An antigen recognized on cells in apoptosis detected by monoclonal antibody 2E12*

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Monoclonal antibody 2E12 was prepared by immunization of mice with cells of a chronic myeloid leukemia cell line MOLM-7. Human hematopoietic cell lines JURKAT, HPB-ALL, RC2A and MOLM-7 were induced to receptor mediated apoptosis by the treatment with anti-Fas monoclonal antibody 7C11 and subsequently tested for reactivity with 2E12 antibody in comparison to staining with annexin V-FITC and PI in the two-color immunofluorescence and flow cytometry. After 2, 5, 24, and 48 hours of induction, a gradual increase of the percentage of 2E12 positive cells in all cell lines was observed, which partially correlated with an increase of annexin V-FITC binding with a delay of about 12 hours. In the two-color fluorescence microscopy the 2E12 antibody positivity was restricted to the annexin V positive cells, but their number was lower. The binding of 2E12 did not induce apoptosis nor influenced the binding of annexin V. We suppose that the antibody 2E12 detects an antigen expressed on a subpopulation of cells in death. Therefore it can be useful as a new marker for further dissection between living, apoptotic and necrotic cellular populations *in vitro*.

Key words: antibody 2E12, cell lines, apoptosis, anti-Fas, annexin V

Programmed cell death is an essential process in the development and regulation of tissue homeostasis. Failure of the cell death machinery contributes to the pathogenesis of many diseases and also to cancer. The prevalent form of programmed cell death, the apoptosis, is tightly regulated physiological process which is dependent on the expression of intrinsic-cellular suicide program machinery [3].

Apoptosis is morphologically characterized by cell shrinkage, chromatin condensation, fragmentation of the nucleus, and cleavage of chromosomal DNA at internucleosomal sites, resulting in the generation of characteristic ladder pattern of DNA fragments in electrophoresis. Nuclear fragmentation is usually followed by the formation of apoptotic bodies, which are *in vivo* phagocytosized by other cells [4, 10].

Two of several well characterized death receptors – CD95 (Apo-1/Fas) and tumor necrosis factor receptor-1 (TNFR-1) are known to function in the induction of apoptosis in lymphoid cells [1].

After binding of Fas ligand (FasL) to Fas, Fas assembles signaling complex called DISC (death inducing signaling complex) which involves a death domain – containing adaptor protein FADD (Fas associated death domain protein) and initiator of caspase 8 and caspase 10 [5].

Dependent on the cell type, Fas activation evokes two general activation pathways of effector caspases resulting in apoptosis [9, 11].

In the previous report we described a new monoclonal antibody (2E12), which was prepared by immunization of mice with cells of chronic myeloid leukemia cell line MOLM-7. We detected a new antigen expressed on cells at the late stage of cell death in cultured patient's cells and in the cell lines induced to apoptosis by camptothecin [2].

In presented report we studied the reactivity of monoclonal antibody 2E12 on T-cell lines JURKAT and HPB-ALL and on nonlymphoid cell lines MOLM-7 and RC2A, induced to apoptosis by monoclonal antibody anti-Fas (CD95) clone 7C11. We compared the kinetics of the appearance of the antigen 2E12 with the annexin V-FITC binding to apoptotic cells using the annexin V-Fluos test.

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Material and methods

Preparation of monoclonal antibody 2E12. Monoclonal antibody 2E12 was prepared by immunization of BALB/c mice with cells of the chronic myeloid leukemia cell line MOLM-7. Cellular population of MOLM-7 consisted of a high number of cells in the process of spontaneous cell death, which has been apparent during the entire cultivation period. After fusion of mouse spleen cells with SP 2/0 mouse myeloma cells, three hybridoma clones were obtained. One clone (2E12) produced this monoclonal antibody [2].

Cultivation of cell lines. T-lymphoid (JURKAT, HPB-ALL), myeloid (MOLM-7) and myelomonocytic cell lines (RC2A) were maintained in suspension culture in medium RPMI 1640 containing 10% fetal bovine serum (FBS), 150 mg/l L-glutamine, 50 000 U/ml penicilin and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. Medium was changed three-times a week. Cells were harvested in the exponential growth phase. Viability was assessed by the Trypan blue exclusion test.

Induction of apoptosis. Apoptosis was induced by mouse anti-human Fas monoclonal antibody clone 7C11 (Immunotech, Czech Republic) using the final antibody concentration 1 μg/ml in the cultivation medium. Cells were treated with anti-Fas for up to 48 hours, and harvested after 2, 5, 24, 48 and 72 hours. The untreated controls were cultured without anti-Fas under the same conditions. The cell number and viability was assessed by the Trypan blue exlusion test.

Reactivity of 2E12 antibody with induced and control cells. Total 1x10^6 harvested cells were washed in PBS and incubated with 2E12 antibody for 20 minutes at 4 °C. Then the cells were washed twice in PBS and stained by a second layer antibody labeled with the fluorochrome. As the secondary antibody either phycoerythrin (PE)-conjugated goat F(ab)'$_2$ anti-mouse immunoglobulins (DAKO, Denmark) or FITC-conjugated swine anti-mouse IgG antibody (SEVAC, Prague, Czech Republic) were used both in the fluorescence microscopy and flow cytometry experiments.

Annexin V fluorescence test. Both induced and control cells (1x10^6) were harvested, washed in PBS and centrifuged at 200g for 5 min. Cell pellets were suspended in 100 μl of staining solution (100 μl HEPES buffer, 2 μl propidium iodide and 2 μl annexin V-FITC labeling reagent) and incubated for 15 min. at room temperature. Distributions for annexin V-FITC and PI labeled cells were analyzed by flow cytometry and by fluorescence microscopy.

Pretreatment of cells with 2E12 antibody followed by annexin V assay. To assess an influence of 2E12 antibody on the distribution of living and dead cells the anti Fas induced and control cells were treated by 2E12 antibody for 20 minutes, washed twice in PBS, stained by using annexin V-FITC assay and analysed by flow cytometry and fluorescence microscopy.

Two color 2E12 (PE)/annexin V-FITC fluorescence test. Total 1x10^6 harvested cells were washed in PBS and incubated with 2E12 antibody for 20 minutes at 4C, washed twice in PBS and incubated for 20 minutes with a second layer antibody labeled with PE. Then the cells were washed twice in PBS and the cell pellet was resuspended in 100 μl of staining solution (100 μl HEPES buffer, 2 μl annexin V-FITC labeling reagent) and incubated for 15 minutes at the room temperature. Samples were analyzed both by flow cytometry and fluorescence microscopy.

Results

Comparison of the Trypan blue exclusion test with the expression of 2E12 antigen in the induced and control cells. Reaction of monoclonal antibody 2E12 and Trypan blue exlusion test were performed on the series of uninduced

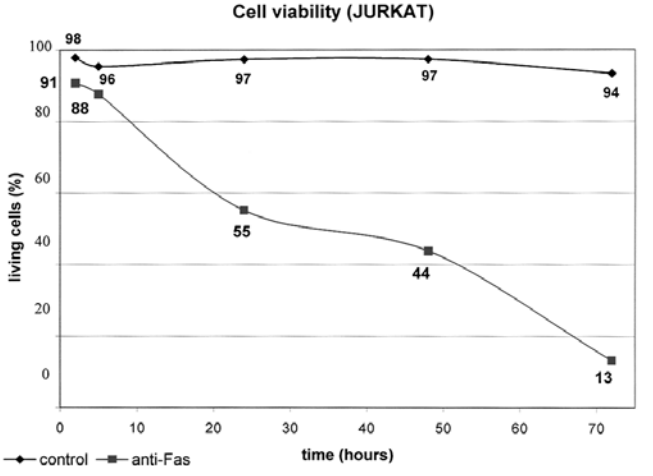

Figure 1. Cell viability of JURKAT cell line induced by anti-Fas and uninduced controls after 0, 2, 5, 24, 48 and 72 hours.

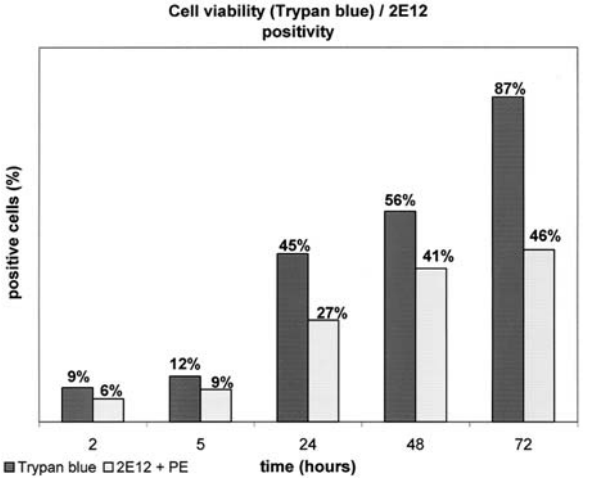

Figure 2. Cell viability assessed by Trypan blue exclusion test compared with positivity for 2E12 fluorescence of anti Fas (CD95) induced JURKAT cells.

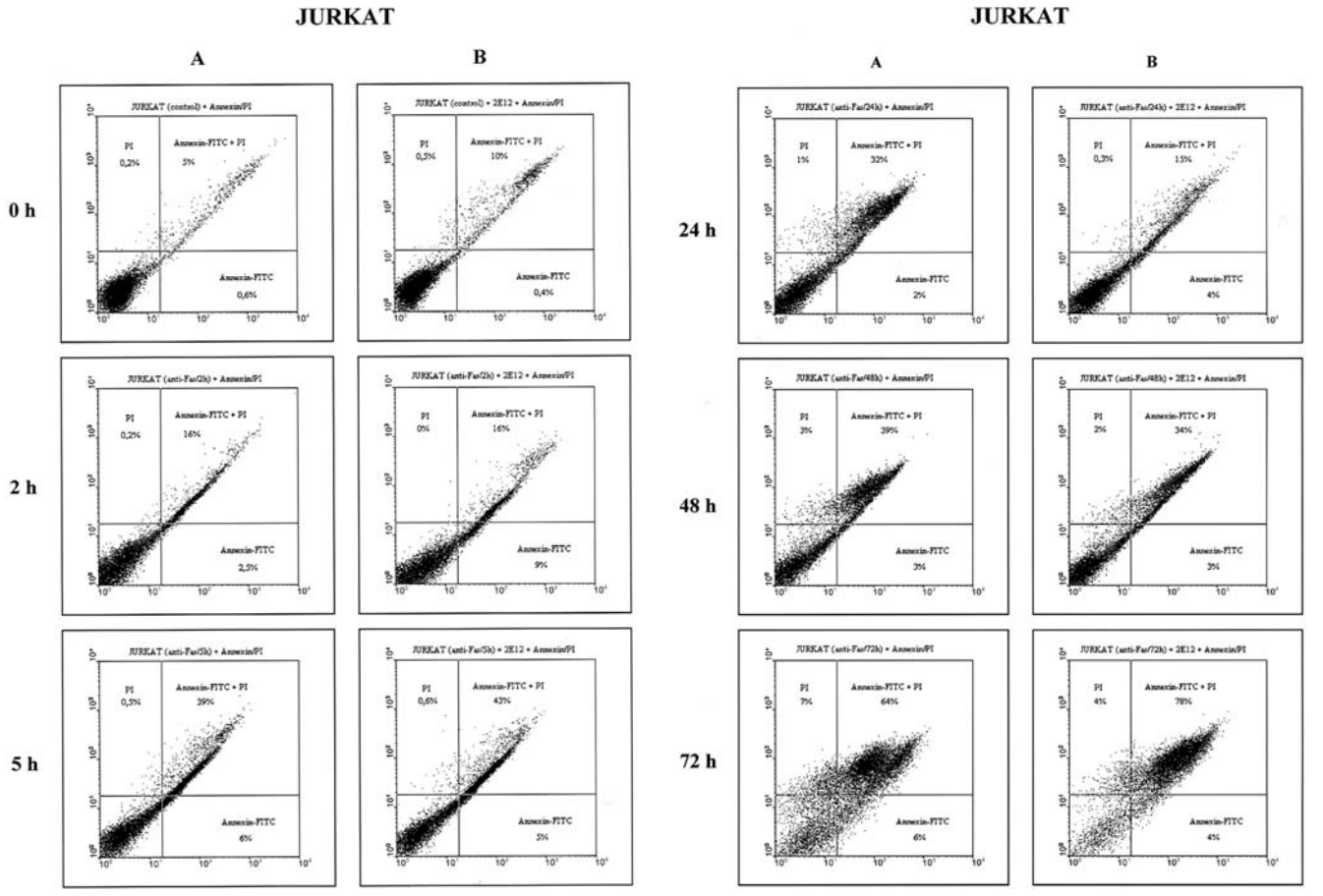

Figure 3. 1) Flow cytometry analysis of the annexin V-FITC binding to JURKAT cells induced by anti-Fas (clone 7C11)(log of green fluorescence intensity on the x-axis) versus binding of propidium iodide (PI) (log of red fluorescence y-axis) after 0, 2, 5 hours (left) and the same culture preincubated with antibody 2E12 before staining with annexin-V FITC (right). Preincubation of cells with 2E12 antibody did not influence the binding of annexin V to cells. 2) Flow cytometry analysis of JURKAT cells (as in Fig. 3.1) after 24, 48 and 72 hours.

and anti-Fas induced human hematopoietic cell lines (JURKAT, HPB-ALL, MOLM-7 and RC2A). Figure 1 shows that the viability of JURKAT cells assessed by Trypan blue exclusion test was rapidly but sequentially decreased with time in the anti-Fas (CD95) treated cells compared to untreated cells. Percentage of Trypan blue positive cells gradually increased (9%, 12%, 45%, 56%, 87%) with increasing time of anti-Fas treatment (2, 5, 24, 48 and 72 h). Figure 2 shows that the number of 2E12 positive JURKAT cells increased simultaneously with the number of Trypan blue positive cells, but it was always lower. 2E12 positive cells of all cell lines were morphologically in the process of cell death.

Results of the annexin V-FITC /PI test. The number of annexin V positive versus PI positive JURKAT cells after 2, 5, 24, 48 and 72 hours of apoptosis induction by anti-Fas antibody is shown in the Figures 3.1A and 3.2A, where x-axis shows intensity of green (annexin V-FITC) and y-axis of red (PI) fluorescence. Percentage of positive cells by both markers (annexin V-FITC and PI) gradually increased from 16% (2 h) to 64% (72 h). The untreated control cells contained 5% cells positive by both annexin V-FITC and PI. To assess an influence of 2E12 antibody on the induced and uninduced JURKAT cell population and to exclude the possibility that antibody 2E12 reacts with the same structure as annexin V-FITC, the aliquots of control and anti-Fas induced cells were preincubated with the 2E12 antibody for 20 min. at +4 ˚C and subsequently subjected to the annexin V-FLUOS test, as it is shown in the Figures 3.1B and 3.2B. Samples were analyzed both by flow cytometry and by fluorescence microscopy to confirm the cytometry results at a single cell level. Figures 3.1B and 3.2B also show that the preincubation of cells with the monoclonal antibody 2E12 and their subsequent staining by annexin V-FITC did not substantially alter the results obtained by annexin V-FITC/PI test alone. The preincubation of cells with the antibody 2E12 neither prevented binding of annexin V-FITC to apoptotic or necrotic cells, nor induced the cell death itself.

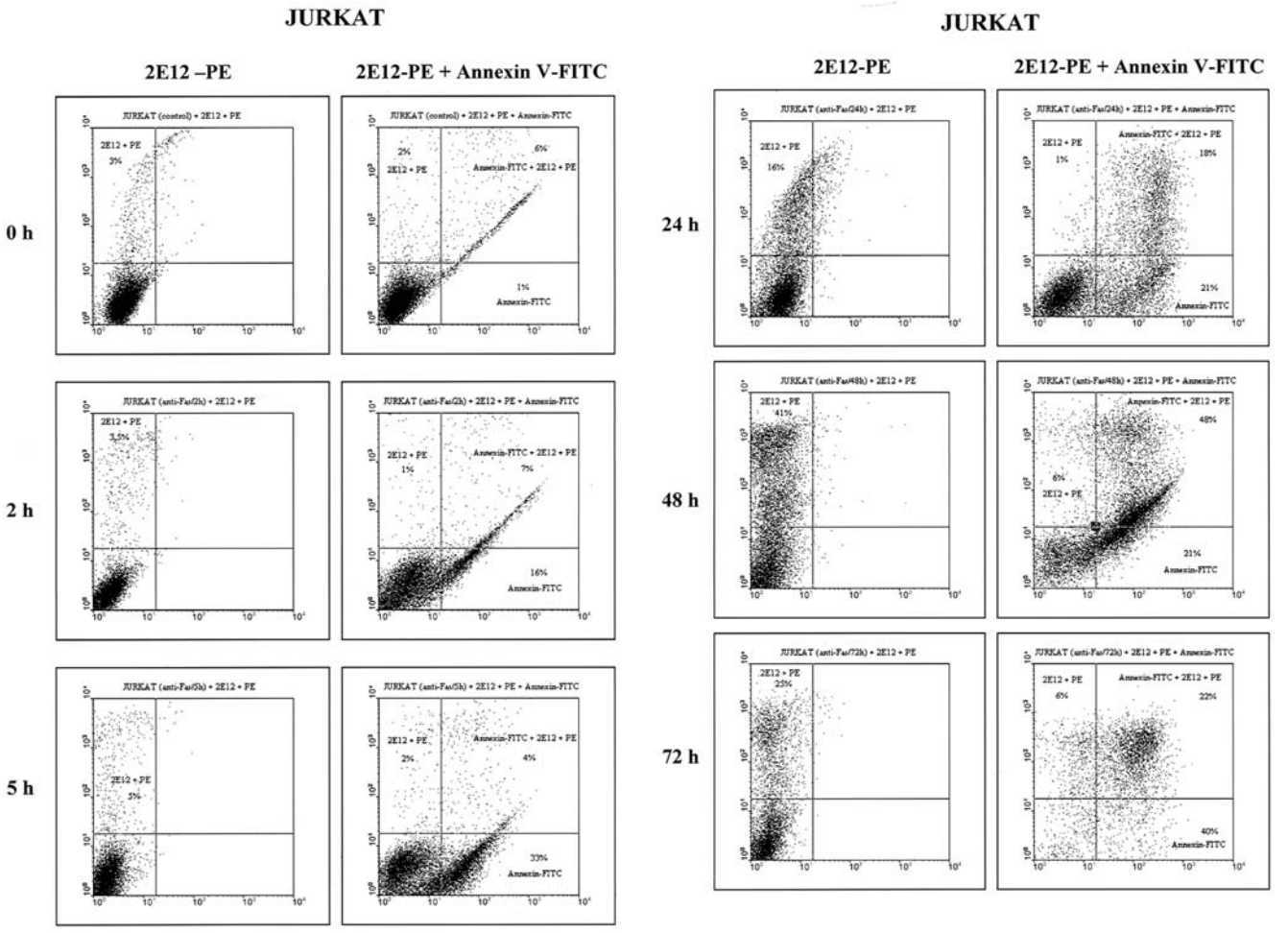

Figure 4. 1) Flow cytometry analysis of JURKAT cell line induced to apoptopsis by anti-Fas (7C11). Positivity of annexin V-FITC binding (x-axis) in comparison to positivity by 2E12 antigen expression (PE) (y-axis) after 0, 2, 5 hours. The cellular population can be dissected into 4 quadrants according to the positivity with one or both markers (annexin Vor 2E12). 2) Flow cytometry analysis of JURKAT cell line induced to apoptosis by anti-Fas (7C11). Positivity of annexin V-FITC binding (x-axis) in comparison to positivity by 2E12 antigen expression (PE) (y-axis) after 0, 24, 48, 72 hours of induction.

Two color fluorescence test for the simultaneous detection of 2E12/PE and annexin V-FITC positive cells. Reactivity of antibody 2E12 alone and together with annexin V-FITC was regularly tested on leukemic cells both by immunofluorescence and flow cytometry. Annexin V-FITC positive population during the anti-Fas treatment always gradually increased with the maximum after 24 hours of induction in HPB-ALL, MOLM-7, RC2A cells and after 48 hours of induction in JURKAT cells. The antibody 2E12 always reacted with a subset of annexin V-FITC positive cells, however an increase of 2E12 positive cells was always detected later with the maximum of 41% in JURKAT cells (Figs. 4.1 and 4.2) and 61% in the HPB-ALL cell line, which is highly sensitive to anti-Fas treatment. Results of two colour flow cytometry analysis of binding of monoclonal antibody 2E12 in combination with annexin V-FITC assay to HPB-ALL cell line are summarized in the Figure 5. Similar results were obtained in nonlymphoid leukemia cells lines MOLM-7 (21, 53, and 77% both 2E12 and annexin V positive cells) and RC2A (10, 59, 72 and 72% both 2E12 and annexin V positive cells), as shown in Figure 6. In the two color fluorescence microscopy we regularly observed only few or no double positive cells by both 2E12/FITC and PI (green fluorescence of 2E12 and red fluorescence of PI) in all cell lines tested as is shown in the Figure 7. Furthermore majority of cells positive by 2E12 antibody was simultaneously positive also by annexin V. The 2E12 positive cells, however, created frequently a subpopulation of annexin V positive cells, as is shown in flow cytometry (Fig. 5) and in the fluorescence microscopy (Fig. 8 and 9).

Discussion

We constructed the mouse hybridoma cell line producing monoclonal antibody 2E12 by immunization of mice with

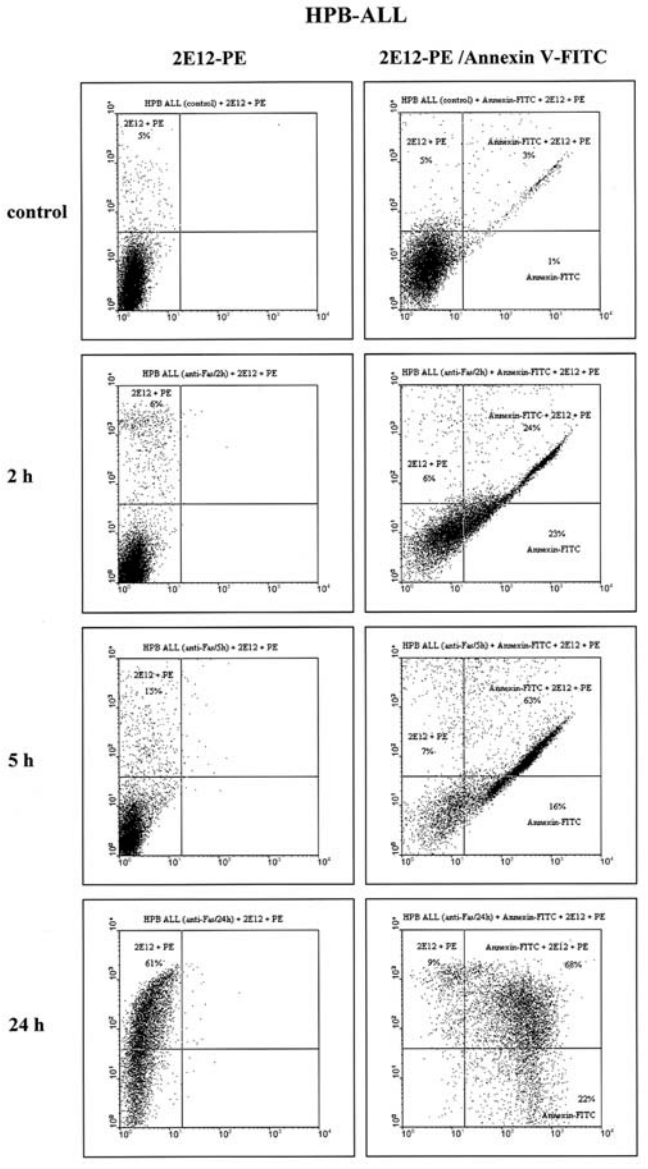

Figure 5. Flow cytometry analysis of HPB-ALL cells induced to apoptosis by anti-Fas (7C11). Positivity of annexin V-FITC binding (x-axis) in comparison to simultaneous positivity with 2E12 antibody (PE) (y-axis) after 0, 2, 5 and 24 hours of induction.

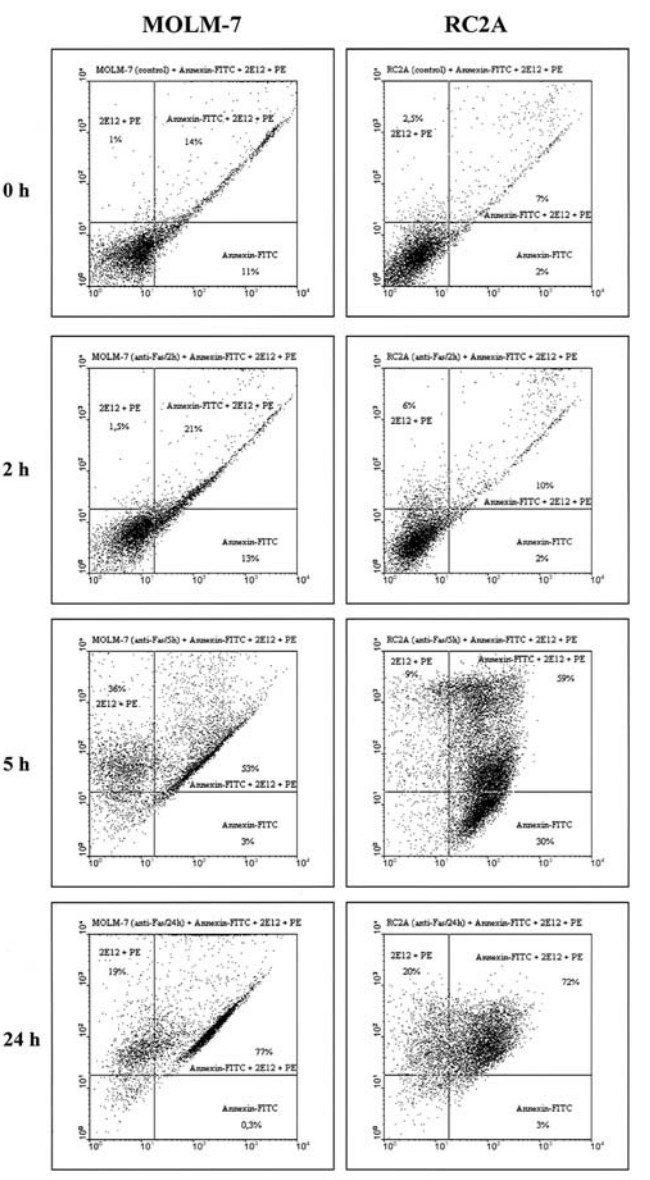

Figure 6. Flow cytometry analysis of MOLM-7 and RC2A cells induced to apoptosis by anti-Fas (7C11). Positivity of annexin V-FITC binding (x-axis) in comparison to simultaneous positivity with 2E12 antibody (PE)(y-axis) after 0, 2, 5 and 24 hours of induction.

fresh cells of chronic myeloid leukemia cell line MOLM-7. This antibody has not been assigned to any definite CD, despite of being examined in the last International workshop of human leukocyte differentiation antigens. Our preliminary hybridoma screening has shown the strong reactivity of the 2E12 antibody with a small percentage of cells of all cultured hematopoietic cells and cell lines used, which were at different stages of cell death. We therefore assumed that the 2E12 antibody reacts with a non-lineage antigen present on all hematopoietic cells which are in the process of cell death by apoptosis. Furthermore we have shown that the 2E12 antibody reacts with the majority of apoptotic bodies [2]. We expected that 2E12 antibody detects an intracellular antigen which becomes exposed at the cell surface after the cell membrane damage during apoptosis or necrosis. However, we did not find any increase of 2E12 positive cells after the cell membrane permeabilization of several cell lines (unpublished results). In the previous report we confirmed an increase of the 2E12 reactivity in several cell lines induced to apoptosis by camptothecin [2].

One of the earlierst indication of apoptosis at a single cell level is the translocation of membrane phosphatidylserine from the inner side to the outer layer of the plasma mem-

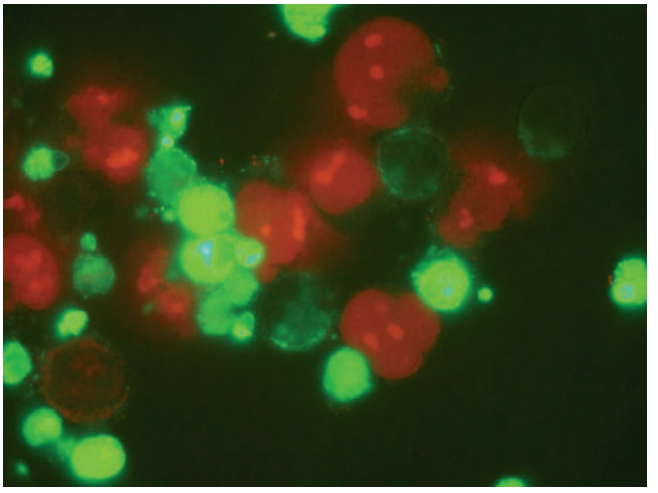

Figure 7. Two color fluorescence microphotograph of MOLM-7 cells induced to apoptosis by anti-Fas antibody for 2 hours. Majority of apoptotic cells stains by anti 2E12 antibody (green fluorescence) or by propidium iodide (PI) red fluorescence. Only few cells are weakly positive by both markers. Majority of 2E12 strongly positive cells are PI negative, so they do not represent population of necrotic cells (microscope Olympus IMT2, 1200x).

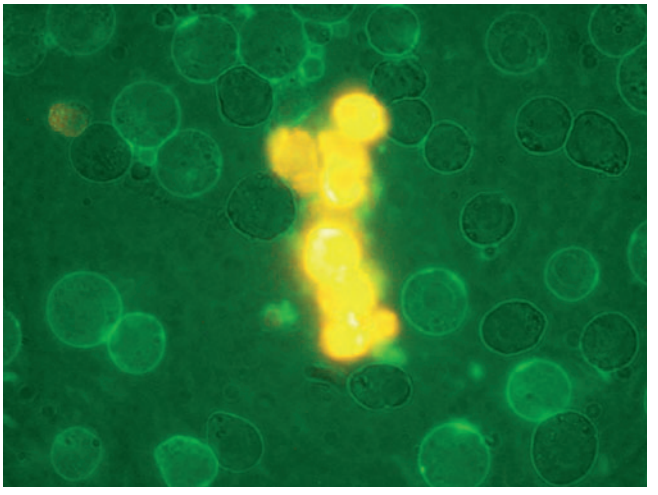

Figure 8. Two color fluorescence microphotograph of JURKAT cells induced to apoptosis by anti-Fas antibody for 2 hours. Cells are stained by annexin V-FITC (green fluorescence) and 2E12 followed by a second layer antibody (PE)(orange fluorescence). Majority of cells stains by annexin-FITC (green) and a small colony of dying cells by 2E12 (orange), some living cells are visualized by weak normal light and are dark grey (microscope Olympus IMT2, 1200x).

brane, by which it exposes at the cell surface. Annexin V is the protein which can be used as a sensitive probe of the phosphatidylserine exposure to the outer leaflet of the cell membrane and is therefore well suited to detect both apoptotic and necrotic cells [7, 8, 9].

Control cells and cells induced with anti-Fas (CD95) were subjected to the annexin V-FITC binding test combined by PI staining of necrotic cells. This approach shows

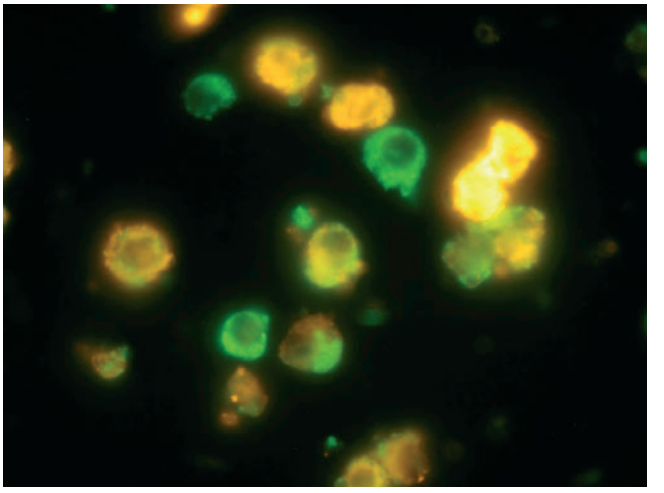

Figure 9. Two color fluorescence microphotograph of JURKAT cells induced to apoptosis by anti-Fas antibody for 24 hours. Cells are stained by annexin V-FITC (green fluorescence) and 2E12 antibody followed by a second layer antibody (PE)(orange fluorescence). Many cells are both Annexin Vand 2E12 positive (microscope Olympus IMT2, 1200x).

the typical pattern, which dissects the resulting cellular population into the four quadrants according to their fluorescence pattern in the two color flow cytometry (Figs. 3.1, 3.2), distinguishing living cells from apoptotic, necrotic and secondary necrotic cells [7].

With a similar approach in the two color fluorescence cytometry by plotting intensity of orange fluorescence of 2E12 (labeled by second layer antibody with PE) as parameter on y-axis and green fluorescence of annexin V-FITC on x-axis, we show, that the anti-Fas induced cellular population can be similarly dissected into the 4 groups according to their expression of both markers (Figs. 4, 5, 6 – right side):

1. subpopulation containing living non-apoptotic cells (both annexin V and 2E12 negative) (lower left quadrant).

2. subpopulation containing 2E12 positive and annexin V negative cells, which is morphologically characterized by cells shrinkage, chromatin condensation and high nuclear fragmentation containing the late apoptotic cells and bodies (upper left quadrant).

3. subpopulation of both annexin V-FITC positive and 2E12 positive cells containing majority of apoptotic bodies, some damaged cells and cells in apoptotic death (upper right quadrant).

4. cells exposing phosphatidylserine at the cell surface and reactive with annexin V-FITC but negative with 2E12 antibody. This subpopulation represents cells in the early stages of apoptosis or in necrosis (lower right quadrant) [8, 9].

We have also shown, that pretreatment of cells by 2E12 antibody prior to annexin V-FITC assay did not influence the results of annexin V/PI test in the induced and control cells, neither the 2E12 antibody induced the cell death itself.

Positivity with 2E12 antibody was detected on dying cells (PI negative) of all cultured hematopoietic and also nonhematopoietic cultures, but has never been detected on freshly separated patient's or normal cells tested. After few days in culture, however, the 2E12 positive cells always appeared. Therefore we suggest that the antibody 2E12 can be used as a new additional marker of the programmed cell death in cultures of cancer and normal cells. This new additional parameter of programmed cell death can be utilized in studies examining the different action of various biological agents and chemical compounds on cell death, differentiation and senescence with a potential for the treatment of cancer, leukemia and other diseases. The biochemical nature of this antigen however remains to be elucidated.

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