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Case report

Apoptosis of Leukemic Cells: A Case Report

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A B S T R A C T

Transformation of leukemic cells is associated with delay in maturation and in apoptosis, and to altered responsiveness to growth factors. However, some studies have revealed that Fas (CD95/APO1) which mediates apoptotic signal and decrease of anti-apoptotic Bcl-2 are frequently observed in acute myeloid leukemia (AML) M4/M5 leukemic cells. The aim of the study was to compare cytomorphology and cytochemistry of bone marrow (BM) apoptotic leukemic cells to preserved peripheral blood (PB) leukemic cells in our patient, a 76-year-old man with AML-M5b treated at Zagreb University Hospital Center. BM and PB of the AL patient were analyzed after Pappenheim and cytochemical stainings, and leukemic cells were classified according to FAB and WHO classification. Analysis of PB revealed leukocytosis and 80–90% monocytic cells (46% monoblasts, 29% promonocytes and 11% monocytes). Only a few preserved monoblasts and promonocytes were found in BM, together with numerous morphologically altered cells with characteristic chromatin condensation and pyknosis of nucleus, as well as nuclear fragmentation and formation of apoptotic bodies. Thus, cytomorphology of PB leukemic cells pointed to proliferation of immature monocytic cells, and cytomorphology of BM to cell apoptosis. Cytochemistry of PB monocytic cells and BM apoptotic cells confirmed monocytic cell lineage because esterase was strongly positive in almost all BM apoptotic leukemic cells and PB leukemic cells, and esterase was completely inhibited with sodium fluoride. On the basis of these findings, AML-M5b was diagnosed in our patient. There are many possible explanations for our observation of BM leukemic cell apoptosis in a patient with AML-M5. The most reliable one is that apoptosis was induced ex vivo after BM aspiration in course of the air drying of BM specimen before staining. Mass BM leukemic cell apoptosis that was recorded in contrast to numerous preserved leukemic cells in PK could be probably connected to unfavorable ratio of relatively low concentration of cytokines in relation to high leukemic cell number in BM aspirated cytologic specimen.

Key words: *acute myeloblastic leukemia, apoptosis, Bcl-2, C*

Introduction

Apoptosis is a programmed cell death mechanism for removal of unwanted cells from tissues and is an important event in tissue development and homeostasis. There are two main apoptotic pathways: extrinsic or death receptor pathway and intrinsic or mitochondrial pathway. However, there is evidence now that the two pathways are linked and that molecules in one pathway can influence those in the other. There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme dependent killing of the cell. Thus, the process of apoptosis is controlled by various cell signals which may originate either extracellularly (extrinsic inducers), intracellularly (intrinsic inducers) or by inducing granzyme pathway[1,2]. Extracellular signals (toxins, growth factors, cytokines, etc.) may trigger or inhibit apoptosis[1–4]. Tumor necrosis factor (TNF) is the major extrinsic mediator of apoptosis and binding to one of its receptors, TNF-1 or Fas (CD95), leads to caspase activation[1–4]. A cell initiates intracellular apoptotic signaling in response to stress (heat, radiation, nutrient deprivation, hypoxia, glucocorticoids)[1–4]. Apoptotic proteins induce mitochondrial protein SMAC (second mitochondria-derived acti-

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vator of caspases) and cytochrome c release from mitochondrial membrane, which are involved in the intrinsic pathway of apoptosis. Extrinsic, intrinsic and perforin-dependent pathways mediate apoptosis via caspases[1–5]. Apoptosis is distinguished from necrosis biochemically and by a unique series of morphological changes, including a decrease in cell volume and budding of cell contents into membrane-enclosed vesicles[1–6]. The Bcl-2 protein family plays a central role in the regulation of apoptosis. To date, a total of 25 genes have been identified in the Bcl-2 family. Some of anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, Bag, and some of pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. The wild type of p53 tumor suppressor gene inhibits cell division and is also essential in inducing apoptosis in cells with damaged DNA[1–6]. Programmed cell death appears to be an integral component of the hematopoietic cell lineage commitment and differentiation. Apoptosis also participates in the physiology of more mature hematopoetic cells[1–4]. In lymphopoesis, generation of the T and B lymphocytes is dependent on recognition and the apoptotic deletion of cells with inappropriate specifics. Thus, T-lymphocytes undergo the selection process that uses apoptosis to eliminate cells that recognize self antigen. Granulocyte and monocyte elimination from circulation may in part be mediated by apoptosis[1–4]. Morphologic evidence of increased apoptosis has been demonstrated in MDS, particularly in early subtypes. In contrast, decreased apoptosis is associated with enhanced leukemic cell survival as MDS progressed toward AML[7,8]. Thus, transformation of leukemic cells is linked to delay in maturation and in apoptosis, and to altered responsiveness to growth factors[7,9]. In acute monoblastic leukemia (M5a) and acute monocytic leukemia (M5b), the types of acute myelod leukemia (AML), most leukemic cells are of monocytic lineage. In M5a the majority of monocytic cells are monoblasts (≥80%), while in M5b there are numerous promonocytes and monocytes but monoblasts must comprise ≥20% of all nucleated cells. AML-M5a is usually observed in young individuals and AML-M5b is more common in adults with male:female ratio 1.8:1[9]. In many malignant tumors and hematological malignancies, induction of c-myc oncogene is not only linked to uncontrolled cell proliferation but also to apoptosis, depending on the presence or absence of second survival signals such as growth factors and anti-apoptotic factors such as Bcl-2[1–4,9]. Indeed, some reports pointed to the involvement of upregulation of antiapoptotic proteins, p21 and spliced isoform of p73 (deltaN-p73), in pathogenesis of myelomonocytic and monocytic leukemias[10,11]. Yet, although apoptosis in leukemic cells is a very rare spontaneous event, there have also been reports with findings that pro-apoptotic Fas (CD95/APO1), as well as a decrease in anti-apoptotic Bcl-2, are more frequently observed in M4/M5 blasts[12–14].

However, to our knowledge the almost complete bone marrow (BM) apoptosis of leukemic cells before therapy has not been described in literature in AML patients. In this report we present a patient with AML-M5b, bone marrow leukemic cell apoptosis, and numerous preserved immature monocytoid cells in peripheral blood (PB).

Case Report

A 76-year-old man with previously diagnosed heart disease was admitted to Zagreb University Hospital Center because of progressive weakness and fever. BM (FNA) was done because of leukocytosis and thrombocytopenia. After standard Pappenheim staining, the cytomophological analysis of BM revealed excessive number of apoptotic cells (96% of all BM nucleated cells) and only several cytomorphologically preserved cells (few monoblasts and promonocytes and only sparse erythroblasts and megakariocytes) (Figures 1 and 2). BM apoptotic cells showed characteristic morphological changes of apototosis: cell shrinkage, nuclear chromatin condensation with formation of pyknotic nuclei, convolution, invagination and fragmentation of nuclei, as well as budding of cytoplasm (Figures 1–4). Besides apoptotic cells, apoptotic bodies – small, roughly spherical or ovoid cytoplasmic fragments were also observed, some of which contained nuclear particles (Figure 4). Quantitative analysis of apoptotic changes was also done by cytomorphological

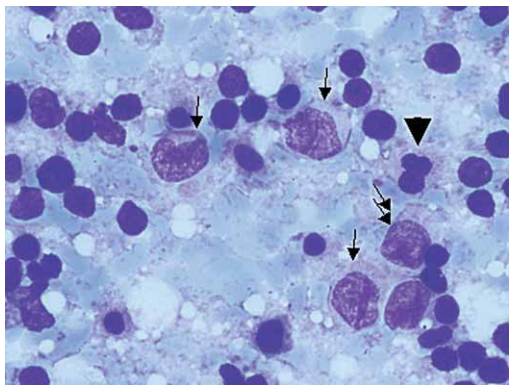

Fig. 1. Bone marrow: numerous apoptotic cells with pyknotic nuclei, one cell with early nuclear fragmentation (small arrow), promonocytes (arrows), one monoblast (two arrows) (Pappenheim, x1000).

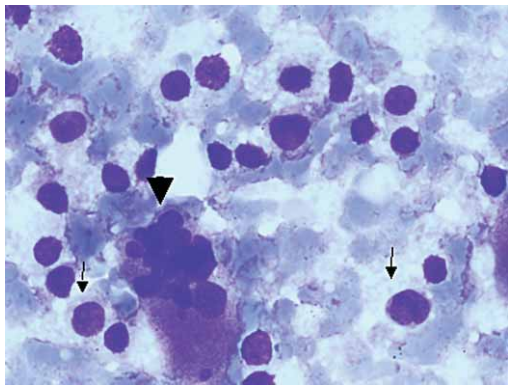

Fig. 2. Bone marrow: numerous apoptotic cells, two promonocytes (arrows), one megakaryocyte (small arrow)(Pappenheim, x1000).

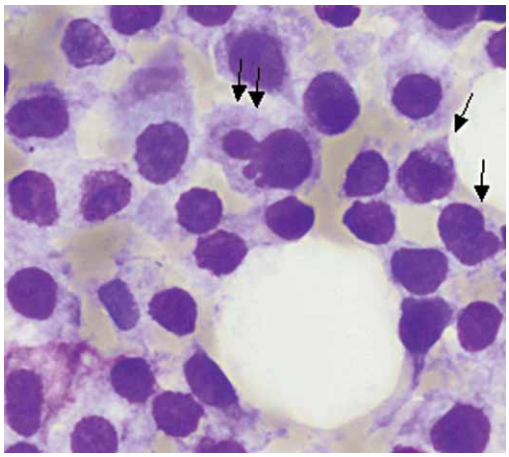

Fig. 3. Bone marrow: numerous apoptotic cells. Some cells have convoluted nuclei (arrow) and one cell showed cytoplasm budding, invagination and early fragmentation of the nucleus (two arrows) (Pappenheim, x1000).

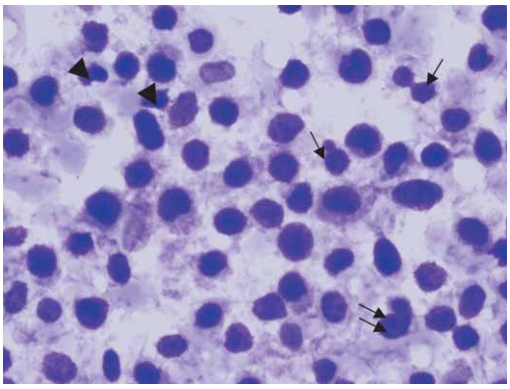

Fig. 4. Bone marrow: numerous apoptotic cells with pyknotic nuclei and budding of cytoplasm¸ cells with nuclear invagination (two arrows) and early nuclear fragmentation (arrow); apoptotic bodies (small arow)(Pappenheim, x1000).

analysis of Pappenheim stained BM smears and was defined as apoptotic cell index, apoptotic body index and apoptotic index (apoptotic cells and apoptotic bodies) per 1000 BM nucleated cells. In our patient, all the mentioned apoptotic indices were very high: apoptotic cell index was 963, apoptotic body index was 24, and apoptotic index was 987. In PB, numerous (80–90%) monocytic cells were found besides leukocytosis (Figure 5). Differential blood count estimated 46% monoblasts, 29% promonocytes, and 11% monocytes. In almost all (about 95%) PB monocytic cells and BM apoptotic cells, alpha-naphthyl-acetate esterase (ANAE) was strongly positive (Figures 6 and 7) and ANAE activity was completely inhibited with sodium fluoride (Figure 7) which is specific for monocytic cell lineage. Sudan reaction was positive in 5% of monoblasts and in 15% of promonocytes. Blasts were negative for myelopexidase and PAS-reaction. Thus, cytomorphology and cytochemistry of PB leukemic cells point to proliferation of leukemic immature monocytic cell. Cytochemistry of BM apoptotic cells confirmed monocytic cell lineage and AML-M5b was diagnosed in our patient. BM flow cytometry (FC) analysis indicated apoptosis, i.e. the analyzed BM cell population showed a decrease in forward light scatter which was not paralleled by a decrease in side scatter. Moreover, specific lineage cell antigens could not be detected in BM cells due to unspecific binding of different lineage moAbs. By standard cytogenetic analysis, it was not possible to identify specific cytogenetic changes, and FISH was also negative for t(9;22) and 11q23. Because of age and heart disease in our patient, reduced dose cytostatic therapy was commenced leading to clinical improvement and reduction of leukocytosis in PB. Control BM FNA after therapy was planned during continuous follow up, but the patient did not present for scheduled control examination.

Discussion

Leukocytosis and numerous monoblasts and promonocytes in PB, as well as monocytic PB cell ANAE positivity (Figures 5 and 6) with complete ANAE activity inhibition with sodium fluoride pointed in our patient to AML-M5b[9,15]. However, cytological analysis of BM revealed numerous morphologically altered cells and in

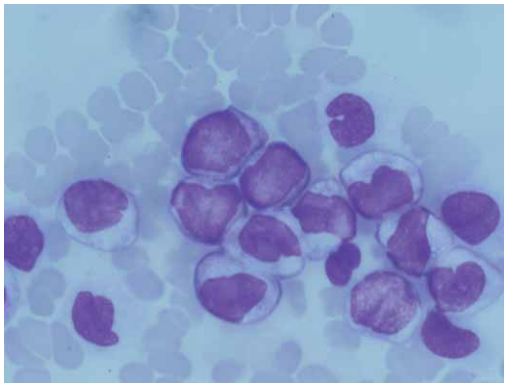

Fig. 5. Peripheral blood: numerous monoblasts and promonocytes (Pappenheim, x1000).

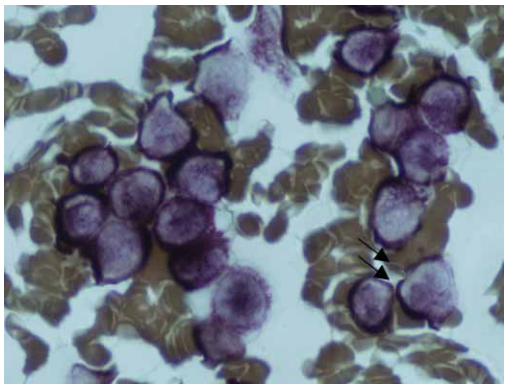

Fig. 6. Peripheral blood: numerous alpha-naphthyl-acetate esterase (ANAE) positive monoblasts and promonocytes (ANAE staining, x1000).

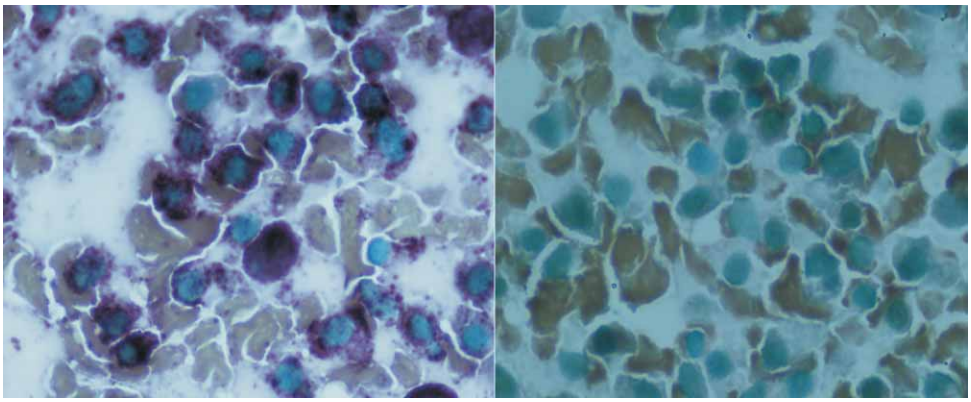

Fig. 7. a) Left: numerous alpha-naphthyl-acetate esterase (ANAE) positive apoptotic cells are evident in bone marrow smear (ANAE staining x1000); b) Right: inhibition of alpha-naphthyl-acetate esterase (ANAE) with sodium fluoride in apoptotic cells is evident in bone marrow smear (ANAE staining x1000).

majority of BM cells it was not possible to determine hematopoietic lineage commitment and the stage of maturation. Such characteristic cytomorphological changes firstly pointed to apoptosis (Figures 1–4). However, cytomorphologic alterations of BM cells resulting in loss of specific hematopoietic characteristics could also be caused by necrosis. Cell necrosis differs from apoptosis both biochemically and morphologically[1–6]. Cell necrosis is associated with initial cell swelling, followed by plasma membrane rupture and leakage of the cell's content[1–6]. Nuclear degeneration of necrotic cells may undergo-progressive nucleus dissolution (karyolysis) which is morphologically evident as nuclear swelling and loss of nuclear texture or progressive nuclear shrinkage or pyknosis of nucleus[1–6]. Biochemically, cell necrosis is characterized by enzymatic digestion of the cell and denaturation of proteins[1–6]. On the other hand, apoptotic BM cells in our patient showed other morphologic changes that are characteristic for the process of apoptosis: cell and nucleus shrinkage with preserved integrity of membrane; chromatin condensation and pyknosis of nucleus; convolution, invagination and nucleus fragmentation; cytoplasm budding; and formation of apoptotic bodies (nuclear fragments surrounded with cytoplasm which dropped from the cell) (Figures 1–4)[1–6]. Biochemically, cellular organelles in early apoptotic cells are not damaged and there is no enzymatic digestion of cytoplasm and deneaturation of cytoplasmic proteins[1–6]. As in our patient, almost all BM apoptotic cells were positive for the specific assay for monocytic cell lineage, i.e. ANAE with inhibition of ANAE activity with sodium fluoride (Figure 7), thus both excluding cell necrosis and confirming monocytic cell lineage of BM apoptotic cells[9,15]. One assay for determination of viable cells shares similar test principle as alpha-nyphtyl-acetate esterase (ANAE) cytochemistry and employs the nonfluorescent esterase substrate, fluorescein diacetate. This substrate, after being taken up by live cells, is hydrolyzed by intracellular esterases which are ubiquitous to all types of cells. The product of the hydrolysis, fluorescein, ensures highly fluorescent labeling of live and early apoptotic cells[16].

Flow cytometry (FC) analysis in our patient indicated apoptosis of BM cells, with concurrently observed decrease in forward light-scatter as a result of cell shrinkage, which was not paralleled by a decrease in side scatter in most BM cells[17,18]. However, light scatter changes are not specific to apoptosis because mechanically broken cells, isolated cell nuclei and necrotic cells also have diminished ability to scatter light. Identification of apoptosis or necrosis by light scatter therefore requires additional confirmation and should be accompanied by another, more specific FC assays (flurochromes, labeling, annexin labeling, loss of F-actin, etc.)[17,18]. Mostly, FC that detect cell viability is based on the characteristic of flurochromes to stain cells which have lost the integrity of their plasma membrane, i.e. necrotic and late apoptotic cells, while early apoptotic cells are not stained[17,18]. Apoptosis is accompanied by a loss of membrane phospholipid asymmetry, resulting in the exposure of phoasphatidylserine at the cell surface, which is a principle for the detection of apoptosis by annexin[17,18]. In our patient, additional FC tests for apoptosis of BM cells were not done since in this patient it was of primary importance to identify cell lineage of BM leukemic cells. Cell lineage of BM leukemic cells could not be estimated by FC analysis with standard moAbs panel because of unspecific binding of different moAbs on apoptotic cells. Many other different methods can also be used to determine apoptosis: TUNEL (TdT-mediated dUTP Nick-End Labeling) analysis, ISEL (in situ end labeling) or detection of apoptosis-related proteins, such as caspase-3, caspase-9, etc.[1–6]. Fragmentation of the nucleus and cleavage of chromosomal DNA in course of apoptosis result in generation of DNA fragments on electrophoresis. Apoptotic DNA fragmentation could also be detected with greater sensitivity with PCR techniques, such as semiquantitative ligation nucleotide PCR of blunt DNA ends[1–6].

Despite high accuracy of FC analysis and other refined techniques for apoptosis detection, characteristic morphological features also enable recognition of apoptotic cells in histological and cytological specimens[19,20]. BM cells in our patient showed all morphologic changes

characteristic for apoptosis, and apoptotic indices linked to these changes in apoptotic cells were extremely high. Some of the highest apoptotic indices (AI) found in some studies[19–21] for small cell lung carcinoma (AI- 10.6), non Hodgkin lymphoma (AI 8.8) and in acute leukemia (AI 19) were far beneath AI (987) found in the BM cell specimen of our patient. An interesting finding in hematological malignancies is that indices of high proliferative cell activity, such as Ki-67, correlate with AI[19]. Apoptosis is also an integral part of normal hemopoiesis and is required for its homeostasis[3,4]. It has been confirmed that BM stroma and hematopoietic growth factors (GF) are not only required for hematopoietic stem cell (HSC) proliferation and differentiation, but are also necessary for cell survival and for prevention of apoptosis[3,4]. Indeed, in normal BM almost all nucleated cells showed no evidence of apoptotic changes and only a single apoptotic cell is a very rare finding. Terminally differentiated cells, such as senescent neutrophils and monocytes cannot re-enter the cell cycle and therefore apoptotic death is a natural fate at the end of their life spans[3,4]. Thus, a single or only a few granulocytes in apoptosis could be occasionally observed in PB, and they mostly present cells with pyknotic, completely separated ovoid fragments of nucleus.

Evidence is available that apoptosis is responsible for the premature intramedullar death, which accounts for peripheral cytopenias in myelodisplasia (MDS)[7,8]. Certain reports point to various factors involved in MDS apoptosis, like CD40-CD40L interactions, low Bcl-2, upregulated Toll-like receptor-4, B lymphocyte alterations and overexpression of some cytokines such as TNF-related apoptosis inducing ligand -TRAIL[22–26]. In contrast, decreased apoptosis is associated with enhanced leukemic cell survival as MDS progresses toward-AML. Many molecules that are expressed in leukemic cells have anti-apoptotic activities (altered p73, upregulated p21, Bcl-2, Bcl-x)[7,9–11]. After considering this literature data, the finding of leukocytosis in PB with monoblasts and promonocytes without apoptotic morphological changes despite massive BM apoptosis is highly unusual. Complex alterations of leukemic cells that influence their proliferation, differentiation and apoptosis[1–4,9] implicate many possible explanations for such finding and some of them are proposed as follows: 1) The cascading network of pleitrophic genes influencing both cell-cycle events and cell death is consistent with hypothesis that c-myc (which can be occasionally overexpressed in AML-M5)[27,28] induces a two-way state in cells so that the cell chooses either apoptosis or proliferation, depending on the presence or absence of second survival signals such as growth factors and Bcl-2[1–4,14]. Thus, the cells overexpressing c-myc may proliferate relatively unchecked while nutrients are available, but once these become limited, as possible in our patient due to extensive leukemic BM cell proliferation if c-myc was overexpressed and/or Bcl-2 was decreased, such cell may undergo apoptosis[1–4,14]. 2) According to some reports, pro-apoptotic Fas (CD95) molecule (member of tumor necrosis factor – TNF family receptors including also TNFR1, TNFR2, CD30 and CD40), which is a bad prognostic marker, is much more frequently expressed in some AML subtypes, especially M4 and M5[12,13,29]. Thus, there is a possibility that blasts in our patient with AML-M5 also have had CD95 that could be spontaneously triggered under some circumstances before chemotherapy and induce apoptosis of BM leukemic cells. Moreover, Riconi et al.[30] found that especially AML-M4/M5 display a high sensitivity to Bortezomib-mediated apoptosis. Other studies have also indicated that some molecules which could be expressed on AML blasts (LAIR-1, CD95) could be triggered with various agents, resulting in apoptosis of leukemic cells[31–33]. 3) Although stromal environment and some cytokines (IL-1β, TNF-α, GM-CSF) provide proliferative and differentiation signals, BM stromal cells were found to induce cell death programme in the presence of some cytokines such as INF-γ, TNF, IL-6 and TG-β1[3,4,34]. It could be explained that a difference in viability between BM and PB leukemic cells in our patient is caused by BM stromal cells' ability to induce apoptosis together with BM cytokines[3,4,34]. On the other hand, cytokines in PB (TNF, INF-γ) without stromal cell interaction and other cytokines that could be found in PB (GM-CSF, G-CSF, IL-2) delay apoptosis[3,4,35–38]. 4) It was also shown that PB myeloblasts in AML had downregulated genes involved in apoptosis with significantly better homing and engrafment of PB CD34$^+$ cells in comparison to the same immunophenotypical BM myeloblasts[39]. This finding allowed the possibility that some differences also exist between BM and PB leukemic cells in our patient resulting only in apoptosis of BM leukemic cells. However, all the above numbered data are only possible explanations because additional analyses, such as CD95 or detection of other death cell receptors, or the assay employing inhibition of apoptosis by blocking Fas/FasL interactions, or detection of caspase-9 involved in intrinsic apoptosis pathway, as well as cytogenetic or molecular c-myc, detection were not performed[1,33]. Actually, there is one more possible reliable explanation of the difference in apoptosis between BM and PK leukemic cells that was found in our patient. It is known that the entire process of apoptosis occurs rapidly, with only a few hours between reception of a death signal and complete degradation of cell via apoptosis[3,4]. Thus, it could be considered that apoptosis process with such high AI was induced ex vivo after BM aspiration, because of cytokine deprivation, in course of the air drying of BM specimen for two hours before cell fixation and Pappenheim staining. Difference in leukemic cell apoptosis between BM and PK could be probably ascribed to unfavorable ratio of high cell number and concentration of cytokines in BM aspirated cytologic specimen.

Conclusion

Cytomorphology and cytochemistry of PB leukemic cells pointed in our patient to proliferation of immature monocytic cells and cytomorphology of BM to cell apoptosis. Cytochemistry (positive ANAE which was inhib-

ited with sodium fluoride) of cytomorphologically changed BM cells confirmed monocytic cell lineage of apoptotic BM cells. Such finding presents a diagnostic challenge and provides insight in complex interactions of various factors linked to proliferation and programmed cell death of leukemic cells. It also allows that some molecules expressed on leukemic cells may be possible targets for new therapeutic approaches to leukemia by inducing cell apoptosis.

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APOPTOZA LEUKEMIJSKIH STANICA – PRIKAZ BOLESNIKA

S A Ž E T A K

Transformacija leukemijskih stanica povezana je s odgodom sazrijevanja, odgodom apoptoze i promijenjenim odgovorom na faktore rasta. Međutim, neke studije su pokazale da se molekula Fas (CD95/APO1), koja provodi signal apoptoze – programirane stanične smrti, te snižene vrijednosti anti-apoptotičke molekule Bcl-2, češće nalaze na leukemijskim stanicama akutnih mijeloičnih lekemija (AML) tipa M4 i M5. Cilj rada bio je usporediti citomorfologiju i citokemiju apoptotičkih leukemijskih stanica koštane srži (KS) i očuvanih leukemijskih stanica periferne krvi (PK) u našeg 76 godina starog bolesnika s M5b koji je liječen u Kliničkom bolničkom centru Zagreb. Koštana srž i periferna krv

bolesnika analizirane su nakon standardnog bojenja po Pappenheimu i citokemijskih bojenja i akutna leukemija (AL) je klasificirana prema kriterijima FAB (French-American-British) i SZO (Svjetska zdravstvena organizacija). Analiza PK je pokazala leukocitozu i 80–90% monocitnih stanica (46% monoblasta, 29% promonocita i 11% monocita). U KS su nađene brojne morfološki promijenjene stanice s karakterističnom kondenzacijom kromatina i piknotičkim jezgrama, fragmentacijom jezgri, apoptotička tjelešca, te tek nekoliko očuvanih monoblasta i promonocita. Citomorfologija leukemijskih stanica PK upućivala je na proliferaciju monocitnih stanica, dok je citomorfologija KS upućivala na apoptozu stanica. Citokemija monocitnih stanica PK i apoptotičkih stanica KS potvrdila je monocitnu liniju stanica jer je u gotovo svim leukemijskim apoptotičnim stanicama KS i leukemijskim stanicama PK nespecifična esteraza bila izrazito pozitivna i njena je aktivnost potpuno inhibirana natrijevim fluoridom. Na temelju morfologije i citokemije leukemijskih stanica postavljena je dijagnoza AL-M5b. Brojni poremećaji u leukemijskim stanicama teoretski bi mogli potaknuti apoptozu u stanicama KS u našeg bolesnika s M5b. Do apoptoze je, međutim, najvjerojatnije došlo ex vivo nakon što je učinjena dijagnostička punkcija koštane srži, dok su se citološki uzorci sušili na zraku tijekom 2 sata prije postupka bojenja. Objašnjenje za tako velik broj apoptotičkih leukemijskih stanica u koštanoj srži nasuprot brojnim očuvanim leukemijskim stanicama u PK moglo bi biti povezano s nepovoljnim odnosom relativno niske koncentracije citokina u odnosu na izrazito velik broj leukemijskih stanica u aspiratu koštane srži.