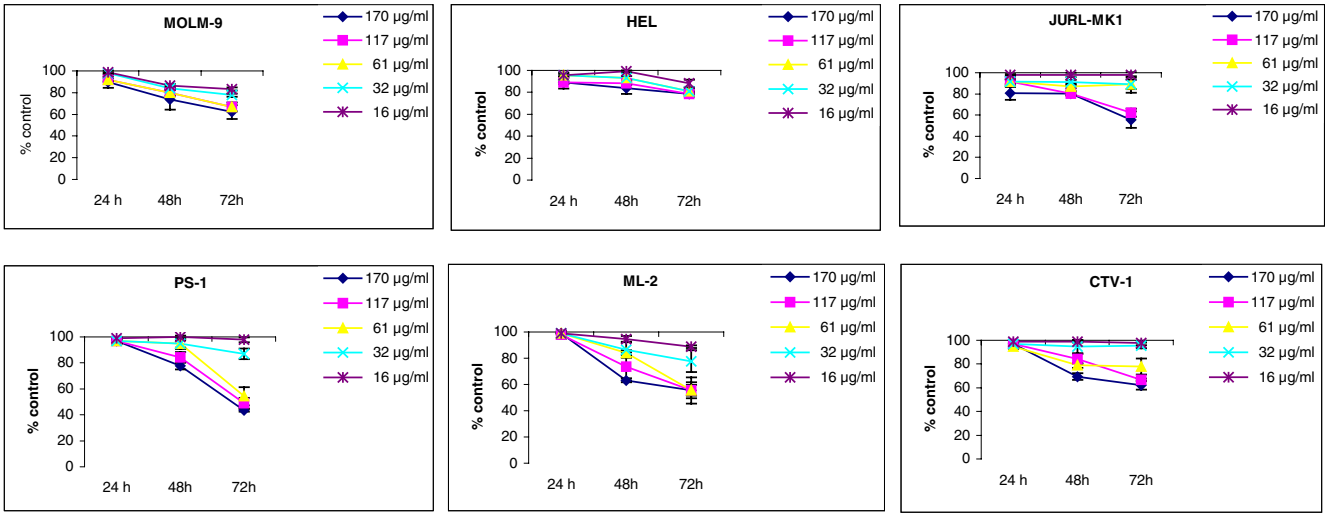


Figure 2. Viability of CD34+ cell lines (MOLM-9, HEL, JURL-MK1) and CD34- cell lines (PS-1, ML-2, CTV-1) after treatment with 4H11 antibody. Cells were incubated in the presence of purified imunoglobulin from hybridoma cell line 4H11 (anti-CD34) in concentrations of 16 – 170 µg/ml for up to 72 h. Viability was assessed by Trypan blue exclusion test. Results are expressed as percentage of untreated control cells and represent mean of three experiments.

molecule of normal hematopoietic stem and progenitor cells, CD34. We have previously prepared a monoclonal antibody to this antigen by immunization of BALB/c mice with cells from the chronic myeloid leukemia cell line MOLM-7 [4]. After fusion of mouse spleen cells with SP2/0 mouse myeloma cell line, we obtained the hybridoma clone, which produced monoclonal antibody to hematopoietic stem and progenitor cells, clone 4H11(APG)(70066) of mouse IgG1 class. This antibody reacts with the majority of myeloid progenitor cells and cell lines (MOLM-6, MOLM-7, MOLM-9, HEL, KG-1) and detects the class III epitope of the CD34 molecule. Specifity of this antibody was defined at the 6th international workshop on the human leukocyte differentiation antigens [6]. Our results show that this antibody in purified form inhibits proliferation and specifically kills cells of CD34+ leukemic cell lines.

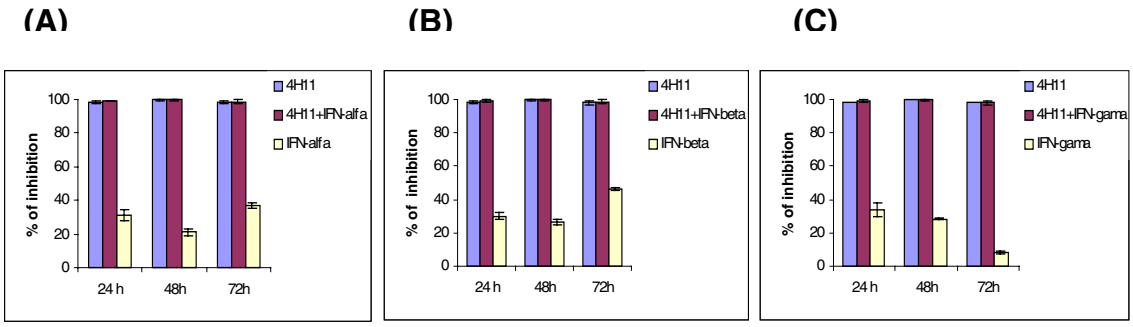


Figure 3. The effect of IFN-α, IFN-β, IFN-γ and purified immunoglobulin from the hybridoma clone 4H11 (anti-CD34) on the proliferation of MOLM-9 cells assayed by ^{3}H-thymidine incorporation. The cells were incubated in the medium containing IFN-α (0.075 μg/ml), antibody alone (61 μg/ml) and by both agent simultaneously (A), IFN-β (0.375 μg/ml), antibody alone (61 μg/ml) and by both agent simultaneously (B), IFN-γ (0.125 μg/ml), antibody alone (61 μg/ml) and by both agent simultaneously (C) for up to 72 hours. The cell aliquots in triplicates were incubated in the medium containing ^{3}H-thymidine for 4 h. The cells were harvested and the ^{3}H-thymidine radioactivity was measured. Results are expressed as the percentage of inhibition of proliferation (mean of three experiments).

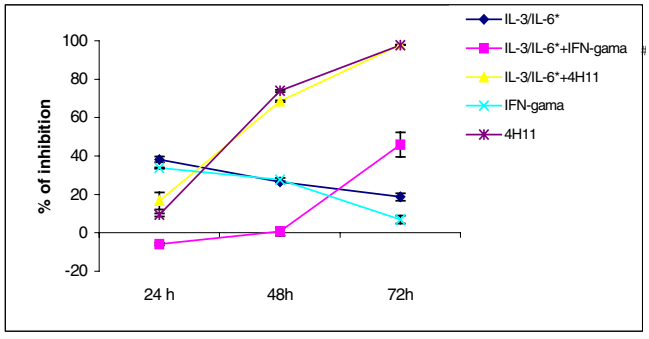
Material and methods

Reagents. Recombinant human interleukin-3 (IL-3), recombinant human interleukin-6 (IL-6), recombinant human granulocyte colony stimulating factor (G-CSF), recombinant human granulocyte-monocyte colony stimulating factor (GM-CSF), recombinant human stem cell factor (SCF) were purchased from BioSource Europe, S.A., Nivelles, Belgium. Recombinant human interferon-alpha-2c (IFN-α), recombinant human interferon-beta (IFN-β), recombinant human interferon-gamma (IFN-γ) were purchased from Boehringer Ingelheim, Ingelheim, Germany. Anti-human monoclonal antibodies CD14 (61D3), CD38 (HIT2) were purchased from eBioscience, San Diego, USA. Affinity purified mouse anti-human monoclonal antibody to CD34 molecule of IgG1 class was previously prepared on Prosep-A (Bioprocessing, Ltd., Scotland) from collected serum free culture medium of 4H11 hybridoma clone. Mouse anti-human monoclonal antibody to HLA-DR (B33.1) was gifted by Dr. Bice Perrusia, (Wistar Institute, Philadelphia, USA). RPE-conjugated F(ab')$_2$ fragment of goat anti-mouse immunoglobulins was purchased from DAKO, Glostrup, Denmark. The APO-BrdU™ TUNEL assay kit was purchased from Invitrogen, Carlsbad, California, USA.

Culture of cell lines. Myeloid (MOLM-9 [4], JURL-MK1 [5]), myelomonocytic (ML-2, CTV-1), lymphomyeloid (PS-1) and erytroid-megakaryocytic cell lines (HEL) were maintained in suspension culture in medium RPMI 1640 containing 10% fetal bovine serum (FBS), 150 mg/l L-glutamine, 50 000 U/ml penicillin and 50 mg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. The culture medium was changed three-times a week.

Detection of cell viability and proliferation. Cell lines were seeded and allowed to reach exponential growth for 24 hours. Cells were plated at the initial density of 2 x 10^5 cells/ml. Purified mouse IgG1 antibody to CD34, clone 4H11, was used in concentrations 170 μg/ml, 117 μg/ml, 61 μg/ml, 32 μg/ml, and 16 μg/ml in culture medium. In some experiments, also recombinant IFN-α (0.075 μg/ml), IFN-β (0.375 μg/ml), IFN-γ (0.125 μg/ml) or a coctail of recombinant cytokines consisting of IL-3 (1 ng/ml), IL-6 (10 ng/ml), SCF (5 ng/ml), G-CSF (1 ng/ml), GM-CSF (1 ng/ml) were added to growing cells to potentiate differentiation or to inhibit proliferation and induce cell death. After 24, 48 and 72 hours of drug treatment, adherent and non-adherent cells were harvested and viable cells were counted by trypan blue dye exclusion method (at least 200 cells were counted in each assay). Cell viability was calculated as the percentage of values of untreated adequate control cells.

Cell proliferation was assayed by measuring uptake (incorporation) of ^{3}H-thymidine. The treated cells as well as the appropriate controls in triplicates were pulsed with 24 kBq



* IL-3/IL-6 = mixture of IL-3+IL-6+G-CSF+GM-CSF+SCF

Figure 4. The effect of cytokines (IL-3, IL-6, SCF,G-CSF, GM-CSF, IFN-γ) and antibody from hybridoma clone 4H11 (anti-CD34) on proliferation of MOLM-9, cells assayed by ^{3}H-thymidine incorporation. Cells were incubated in medium containing IL-3 (1 ng/ml), IL-6 (10 ng/ml), SCF (5 ng/ml), G-CSF (1 ng/ml), GM-CSF (1 ng/ml), IFN-γ (0.175mg/ml) and antibody from clone 4H11 (61μg/ml) for up to 72 hours. The cell aliquots in triplicates were incubated with ^{3}H-thymidine for 4 hours.

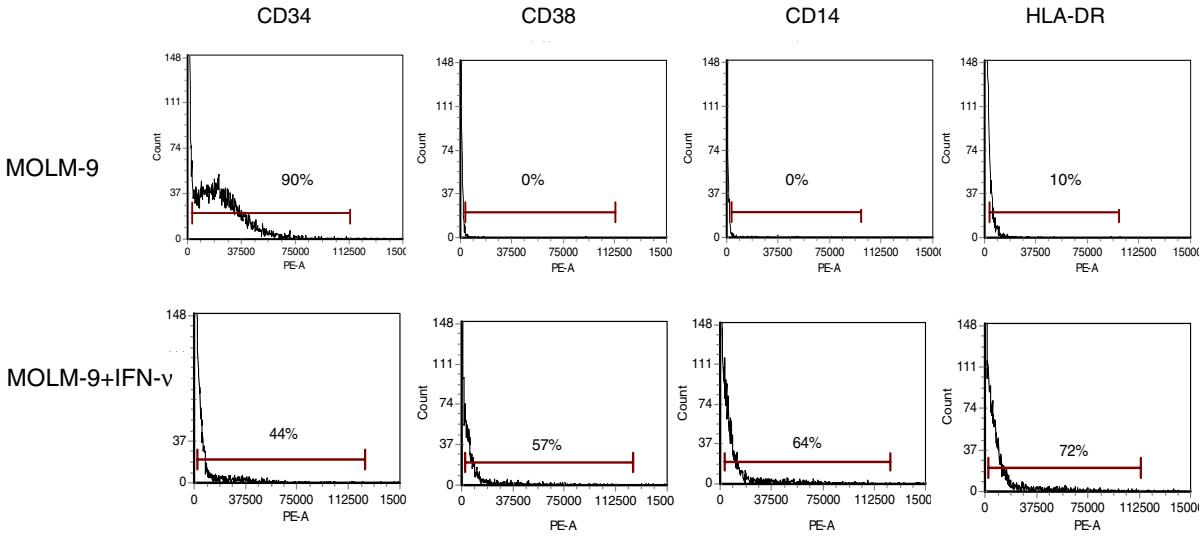


Figure 5. Expresion of CD34, CD38, CD14 and HLA-DR determined by flow cytometry in untreated and IFN-γ treated MOLM-9 cells after 72 hours of treatment.

Table 1. Expression of cell surface molecules of untreated, antibody, cytokines or both antibody + cytokine treated cells after 72 hours of induction (in percentage of positive cells detected by flow cytometry)

MOLM-9	CD34	CD38	CD14	HLA-DR
IL-3/IL-6*	89	0	0	7
IL-3/IL-6* + IFN-g	91	0	0	41
IL-3/IL-6* + 4H11	76	0	0	8
IFN-g	33	48	54	63
4H11	87	0	0	5
untreated control	88	0	0	8

* IL-3/IL-6 = mixture of IL-3+IL-6+G-CSF+GM-CSF+SCF

(6-^{3}H)-thymidine (Institute for Reasearch, Development and Aplication of Radioisotopes, Prague, Czech Republic) of the specific activity 980 (Gbq/mmol) for 4 h and the cells were collected using Scatron cell harvester. The incorporated radioactivity into the newly synthesized DNA was measured on the beta scintillation counter. The mean values of triplicate experiments expressed in counts per minute (cpm) were calculated. The inhibition of proliferation was expressed as percentage of cpm of cells that were treated in relation to untreated cells according to the formula:

Inhibition of proliferation [%] = [cpm (untreated) – cpm (treated)] / cpm (untreated) x 100]

Cell cycle analysis. MOLM-9, JURL-MK1, PS-1 cells (10^6 cells/ml) treated by 4H11 antibody (61 mg/ml) were collected by centrifugation after 24, 48 and 72 hours, suspended in 4.5 ml of ice-cold 70% ethanol, incubated for 30 min at 10 °C and kept for 5-7 days at -20 °C. The samples were then washed once in PBS and incubated for 30 min at room temperature in 1 ml of the modified Vindelov's propidium iodide buffer (10 mM Tris, pH 8, 1 mM NaCl, 0,1% Triton X-100, 20 mg/ml PI and 10 K units of ribonuclease A). The fluorescence excited at 488 nm was then measured using Coulter Epics XL flow cytometer. Distribution of DNA during the treatment by 4H11 antibody was indicated by G1/G0-G2/M transition.

Assessment of apoptosis by TUNEL assay. The tunel assay was performed employing the APO-BrDU TUNEL Assay Kit following the standard manufacturer's protocol. Briefly, MOLM-9, HEL, JURL-MK1, PS-1 cells treated with 4H11 antibody (61 μg/ml) were collected after 24, 48 and 72 hours. The cells were washed by PBS, adjusted to a density of 1-2 x 10^6 cells/ml and fixed with 1% paraformaldehyde solution in PBS for 15 min. Subsequently, the cells were pelleted and permeabilized with the ice-cold 70% ethanol for 18 hours at -20 °C. To label DNA strand breaks, 1-2 x 10^6/ml cells were incubated with 50 μl of TUNEL reaction mixture for 60 min. at 37 °C. The extent of DNA labeling with Alexa Fluor 488 dye-labeled anti-BrdU antibody was determined by flow cytometry.

Morphological analysis. Cytospin preparations were made after 24, 48 and 72 hours of incubation of MOLM-9 cells with a coctail of recombinant cytokines IL-3 (1 ng/ml), IL-6 (10 ng/ml), SCF (5 ng/ml), G-CSF (1 ng/ml), GM-CSF (1 ng/ml) or with recombinant IFN-γ (0,125 μg/ml) in combination with 4H11 antibody or without.

Immunophenotype analysis. The expression of surface antigens on MOLM-9 cell line was determined by indirect immunofluorescence using a panel of monoclonal antibodies and RPE-conjugated F(ab')$_2$ fragment of goat anti-mouse

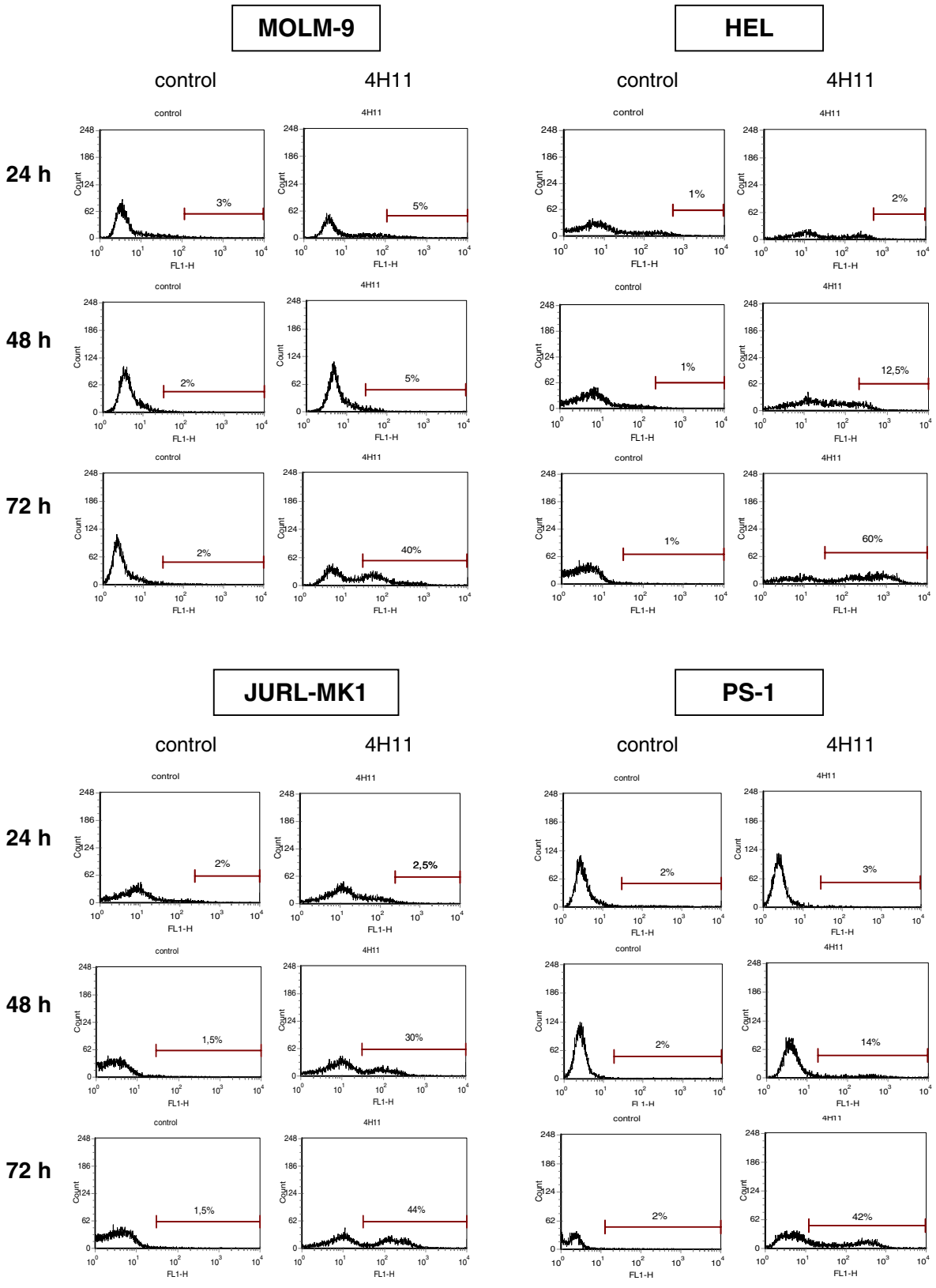


Figure 6. Flow cytometry analysis of MOLM-9, HEL, JURL-MK1, PS-1 cells induced to apoptosis by 4H11 antibody (61μg/ml). Fractions of apoptotic cells were determined by TUNEL assay after 24 h, 48 h and 72 h.

immunoglobulin as a secondary reagent. The following monoclonal antibodies were used: mouse anti-human CD14 (61D3), CD34 (4H11), CD38 (HIT2), HLA-DR (B33.1). The percentage of labeled cells was determined by flow cytometry.

Results

The effect of anti-CD34 antibody 4H11 on proliferation and viability of CD34+ cell lines (MOLM-9, HEL, JURL-MK1) and CD34- lines (PS-1, ML-2, CTV-1). As shown in the Fig. 1, the concentrations of antibody at 16 mg/ml and higher strongly inhibited the proliferation of all CD34+ cell lines. However the growth of CD34- cell lines was also inhibited, but only in the antibody concentrations at 32 mg/ml and higher. The strongest growth-inhibitory effect on all cell lines was detected after 72 hours of treatment.

The number of viable cells gradually decreased with the increasing concentration of antibody during the period of treatment. The effect of the antibody at the lowest concentrations (16-32 μg/ml) on the viability of CD34- cell lines (JURL-MK1, PS-1, CTV-1) was negligible (Fig. 2).

The effect of IFN-α, IFN-β, IFN-γ and antibody 4H11 on proliferation of MOLM-9 cell line. The effect of IFN-α, IFN-β, IFN-γ and of the antibody 4H11 alone on the growth of MOLM-9 leukemic cell line was examined. To assess the effect of IFN-α, IFN-β, IFN-γ and 4H11 antibody on the proliferation of MOLM-9 cells, we used ^{3}H-thymidine incorporation into the cellular DNA. As shown in Fig. 3, both IFN-α or IFN-β alone suppressed the cell growth of MOLM-9 (around 30-40%) compared to untreated control cells. IFN-γ also inhibited the proliferation of MOLM-9 cells but the inhibition of cell growth decreased (from 35% to 10%) within the period of treatment. The course of treatment of MOLM-9 cells by 4H11 antibody in combination with IFN-α or IFN-β or IFN-γ and by antibody 4H11was very similar. Both treatments progressively suppressed the growth of MOLM-9 cells.

The effect of cytokines and antibody 4H11 on cell growth and differentation of MOLM-9 leukemic cell line. We investigated the effect of various cytokines together with anti-CD34 antibody on cell growth of MOLM-9 cell line during the 72 hours of treatment. The possible induction of cell differentiation and inhibition of cellular proliferation by treatment with this agent alone and in combinations was also studied. Cellular differentiation was assessed by morphological analysis of Cytospin preparations and by the loss of expression of CD34 and by the appearance of membrane molecules of more differentiated cells such as CD14 and HLA-DR. Table 1 shows the expression of cell surface molecules on MOLM-9 cells after 72 hours of treatment. Levels of CD38, CD14 were strongly increased only after treatment by IFN-γ in comparison to untreated control cells. However, the expression of CD34 was down-regulated by IFN-γ (Fig. 5). Treatment with other agents did not influence the expression of CD38, CD14 and HLA-DR. By morphological analysis of Cytospin preparations (Fig. 8) and by flow cytometry, we did not find signs of either myeloid or lymphoid differentation. We observed a relative decrease of inhibition of cell growth during the treatment of MOLM-9 cells with the mixture of cytokines (IL-3+IL-6+SCF+G-CSF+GM-CSF) and after treatment of MOLM-9 cells by IFN-γ alone. The combined treatment of MOLM-9 cells by mixture of cytokines (IL-3+IL-6+SCF+G-CSF+GM-CSF+IFN-γ) did not influence the proliferation of MOLM-9 cells within 48 hours of culture, compared to the untreated control cells. The growth of MOLM-9 cells decreased after 48 and 72 hours of treatment. The treatment of MOLM-9 by mixture of recombinant cytokines (IL-3+IL-6+ SCF+G-CSF+GM-CSF) in the presence of 4H11 antibody or the treatment by 4H11 antibody alone inhibited proliferation (Fig. 4).

DNA fragmentation and Tunnel assay. We studied the effect of 4H11 antibody on the induction of apoptosis of CD34+ cell lines (MOLM-9, HEL, JURL-MK1) and CD34- cell line (PS-1). The number of TUNEL-positive cells gradually increased during 72 hours of treatment by 4H11 antibody compared to 2% in control cells (Fig. 6). The majority of TUNEL-positive cells (60%) was detected after 72 hours of treatment of HEL cells. The apoptosis of MOLM-9 cells was delayed up to 72 hours of treatment when the apoptosis of CD34- cell line PS-1 was also detected.

Cell cycle analysis. Induction of apoptosis in leukemic cell lines MOLM-9, JURL-MK1 and PS-1 was also studied by the cell cycle analysis. In the Fig. 7, a series of flow cytometry histograms show pronounced changes of DNA distribution that occured after treatment with 4H11 antibody. An increase of the percentage of cells in G1 phase in comparison with controls was detected in all cell lines treated by 4H11 antibody. It indicates that cells are arrested in the G1 phase of the cell cycle. An increased fraction of cells in subG1 phase (apoptotic cells) was detected during the treatment by 4H11 antibody compared to control cells in all the cell lines studied.

Discussion

Due to morphological and phenotypic heterogeneity observed in leukemia and also in other malignant tumors, two models of tumor etiology have been recently proposed [7, 8]. The stochastic theory postulates that cells within malignant tumors are relatively homogeneous and the genetic changes leading to the development and progression of malignancy are operative in all cells. Therapy to eradicate tumor can be directed to the bulk of the cell population. However, the newly proposed stem cell model (Fig. 9) postulates that a rare population of tumor-initiating cells is biologically and functionally distinct and only the cancer stem cell subset has the ability to proliferate extensively and to form new tumor cells [8, 9]. This model is substantiated by findings of Lapidot et. al [10] and Bonnet et al [11], who have shown that AML, like the normal hematopoietic system, is organized as a hierarchy of distinct, functionally heterogeneous classes of cells that are ultimately sustained by a small num-

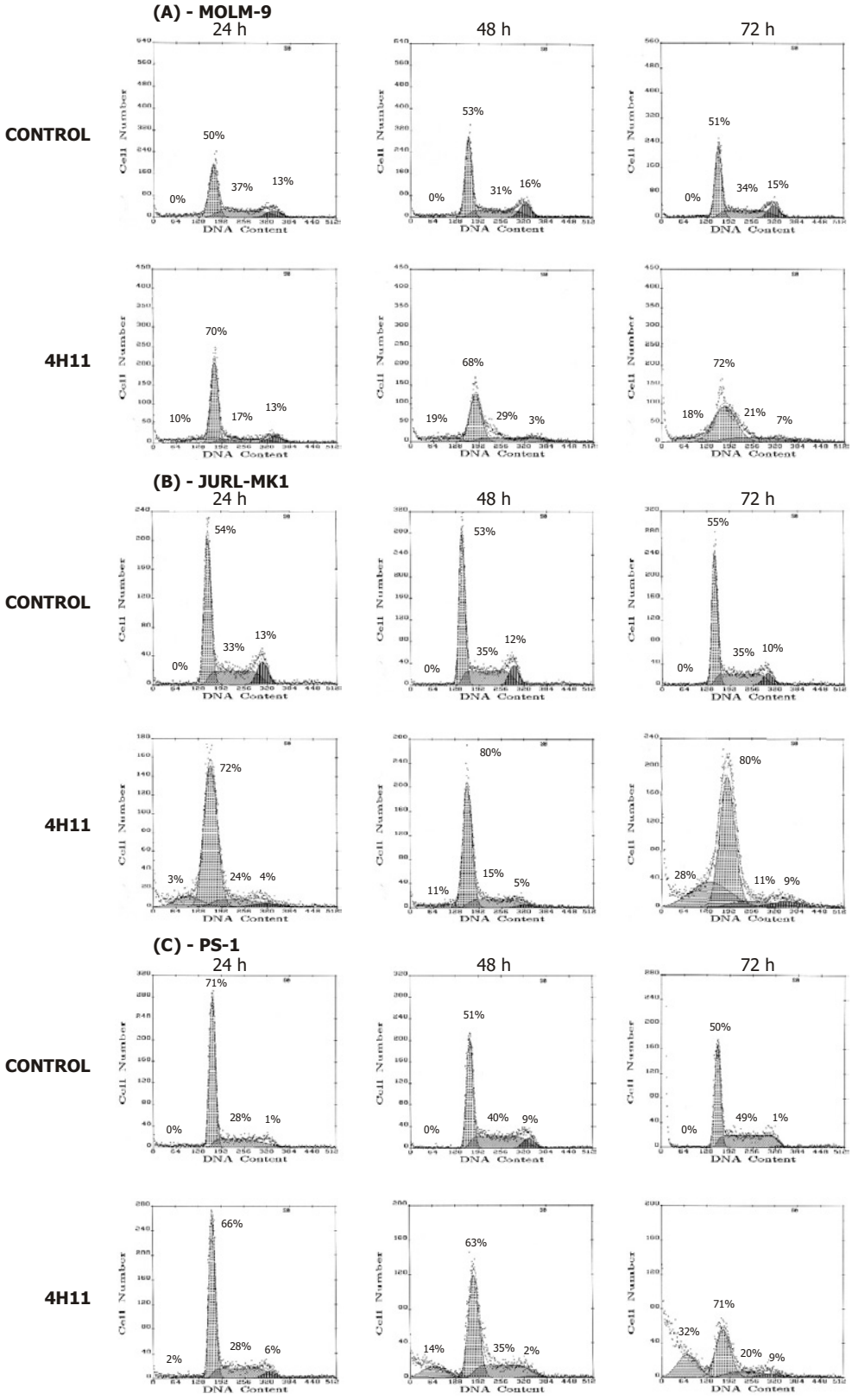


Figure 7. Cell cycle distribution obtained by flow cytometry analysis of MOLM-9 (A), JURL-MK1 (B) and PS-1 cells (C) after 24, 48 and 72 hours of treatment by 4H11 antibody.

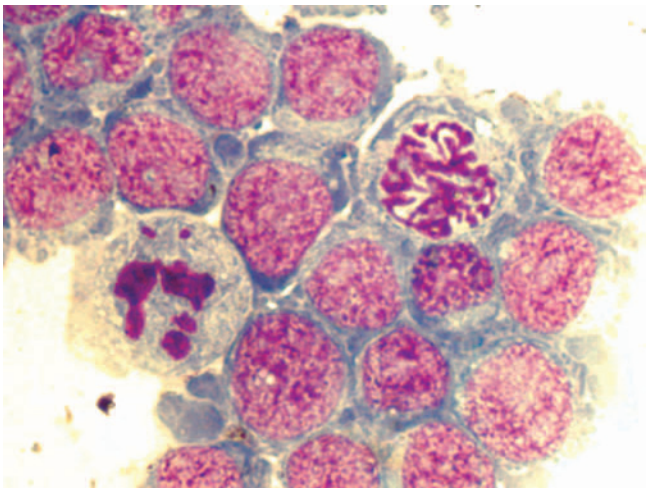


Figure 8. Smear of MOLM-9 cells treated by anti-CD34 clone 4H11 for 24 hours. Cells in mitosis transiting to apoptosis are characterized by chromosome and chromatin condensation.

ber of leukemia stem cells (LSCs). Also in CML, the property of self-renewal has been recently identified within the stem cell population of CML patients in the chronic phase disease and within the stem cell and myeloid progenitor population in patients in blast crisis [12]. LSCs have a primitive immunophenotype that is similar to normal HSCs (hematopoietic stem cells) in many respects. Both LCSs and HSCs express CD34 molecule but not CD38 [11]. However, Thy-1 (CD90) and c-kit (CD117) that are present on normal HSCs are not expressed on LSCs [13, 14] and the IL-3 receptor a chain (CD123) is unique marker for LSCs [15] (Fig. 9). Therefore the targeted therapy of leukemia may be focused and designed to specifically target these LSCs in case it effectively cures and prevents disease relapse. In context with these recent findings, we decided to target the leukemia stem cells in AML and CML with our previously developed monoclonal antibody to hematopoietic stem cell antigen CD34, clone 4H11, with the aim of possible eradication of the rare population of LSCs responsible for maintaining the disease.

In this study, we investigated the effect of purified immunoglobulin from hybridoma clone 4H11 of anti-CD34 alone or in combination with one or more recombinant cytokines (IL-3, IL-6, SCF, G-CSF, GM-CSF, IFN-α, IFN-β, IFN-γ) on proliferation, differentiation and induction of cell death of CD34+ and CD34- leukemic cells in the "*ex vivo*" system. The ultimate goal of this investigation is the development of a new therapeutic agent based on the antibody to the surface molecule of hematopoietic stem cells CD34. For this purpose, we have chosen several CD34+ cell lines, especially Ph1+ CML-derived cell lines (MOLM-9, JURL-MK1), erythroleukemia-derived cell line (HEL), in comparison to CD34- lymphomyeloid cell line (PS-1) and monocytic cell lines (CTV-1, ML-2). The mouse monoclonal antibody to CD34 molecule of IgG1 class, clone 4H11 (APG), was previously developed in our laboratory by fusion of mouse spleen cells immunized with cells of CD34+ cell line MOLM-7 with mouse myeloma cell line SP 2/0 and the purified antibody from the resulting hybridoma clone 4H11 (APG) was used in all experiments. This antibody inhibited proliferation and induced cell death of all studied CD34+ cell lines, however in the concentrations higher than 32 μg/ml exhibited a nonspecific antiproliferative and proapoptotic effect also on CD34- cell lines. As is shown in the Fig.1 and 2, the antiproliferative effect of the antibody on CD34+ cell lines is dose dependent and the optimal concentration of antibody which efficiently mediated inhibition of proliferation and induced the cell death determined as the percentage of trypan blue positive cells was at narrow range between 16 to 32 μg/ml. At concentrations lower than 16 μg/ml the effect was diminished (not shown), but at the antibody concentrations higher than 32 μg/ml the nonspecific inhibitory effect of antibody was prevalent. The cell cycle analysis revealed that after 24 hours of the antibody treatment, cells were arrested in the G1/G0 phase of the cell cycle (Fig.7). DNA fragmentation, another feature of the process related to apoptosis, was also detected (Fig. 6). The results obtained on the MOLM-9 cell line are similar to the results of Zada et al [16] who have shown the growth-inhibitory G1 arrest of anti-CD44 monoclonal antibody A3D8 or H90 on cells of myeloid cell lines. In their study, the percentage of apoptotic cells (cells in subG1 phase) also increased after treatment with anti-CD44 antibody. Charrad et al [17] reported induction of terminal differentiation and inbibition of proliferation of myeloid leukemia cell lines HL-60, THP-1 and NB4 after treatment by anti-CD44 monoclonal antibody A3D8 or H90.

Induction of differentation is generally associated with the cell cycle arrest and with the prolongation of G1 phase of cell cycle in differentiating cells. After treatment of MOLM-9 cells with 4H11 antibody alone, we did not reveal signs of either monocytic or granulocytic differentiation by monitoring morphology (Fig. 8) and expression of the surface antigen CD14 by flow cytometry. 4H11 antibody, however, inhibited the cell growth and arrested the cells in the G1 phase. Therefore, we suggest that 4H11 is able to induce cell senescence, characterized by cessation of cell division while retaining cell viability. Senescent cells are typically arrested in G1 phase of the cell cycle [18]. The mechanism of the direct cell death induction by this antibody in CD34+ cell lines, however, remains to be explained.

Type I IFNs (IFNs α/β) have shown a considerable promise in the treatment of many malignant tumors [19] and have been proven to be effective in several hematological malignacies including chronic myeloid leukemia [20] and hairy cell leukemia [21]. We have shown that both IFN-α and IFN-β suppressed the cell growth of MOLM-9 Ph^1+ chronic myeloid leukemia cells. This observation is in accordance with the finding of Benjamin et al., who reported antiproliferative activity of IFN-α and IFN-β against AML cell lines [22]. IFN-α (0.075 μg/ml)

and IFN-β (0.375 μg/ml) were more effective in the antiproliferative effect than IFN-γ (0.125 μg/ml). The combination of 4H11 antibody with IFN-α or IFN-β or IFN-γ did not have an enhancing effect and did not result in cell growth inhibition to a higher extent than did 4H11 antibody alone. The decreased expression of CD34 antigen detected after treatment by IFN-γ, as shown in Fig. 5, can be explained by the ability of IFN-γ to stimulate the expression of MHC-II and many other surface molecules of more mature cells such as CD38, CD14 and HLA-DR, compared with untreated controls. It has been shown earlier that IFN-γ inhibits proliferation and induces monocytic differentiation of acute myeloid leukemia cells and myeloid cell lines HL-60, ML-1 [23]. After treatment of MOLM-9 cells with a mixture of recombinant cytokines (IL-3+IL-6+SCF+G-CSF+GM-CSF) and with 4H11 antibody simultaneously, the proliferation rate decreased (Fig. 4). The growth of MOLM-9 cells in medium containing mixture of recombinant cytokines was partially inhibited, nevertheless, the growth-inhibitory effect of recombinant cytokines in combination with 4H11 antibody was the same as the treatment with 4H11 antibody alone. This suggests that 4H11 antibody inhibits cell proliferation by a different mechanism, because the cytokines alone or combinations of 4H11 antibody with recombinant cytokines did not show an enhanced antiproliferative effect.

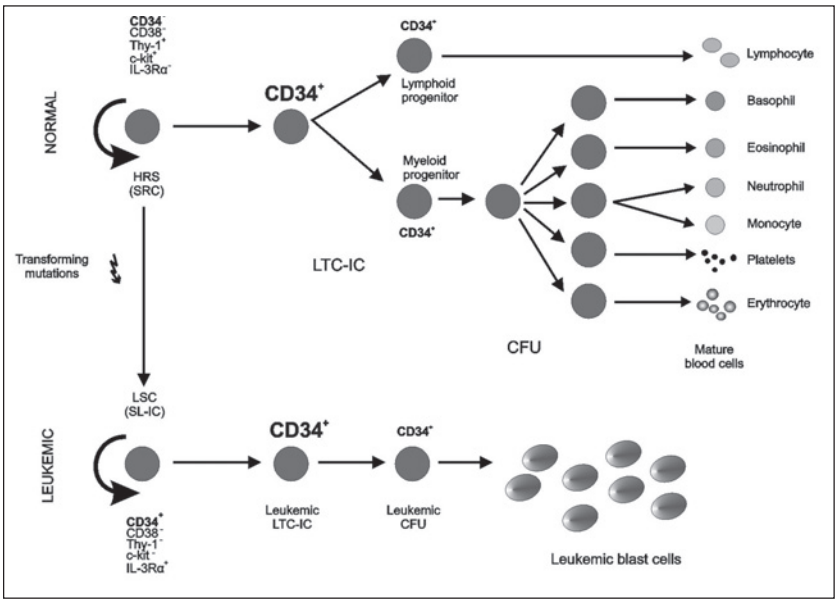


Figure 9. Scheme of the hierarchy of normal and leukemic hematopoietic cells. Human hematopoietic stem cells (HSC) are organized in a hierarchy that is sustained by a small population of self-renewal capacity LTC-ICs (long term culture-initiating cells), CFU (colony-forming units), which produce functionaly mature bood cells. Distruptions of pathways regulating self-renewal and differentiation by transforming mutations give rise to leukemia stem cells (LSCs). *In vivo* reconstitution assay immune-deficient mouse recipients enable detection of HSCs and LSCs as SCID-repopulating cells (SRC) and SCID leukemia-initiating cells (SL-IC) [9].

In conclusion, we demonstrated that the anti-CD34 antibody at concentrations of 16-32 μg/ml produced by clone 4H11 exhibits a strong antiproliferative effect on myeloid (MOLM-9, JURL-MK1) and erythroid-megakaryocytic (HEL) CD34+ cell lines, but only a weak or no antiproliferative effect was detected on CD34- lymphomyeloid cell lines. The proapoptotic effect of 4H11 has been observed in both CD34+ and CD34- cell lines. These results indicate that MOLM-9, JURL-MK1, HEL cell lines are suitable experimental models for laboratory studies of the mechanism(s) involved in 4H11-induced apoptosis and growth inhibition. Our data indicate the induction of G1/G0 arrest of MOLM-9 cells by 4H11 antibody, however without the induction of differentation. Furthermore, recombinant cytokines (IL-3, IL-6, SCF, G-CSF, GM-CSF, IFN-α , IFN-β, IFN-γ) do not significantly potentiate the growth-inhibitory effect of 4H11 antibody. Despite that the exact mechanisms of cell death induction and of the antiproliferative effect of this antibody remains unknown, this antibody (4H11), after sufficient preclinical testing on laboratory animals, may represent a new alternative to the spectrum of surface molecules chosen for targeted therapy of human myeloid leukemia. It might be especially useful in cases where therapy by gemtuzumab-ozogamicin fails, due to the late expansion of leukemic stem cells, which do not express the target antigen CD33, but do express the CD34 molecule. There are, however, still many problems to be solved. In the first place is the cross-reactivity of anti CD34 antibodies with other types of cells, mainly cells of nonhematopoietic origin such as endothelial and some neural cells. Also the possible damage to normal hematopoietic cells must be considered and carefully examined.

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