ORIGINAL ARTICLE

Expression of programmed cell death proteins in patients with chronic myeloid leukemia

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Summary

Purpose: Chronic myelogenous leukemia (CML) is a malignant myeloproliferative disease developing out of pluripotent hematopoietic stem cells that contain the fusion Bcr-Abl gene. The mechanisms that lead to these changes at molecular level are still unknown as are the mechanisms that increase the proliferative capacity of these cells. Disorders that occur in the process of apoptosis represent one of the possible molecular mechanisms that bring about disease progress. In our study we analyzed the presence of mutated (mut) p53 gene and the amplification of Bax proteins in patients with CML.

Patients and methods: This study included 30 patients with CML (23 in chronic phase, 7 in blast transformation). Using immunohistochemistry with alkaline phosphatase / anti-alkaline phosphatase (APAAP) method we analyzed the expression of cell death proteins p53 and Bax in mononuclear bone marrow cells. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method was used to analyze the presence of mut p53 gene in mononuclear peripheral blood cells. Reverse transcription-polymerase chain reaction (RT-PCR) method was used to analyze the presence of Bcr-Abl in peripheral blood cells.

Results: High expression of Bax protein was detected in all analyzed patients, but no significant differences were noticed among them. No mut p53 gene was detected in any of the analyzed samples. Bcr-Abl b3a2 protein form was detected in all patients with variant translocations.

Conclusion: Lack of mut p53 product in the peripheral blood and bone marrow cells in patients with CML suggests that this gene plays no important role in disease pathology. Increased level of Bax protein expression is an essential characteristic of CML cells but it is not related with the clinical stage of disease.

Key words: apoptosis, Bax, chronic myelogenous leukemia, p53

Introduction

CML is a malignant myeloproliferative disease developing out of pluripotent hematopoietic stem cells that typically evolves in 3 distinct clinical stages. It starts with a relatively indolent chronic phase followed by the acceleration phase characterized by increased number of immature cells in the bone marrow and peripheral blood, progressive anemia and thrombocytopenia, as well as reduced response to cytotoxic therapy. Blast crisis is the terminal phase of CML in which the patient has all the symptoms of acute leukemia [1-3].

The Philadelphia (Ph) chromosome plays a central role in the pathogenesis of CML, and results from the reciprocal translocation of the long arms of chromosomes 9 and 22 that generates the Bcr-Abl fusion gene which determines the synthesis of the hybrid protein that is larger than the normal Abl protein and with increased tyrosine-kinase activity [4,5].

It is generally believed that CML develops when a single, pluripotential, hematopoietic stem cell acquires a Ph chromosome carrying the Bcr-Abl fusion gene, which confers on its progeny a proliferating advantage over normal hematopoietic cells and thus

allows the Ph-positive clone to gradually displace the residual normal hematopoietic cells. Nevertheless, the molecular mechanism that leads to these changes is still unknown, as is the mechanism of increased proliferating ability of these cells or the mechanism that leads to disease progression [6-8].

The events at the cellular level, important for the progression of disease, are the increased proliferation of cells, resistance to therapy, reduced levels of apoptosis, as well as the occurrence of stoppage of cell maturation. The changes on the genetic and molecular level encompass activation of oncogenes (c-Myc, Ras, AML-EV1), inactivation of tumor of suppressor genes (p53, p16, RB), genome instability, as well as defective repair mechanisms [9].

There is accumulated experimental evidence that the disbalance in apoptosis has significant impact on the process of neoplastic transformation. Research of the kinetics of the neoplastic tissue growth has shown that the basic cause for the increase of the neoplastic tissue is the reduced number of the dying tumor cells, and not unrestricted tumor cells' proliferation [10-12]. This is the reason why the search and detection of genes significant for the regulation of the programmed cell death could be important in interpreting the still insufficiently clear mechanism of CML progression.

Apoptosis represents the controlled way of cell death in which the cell participates actively, performing a precise gene-regulated self-destruction programme (cell suicide) [13]. Although a whole range of biochemical events that lead to stereotypical morphological changes has been identified so far, many molecular mechanisms that participate in the apoptotic process are still unknown [14,15].

The decision of the cell to enter the process of programmed cell death is very complex and depends on the interaction of the array of intra- and extracellular, as well as pro- and antiapoptotic signals [10]. The protein products of Bcl-2 family gene, p53 tumor suppressor gene, some oncogenes (c-Myc, Ras), genes that regulate the cell cycle, various transcription factors, certain classes of phosphatases and kinases represent important intracellular regulators of the apoptotic process [16,17].

p53 tumor suppressor gene determines the synthesis of a nuclear phosphoprotein, which acts as transcription factor by participating in the regulation of the cell cycle and apoptosis. Mutations within the p53 gene are present in more than 60% of human tumors and are mostly connected with poor prognosis and disease progression. Mutation of p53 gene is one of the most common secondary mutations in blast transformation of CML [18].

The main role of the Bcl-2 protein family is to change the permeability of the mitochondrial membrane, and indirectly, through the release of cytochrome C and activity of caspases, to regulate the process of apoptosis [19]. Some proteins of this family display stimulatory effect (Bax, Bid, Bad), while others have inhibitory effect on apoptosis (Bcl-2, Bcl-Xl). The decision of the cell whether to enter the apoptotic process or not depends on the proportion of pro- and anti-apoptotic members of the family [20-24].

The aim of this work was to determine whether the changes in the expression of Bax protein and mutation of p53 gene are related with the clinical stage and progression of CML.

In our previous work we have shown that the expression of Bcl-2 protein is considerably higher in bone marrow samples of patients undergoing CML blast transformation compared to patients in chronic phase. Also, amplification of c-Myc gene has been detected in patients with CML blast transformation [25].

Patients and methods

Patients

Our study included 30 patients with CML of whom 23 were in chronic phase and 7 in blast transformation. There were 18 men and 12 women aged l8-68 years (median 45). Diagnosis was made on peripheral blood and bone marrow smears using standard cytological and cytochemical methods. Seven healthy volunteers served as controls for comparison of mut p53 and Bax proteins. Patients included in this study were treated at the Clinic of Hematology, Military Medical Academy.

Cytogenetic analysis

Bone marrow samples were used for cytogenetic analysis. Cytogenetic studies were carried out on Giemsa-banded chromosomes obtained directly or from 24 h unstimulated bone marrow cultures as described previously [26]. At least 20 metaphases were analyzed. The karyotypes were described according to the International System for Human Cytogenetic Nomenclature [27].

RT-PCR analysis

Total RNA was extracted from peripheral blood cells using the guanidine thiocyanate phenol-chloroform extraction method [28]. Reverse transcription was performed on 1 μg of total RNA after heating at 65° C

for 15 min. Reverse transcription was performed with 1st Strand cDNA Synthesis Kit for RT-PCR (AMV; Roche Diagnostics Corporation, Indianapolis, IN, USA). A volume of 5 μl cDNA was diluted with 45 μl of PCR mixture, PCR Core Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) and amplified with slight modification as described by Moravcova et al. [29].

Immunohistochemistry

The mononucleated cells (MNCs) from bone marrow were enriched by centrifugation (1600 rpm, 30 min) on Ficol-Hypaque (ICN, USA), washed in phosphate-buffered saline (PBS) and cytocentrifugates were prepared. Bax and p53 proteins in bone marrow MNCs were detected with a commercial antibody (Dako, Denmark) as described previously [30,31]. The data obtained were arbitrarily grouped in 4 classes of positivity: I: + (< 20%); II: ++ (between 20-70%); III: +++ (between 70-90%); and IV: ++++ (> 90%).

PCR-SSCP analysis of p53 gene

p53 mutations in exons 5-8 were evaluated by SSCP-PAGE analysis. Genomic DNA (500 ng) was amplified in a 50 μl reaction mixture (Pharmacia Bio-tech, Sweden). Primers covering the region between exons 5 and 8 of p53 gene were amplified by PCR using p53-specific set of primers [32] under the following conditions: 95° C, 60 sec; 60° C, 60 sec×35 cycles. Samples were denatured by heating at 95° C for 5 min and electrophoresed (LKB, Pharmacia, Sweden) at 4° C on an 8% and 10% non-denaturing polyacrylamide gel stained with silver nitrate.

Statistical analysis

Statistical significance was determined using the x^2 test; $p<0.05$ was considered statistically significant.

Results

Results of cytogenetic analysis

The cytogenetic analysis of bone marrow showed chromosomal abnormalities in all 30 patients. These included translocations [t(9;22)(q34;q11), t(3;21)(q21;p11), t(6;9)(q15;p24), t(9;20;22)(q34;q11;q11), t(9;12;22)(q34;q13;q11), t(12;22)(p11;p11)], deletions [del(20)(p12)], the presence of marker chromosomes, loss of Y chromosome, derivative chromosome and hyperdiploidy and tetraploidy clones. The majority of the patients (n=26, 87%) had characteristic t(9;22)(q34;q11), 3 (10%) had variant translocations and one (3%) was Ph-negative (Figure 1). Clonal evolution was evident in 11 (37%) patients (Figure 2).

Results of RT-PCR

We found that the b3a2 form transcript of Bcr-Abl gene was expressed in MNCs of peripheral blood in all patients with variant translocations. In the peripheral blood MNCs of the Ph-negative patient no expression of Bcr-Abl gene was found (Figure 3).
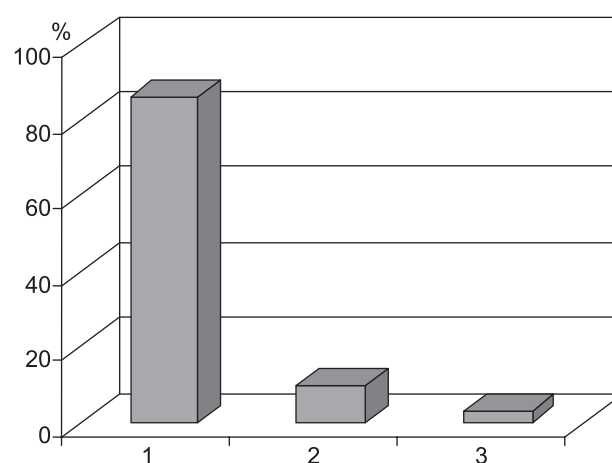

Figure 1. Percent distribution of patients with classic translocations (1), variant translocations (2) and Ph-negative patient (3).
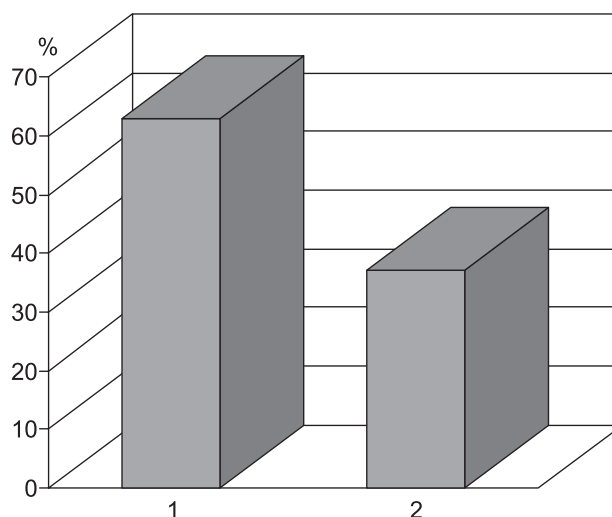

Figure 2. Percent distribution of patients without (1) and with (2) clonal cytogenetic evolution.

Results of the immunohistochemical APAAP

The immunohistochemical reaction for mut p53 protein was negative in all bone marrow MNCs of the analyzed patients with CML and healthy controls. The reaction to Bax protein was positive in all patients in 90% of the analyzed bone marrow MNCs cells and defined as ++++. High statistical significance was seen between patients and healthy controls (p<0.001; Figure 4).

p53 gene mutations

30 samples of CML were examined for mutations in exons 5-8 of the p53 gene by the PCR-SSCP method. No p53 mutation was found in the MNCs of the peripheral blood in CML patients.
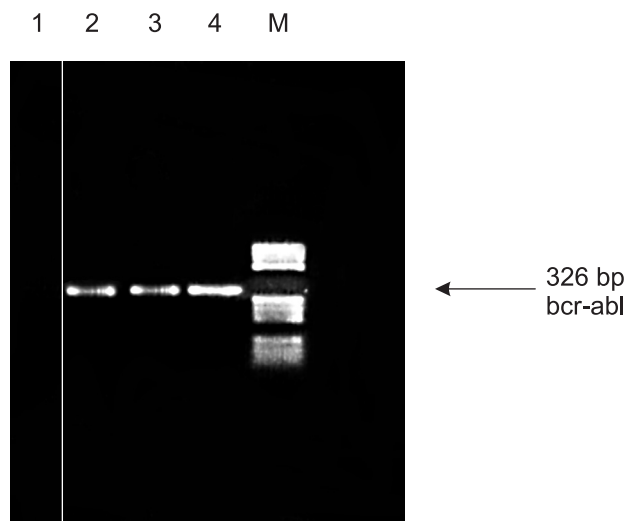

Figure 3. RT-PCR amplification of *Bcr-Abl* mRNA extracted from peripheral blood of the CML patients. Line 1: Ph-negative CML patient; lines 2-4: b_3a_2 transcript (326 bp); line M: molecular-weight size marker.
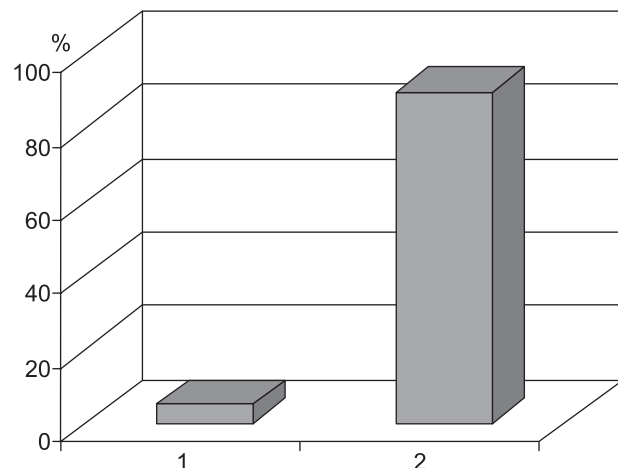

Figure 4. Histogram of average difference in the expression of Bax protein in healthy individuals (1) and patients with CML (2).

Discussion

CML is one of the most studied human malignant diseases. The chimeric gene that is generated through this specific translocation was first characterized on the molecular level so that the detection and analysis of the presence of Ph chromosome represent the first successful study in human neoplasia on the cytogenetic and molecular level [33,34].

Our analyzed group included 26 (86.7%) patients with the classic translocation t(9;22) (q34;q11), and 3 (10%) patients with variant translocations. According to literature data 2-10% of the patients have variant translocations, which, depending on the number of participating chromosomes, can be either simple or complex [1,33]. Some authors suggest that the origin of variant translocations could also be a part of the evolution of karyotypes. There has been simultaneous presence of both variant and classic translocation in several cases [4]. Today's common opinion is that clinical, prognostic and hematologic characteristics of CML with variant translocations are no different from CML with Ph chromosome that is generated through typical translocation [35,36]. In our analyzed group, there was no difference in the clinical course of disease between patients with classic and variant translocations.

So far the research of karyotypes of patients with CML has shown that Ph chromosome is in most cases the only visible chromosomal change in the chronic stage of the disease, while additional aberrations precede the blast transformation. In some cases, these additional chromosomal changes disappear after cytotoxic treatment, which indicates that the evolution of karyotypes and the progress of disease are closely connected [33].

The detection of additional or secondary chromosome alterations is important, because these changes can be detected as the disease progresses in 75-80% of patients. These secondary changes mostly precede the hematologic and clinical progress of disease, which follow after several months, and therefore they are considered to be important prognostic parameters [1]. In our patients, the evolution of karyotypes presented through the numerical and/or structural aberrations and /or the presence of hyperdiploid cells, was detected in 11 (37%) patients.

The evolution of karyotypes was detected in 2 out of 7 patients in blast transformation, whereas 9 out of 23 were in the chronic phase of disease. The results indicate that the occurrence of additional chromosomal changes could represent an important characteristic of neoplastically transformed CML cells. The prognostic importance of secondary aberrations in CML is com-

plex, heterogeneous and depends on many parameters such as the time of appearance, specific changes and treatment modality [9,37].

It is considered that more lesions are needed, probably more related genetic occurrences, for the induction of blast transformation phenotypes. Although Bcr-Abl protein definitely plays an important role in the initiation and development of CML, additional genetic events are necessary for the transition from chronic to terminal stage of disease. The course of the chronic stage of disease is different in different patients, a fact that also indicates the participation and influence of many other factors [9].

Mut p53 is one of the most common secondary mutations in blast transformation of CML. It is more related to the progress than to the initiation of the disease and can be detected also in other types of leukemia and lymphoma [4, 38-40]. The loss of antioncogene function is a common mechanism in clonal evolution of leukemia and lymphoma [4]. In the chronic phase of CML, the structure of p53 gene usually remains unaltered, but in blast transformation p53 mutations are detected in around 30% of the cases. The changes occur more often in myeloid, and only infrequently in lymphoid aberrance of CML [38].

The analysis of all 30 patients with CML showed that there was neither expression in immunohistochemical detectable level of p53 mutated protein in bone marrow cells, nor did the PCR-SSCP method detected the presence of mutation in the exons 5,6,7 or 8. The results of our work are in accordance with the results of previous studies where no detection of mut p53 in transformed CML was registered [41,42]. The detection of mutation within the p53 gene definitely requires larger number of samples on more patients in the blast transformation phase.

Using the immunohistochemical APAAP method we detected the presence of the protein product of Bax gene in all of the analyzed patients. All of them displayed Bax protein expression in > 90% of the analyzed bone marrow MNCs.

There is no clear explanation about the existence of this paradoxical positive correlation in the expression of Bax. Maybe high and constant expression of Bcl-2 leads to the half-life extension of Bax protein through mechanisms of post-translation modification. One group of researchers did not exclude the possibility of mutation in Bax gene that leads to a visible expression of dysfunctional protein [43]. The knowledge of the importance and impact of Bax protein in apoptosis in CML is incomplete and further research is needed [44,45].

The results of this and our previous work have shown that the expression of Bcl-2 protein is better correlated with the phase of CML than other studied proteins and rather represents a negative prognostic parameter in the treatment of CML [25].

In conclusion, the establishment of correlations between the expression of apoptotic proteins and the programmed cell death provides important information for the progress of disease. Better understanding of basic biological mechanisms of the apoptotic process and further analysis of proteins relevant to this process would provide a possibility for detecting key pathways in CML progress. Such investigations might offer further assistance in clinical practice.

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