SPANDIDOS PUBLICATIONS

Transcription of genes of p53-dependent apoptosis in acute leukaemia

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Received July 28, 2008; Accepted September 3, 2008

DOI: 10.3892/ijmm_00000092

Abstract. Tumour suppressor protein p53 prevents cancer development through various mechanisms, including the induction of apoptosis. We demonstrated that acute leukaemia, myeloblastic (AML) and lymphoblastic (ALL), is associated with significantly elevated levels of p53 and Bax mRNA in leukaemic cells. Regarding ALL, significantly elevated levels of Bcl-xL mRNA may explain the relative resistance of ALL cells to p53-dependent apoptosis. Altered alternative processing of Bcl-x and myeloid cell leukaemia-1 (MCL1) primary transcripts were observed in the case of AML and AML and ALL, respectively. We assumed that increased *glyceraldehyde-3-phosphate dehydrogenase (gapdh)* transcription and decreased MCL1s mRNA were not fully responsible for the dysregulation of p53-dependent apoptosis in the case of AML. In addition, transcription of hsp70.1 and Bcl-2 producing anti-apoptotic proteins was not affected in acute leukaemia.

Introduction

Tumour suppressor protein p53, a central player in cell death, prevents cancer development through various mechanisms, including induction of cell-cycle arrest, apoptosis, and the maintenance of genome stability (1). Use of animal models has indicated that attenuation of p53-dependent apoptosis can contribute to the initiation and progression of cancer, but the molecular mechanisms are still not completely known. In solid tumours, p53 is frequently mutated (2,3), but in haematological malignancies this is a rare feature (4). In acute leukaemia, AML and ALL more than 90% of the patients comprise wild-type p53 in the cancer cells (5,6). Mutations of p53 occur especially as haematopoietic abnormalities become more malignant, e.g. going from the chronic phase to the blast crisis of chronic myeloid leukaemia (4), and are often associated with treatment failure due to chemoresistances (7,8). Disturbance of p53-mediated transcriptional activation represent another possible explanation concerning cancer development (9) including leukaemia. Induction of the transcriptional activity of p53 by DNA damage or other forms of cellular stress results in apoptosis mediated by the intrinsic (mitochondrial) pathway. Transcriptional activation of p53 stimulates the expression of several Bcl-2 family genes including pore forming protein Bax and multiple BH3-only proteins, e.g. Bid, Noxa, and PUMA, that facilitate forming of the pore (10). In addition to elevating the levels of proteins that mediate the release of cytochrome c, p53 stimulates apoptosis by a transcription-independent pathway (11). The molecular mechanism by which p53 activates apoptosis independent of transcription apparently involves the direct binding of p53 to one or more anti-apoptotic mitochondrial proteins, e.g. Bcl-xL, thereby inhibiting their ability to suppress Bax- or Bak-mediated pore formation and cytochrome c release (12,13). Regarding acute leukaemia, several other anti-apoptotic mechanisms, including expression of heat shock protein 70 (hsp70)(14) or myeloid cell leukaemia-1 (MCL1)(15), should be considered. In addition, recent studies have documented a relation between metabolic profiles of cancer cells, i.e. enhanced glycolysis, and resistance of these cells to mitochondrial apoptosis (16).

Apoptosis plays an important role in haematopoiesis by controlling haematopoietic stem cell numbers and eliminating non-differentiated haematopoietic cells (17). In this process, Bax appears to play a crucial role since elimination of the *Bax* gene is associated with increased number of lymphocytes (18). It is generally approved that AML and ALL are caused by different genetic aberrations (19,20), which are probably leading to the activation of p53. Therefore, the aim of our work was to study the transcription of genes of p53-dependent apoptosis in relation to acute leukaemia. In addition to pro-apoptotic p53 and Bax, transcription of anti-apoptotic genes such as *Bcl2* and *hsp70.1,* and *Bcl-x* and *MCL1* genes producing pro- and anti-apoptotic proteins, were investigated. Finally, the transcription of *glyceraldehyde-3-phosphate dehydrogenase (gapdh)* gene was investigated, due to the relationship between cancer development and a shift in cellular metabolism.

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Key words: acute leukaemia, apoptosis, p53, transcription, alternative processing.

Materials and methods

Patients. Twenty-two consecutive AML and seven ALL patients were included in this study. They were not selected on the basis of any risk factors or cytogenetic data. The diagnosis of AML and ALL was based on the French-American-British (FAB) classification.

Cell preparation. After obtaining informed consent, clinical samples of whole blood (WB) were obtained from 16 healthy volunteers and patients at diagnosis prior to treatment and during relapse prior to re-induction treatment. Mononuclear cells (MNCs) were separated from WB by centrifugation using lymphocyte separation medium LSM 1077 (PAA Laboratories) according to manufacturer's protocol. Regarding patients, the blast cells in the separated MNCs amounted to more than 90% in most cases.

Isolation of total RNA and semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from isolated MNCs using TRIzol reagent (Invitrogen) following the manufacturer's protocol. Total RNA (3 μg) was reversely transcribed to cDNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to protocol supplied by the manufacturer. Aliquots of resulted cDNA corresponding to 0.35 μg of total RNA were used in PCR reaction. Sequences of primers used for the amplification of particular mRNA were designed and verified using a nucleotide database of National Center for Biotechnology Information. To avoid possible influence of genomic DNA primers were located in different exons of particular genes. Sequences of all primers used in this study are shown in Table I. Amplification of the cDNAs was initiated by denaturation at 95˚C for 2 min, followed by PCR cycles (denaturing at 95˚C for 20 sec, annealing at 60˚C for 40 sec, and extension at 72˚C for 40 sec) and a final extension at 72˚C for 5 min in a DNA thermal cycler (Biometra). The number of cycles (Table I) for each reaction was set up experimentally to observe a linear increase in the intensity level of PCR amplicons. The PCR products were electrophoresed through a 2% agarose gel and then stained with ethidium bromide. Semi-quantification of the photographic signals was performed using GeneTools image analysis system (SynGene, UK). The entire width of the lane was analyzed with appropriate background subtraction. The relative mRNA level was defined as a ratio of the particular amplicon signal to that of ß-actin.

Single-stranded conformation polymorphism (SSCP) gel analysis of the Bax gene mutations. The coding sequence of a complete *Bax* gene was divided into two parts using specific primers (Table I) and amplified by RT-PCR as described above. After PCR, amplicons were diluted 1:1 in 2X SSCP dye (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamid, 20 mM EDTA pH 8.0) and denaturated for 10 min at 95˚C. Following denaturation, samples were immediately chilled in an ice bath and then applied on 12% polyacrylamide gels. Separation was carried out at room temperature for 4 h at a constant voltage. Gels were stained with ethidium bromide and visualised using the GeneTools image analysis system.

Statistical methods. Statistical analyses were performed using GrafPhad InStat V2.04a (GrafPhad Software). For the comparison of changes among all groups, a one-way ANOVA test was first carried out to test for differences among all experimental groups. Additionally, the unpaired Tukey's test was used to determine differences between individual groups. Significance level was set at $p<0.05$.

Results

In order to study the transcription of genes of p53-dependent apoptosis, levels of p53 and Bax mRNA in normal and leukaemic cells isolated from AML and ALL patients were investigated. As shown in Fig. 1, levels of p53 mRNA are significantly elevated in AML (1.19±0.16, $p<0.001$) and ALL cells (1.14±0.15, $p<0.05$) compared with the p53 mRNA level in normal cells (0.9±0.15). Levels of Bax mRNA are even more elevated in AML (3.0±0.41, $p<0.001$) and ALL cells (2.39±0.33, $p<0.001$) as compared to the Bax mRNA level in normal cells (1.34±0.24).

Since transcription of the *Bax* gene is controlled by p53 protein we investigated the correlation between the level of p53 mRNA and the level of Bax mRNA in AML and ALL cells. Significant positive linear correlation ($p<0.0001$) between p53 mRNA level and Bax mRNA level was observed (Fig. 2).

Mutations of the *Bax* gene have been documented in several cell lines derived from acute leukaemia cells (21-23). Therefore, we investigated possible mutations of the *Bax* gene of leukaemia cells by PCR-SSCP (Fig. 3). Any mutation of the *Bax* gene was detected in all investigated samples.

Since Bcl-xL and Bcl-2 proteins are potent inhibitors of p53-induced apoptosis we determined the mRNA level in normal and leukaemic cells. Regarding the *Bcl-x* gene, PCR primers were set up in a way, discriminating between alternative spliced long variant of mRNA (producing anti-apoptotic Bcl-xL protein) and short variant of mRNA (producing pro-apoptotic Bcl-xs protein). As with Yamaguchi and co-workers (24), we observed two amplicons corresponding to Bcl-xS and Bcl-xL mRNA (Fig. 4a). Notably (Fig. 4b), a significantly higher level of Bcl-xL mRNA was observed in ALL cells (4.05±0.48, $p<0.001$) compared to the level of Bcl-xL mRNA in normal cells (2.21±0.28). Although the level of Bcl-xL mRNA in AML cells (2.74±0.83) was higher, it was not significantly different from the level of Bcl-xL mRNA in normal cells. However, higher standard deviation indicates that in some individual cases the level of Bcl-xL mRNA was elevated above normal level. Since we did not discriminate between different cytogenetic backgrounds, the differences in Bcl-xL mRNA level may be attributed to different cytogenetics (25). Levels of Bcl-xS mRNA are elevated in AML (0.49±0.31, $p<0.05$) and ALL cells (1.0±0.24, $p<0.001$) compared to the Bcl-xS mRNA level in normal cells (0.25±0.07). Similar to Bcl-xL mRNA, levels of Bcl-xS mRNA varied significantly among different AML patients. In the case of ALL, levels of Bcl-xS correlated with levels of Bcl-xL mRNA, indicating increased transcription of the *Bcl-x* gene. However, levels of Bcl-xS were independent from levels of Bcl-xL mRNA in AML cells, indicating altered alternative processing of Bcl-x primary transcripts in

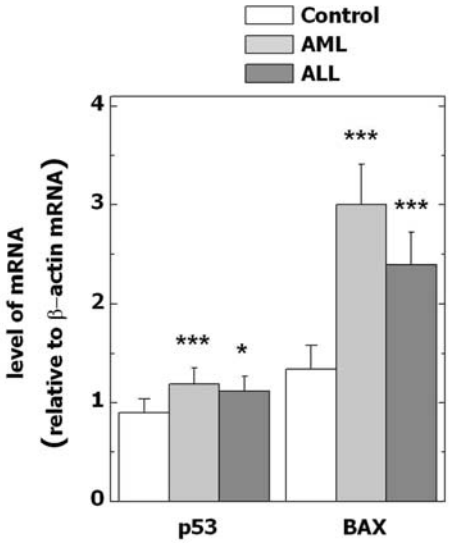

Figure 1. Relative levels of p53 and Bax mRNA in normal, AML and ALL cells.

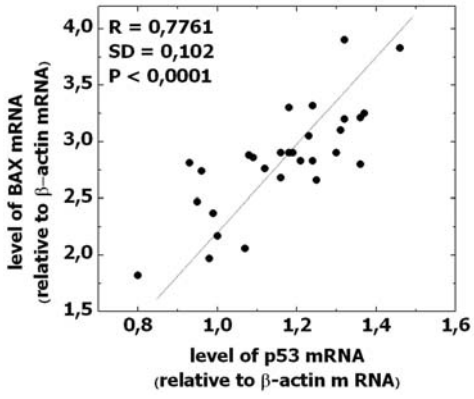

Figure 2. Correlation between p53 and Bax mRNA levels in AML and ALL cells.

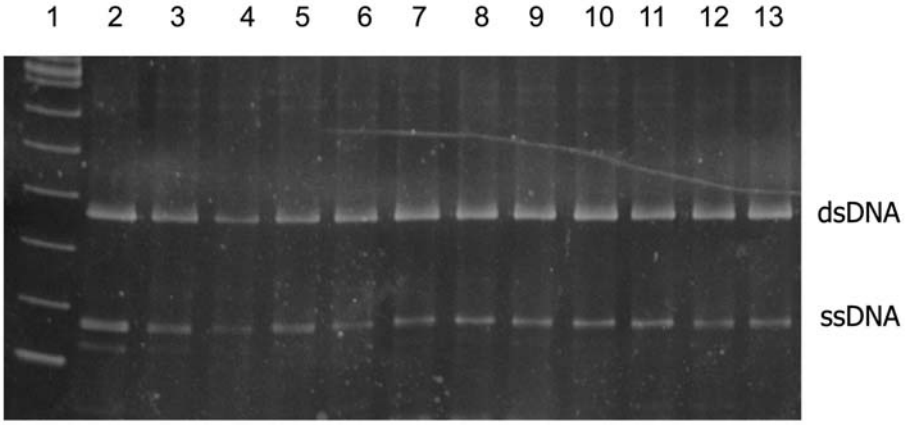

Figure 3. Representative gel of PCR-SSCP analysis of *Bax* gene mutations. Lane 1, 100 bp DNA ladder; lane 2, analysis of Bax5 amplicon of control samples; lanes 3-13, analysis of bax5 amplicon of acute leukaemia samples. ssDNA, single stranded DNA; dsDNA, double stranded DNA.

the case of AML: Although Bcl-2 knockout mice exhibited loss of lymphocytes (26), levels of Bcl-2 mRNA were not significantly different in AML (0.38±0.03) and ALL cells (0.26±0.02) compared to the Bcl-2 mRNA level in normal cells (0.24±0.07).

MCL1 represents another important protein of the Bcl-2 family involved in apoptosis. Conditional knockout in lymphocytes showed complete loss of affected cells (27). Likewise Bcl-x, alternative splicing of MCL1 primary transcript produces two different mRNAs, giving rise to two isoforms with opposite activity regarding apoptosis. While the long isoform is anti-apoptotic, the short isoform promotes apoptosis (28). PCR yields two products corresponding to both isoforms of MCL1 protein (Fig. 5a). As shown in Fig. 5b, the level of long variant of MCL1 mRNA in AML (3.45±0.45) and ALL cells (2.87±0.33) was not significantly different from those in normal cells (3.23±0.43). Notably, the level of short variant was significantly decreased in AML (0.52±0.16, $p<0.05$) and ALL cells (0.41±0.09, $p<0.001$) in comparison to normal cells (0.72±0.11), indicating that acute leukaemia is associated with changes in alternative splicing of the MCL1 primary transcript.

Recent evidence has been documented showing that hsp70 inhibits apoptosis by interfering with events upstream of mitochondrial membrane permeabilisation that ultimately decrease the activation of Bax (29,30). Therefore, we investigated the transcription of the inducible *hsp70.1* gene in normal and leukaemic cells. However, the level of hsp70.1 mRNA in AML (1.67±0.54) and ALL cells (1.8±0.36) was not statistically different from the level of hsp70.1 mRNA in normal cells (2.03±0.2)(Fig. 6). Since a recent study has postulated a hypothesis that the unique metabolic profile of cancer (enhanced glycolysis) might confer apoptosis resistance (16), transcription of the *gapdh* gene in leukaemic and normal cells was investigated. Notably, the level of gapdh mRNA was elevated in AML cells (1.55±0.62) compared to normal cells (1.28±0.15), however, the difference was not statistically significant. Similarly, non-

Table I. Sequences of primers, size of amplicons and number of amplification cycles.

Gene	Primer sequence (5´-3´)	Amplicon size (bp)	No. of cycles
Bcl-x	Forward: CTG GTG GTT GAC TTT CTC TCC Reverse: GCT GCT GCA TTG TTC CCA TAG	Short: 392 Long: 582	28
Bcl-2	Forward: GGC CTT CTT TGA GTT CGG TGG Reverse: GAT AGG CAC CCA GGG TGA TGC	Variant α: 159 Variant ß: 263	40
Bax 5' end	Forward: TGG ACG GGT CCG GGG AGC Reverse: GCA CAG GGC CTT GAG CAC C	377	40
Bax 3' end	Forward: GCC CTT TTC TAC TTT GCC AGC Reverse: TCA GCC CAT CTT CTT CCA GAT	246	30
MCL1	Forward: CGG GGA ATC TGG TAA TAA CAC C Reverse: CCT CTA CAT GGA AGA ACT CCA C	Short: 279 Long: 527	35
p53	Forward: CCT CCT GGC CCC TGT CAT CTT Reverse: ACC TCC GTC ATG TGC TGT GAC	250	28
Hsp70	Forward: CGACCTGAACAAGAGCATCAATC Reverse: CTTGTCGTTGGTGATGGTGATCT	451	35
Gapdh	Forward: GAGCTGAACGGGAAGCTCACTGG Reverse: CAACTGTGAGGAGGGGAGATTCAG	459	25
ß-actin	Forward: GGGTCAGAAGGATTCCTATG Reverse: GGTCTCAAACATGATCTGGG	238	28

significant difference was observed between ALL (1.07±0.25) and normal cells (Fig. 6). Likewise in the case of Bcl-xL, high standard deviation indicates that significant differences in gapdh mRNA exist among individual cases of AML. Therefore, to investigate a possible relationship between *Bcl-xL* transcription and *gapdh* transcription we have investigated the correlation between gapdh and Bcl-xL mRNA level in AML cells. We did not observe any significant correlation between Bcl-xL mRNA and gapdh mRNA (not shown). Thus, it seems that no relationship exists between transcriptions of these genes in AML cells.

Discussion

The main finding of our study was that the transcription of *p53* and *Bax* genes is elevated in the leukaemic cell compared to normal leukocytes. The elevated level of p53 mRNA indicates possible changes in genomic DNA integrity associated with acute leukaemia. Several chromosomal abnormalities that are considered to be main reason of leukaemia development were observed in association with acute leukaemia (19,20). However, transcription of a particular gene represents only one step in serial events involved in the process of production of fully active protein. It is generally approved that the activation of p53, triggering the transcription of genes that are involved in DNA repair, growth arrest and apoptosis, is controlled on a post-translational level (31). The low frequency of p53 mutations associated with acute leukaemia documented previously (4) and our results are more in favour that p53 is transcriptionally active in leukemic cells. In support of this, significantly elevated levels of Bax mRNA and a positive correlation between p53 and Bax mRNA levels was documented in AML and ALL cells. Other ways to disrupt the p53 pathway

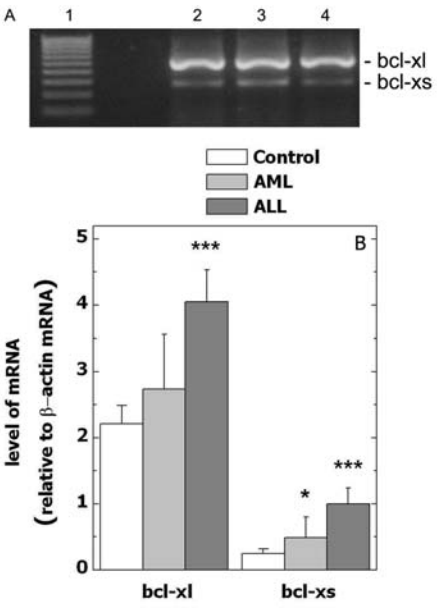

Figure 4. (A) Representative gels showing RT-PCR analysis of *Bcl-x* transcription. Lane 1, 100 bp DNA ladder; lanes 2-4, Bcl-xL and Bcl-xS detected in acute leukaemia samples. (B) Relative levels of Bcl-xL and Bcl-xS mRNA in normal, AML and ALL cells.

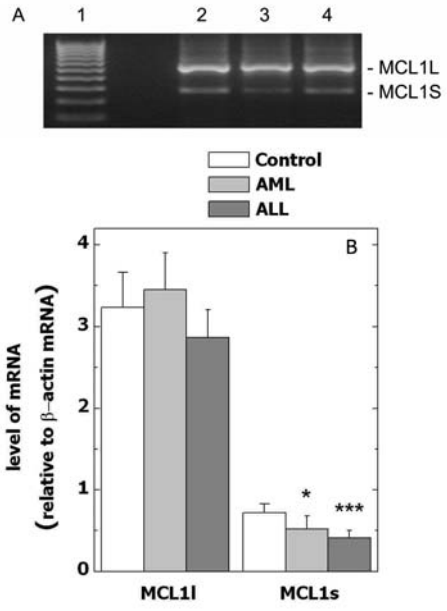

Figure 5. (A) Representative gels showing RT-PCR analysis of *MCL1* transcription. Lane 1, 100 bp DNA ladder; lanes 2-4, MCL1l and MCL1s detected in acute leukaemia samples. (B) Relative levels of MCL1l and MCL1s mRNA in normal, AML and ALL cells.

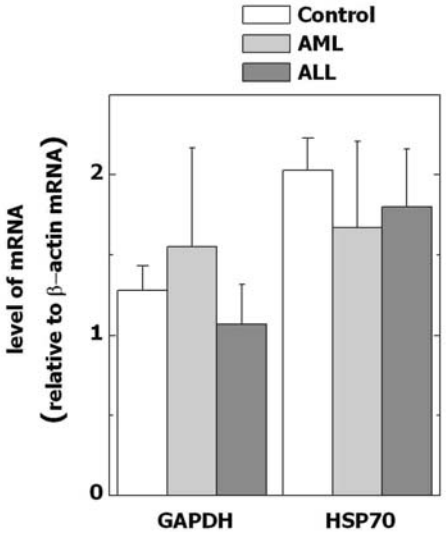

Figure 6. Relative levels of gapdh and hsp70.1 mRNA in normal, AML and ALL cells.

in cancer includes over-expression of murine double minute 2 protein (MDM-2). The level of p53 protein is negatively controlled by MDM-2, which works as ubiquitin ligase, while active p53 increases MDM-2 at a transcriptional level (32). Over-expression of MDM-2 due to hitherto unknown reasons might play a role in some haematopoietic malignancies including acute leukaemia. MDM-2 has been documented to be over-expressed in ~50% cases of AML (33,34) and in childhood ALL, where it was associated with a poor clinical outcome (35-37). However, the possibility that over-expression of MDM-2 is involved in the dysregulation of p53-dependent apoptosis was ruled out since a significant several-fold increase of Bax mRNA level was documented in all investigated cases. Thus, our results open the possibility that p53-dependent apoptosis in the case of acute leukaemia is initiated at least at the level of Bax transcription, however, is not executed. Apoptosis plays an important role in haematopoiesis by controlling haematopoietic stem cell numbers and eliminating non-differentiated cells (38). In addition, evading apoptosis represents one of the hallmarks of cancer cells (39), including leukaemic (17), and inhibition of anti-apoptotic proteins is associated with either restoring the normal apoptotic processes in tumour cells or increasing their sensitivity to chemotherapy (40). Although mutations of the *Bax* gene associated with apoptosis dysregulation were observed in some cell lines derived from acute leukaemia cells (21-23), our experiments did not reveal any mutation of the *Bax* gene in the cases investigated. It seems that the frequency of *Bax* gene mutations is rather low regarding acute leukaemia. Apoptotic activity of Bax protein is significantly affected by a conformational state and oligomerisation of the protein but the mechanisms leading to Bax activation are still not completely known (41). Therefore, transcription of other gene products which are important players in the p53-dependent apoptosis, was investigated. Activation of Bax is opposed by pro-survival anti-apoptotic proteins: MCL1l, Bcl-xL and Bcl-2 (42). The level of Bcl-xL mRNA was significantly elevated in MNCs isolated from ALL patients but not from AML patients. It has been demonstrated that Bcl-xL is capable of protecting cells from p53-mediated apoptosis, and tumours expressing Bcl-xL are able to partly overcome the tumour suppressor functions of p53 (43). Although mRNA level of pro-apoptotic Bcl-xS was also significantly increased, we supposed that increased Bcl-xL mRNA level might be associated with dysfunction of the p53-dependent apoptosis in the case of ALL. This idea is supported by the fact that ALL is preferentially treated by

glucocorticoids, which induce apoptosis of leukaemic cells in a p53-independent manner (44). In addition, elimination of the *Bcl-x* gene in knockout mice led to shortening of the life-span of immature lymphocytes while the life-span of mature lymphocytes was not affected (45). We also found elevated levels of Bcl-xL mRNA in some cases of AML, but the average level was not significantly different to the level in control cells. It was documented previously that expression of Bcl-xL in AML cell depends on the cytogenetic background of AML (25). Whether over-expression of Bcl-xL in some individual cases modulates sensitivity of AML cells to apoptosis is unclear and needs further investigation. In addition, we found elevated levels of Bcl-xS mRNA in AML cells. Association of increased levels of Bcl-xS mRNA with prolonged disease-free intervals and overall survival was documented previously (24), indicating the important association of *Bcl-x* gene products with chemotherapy-induced cell death. In addition to Bcl-x, MCL1 is another Bcl-2 family protein discovered as an early induction gene during myeloblastic leukaemia cell differentiation (15). Alternative splicing occurs at this locus and two transcript variants encoding distinct isoforms have been identified. The longer gene product (MCL1l) enhances cell survival by inhibiting apoptosis while the alternatively spliced shorter gene product (MCL1s) promotes apoptosis and is death-inducing. The naturally occurring MCL1s variant represents a BH3 domain-only protein capable of dimerising with the anti-apoptotic MCL1l. Thus, the fate of MCL1 expressing cells may be regulated through alternative splicing mechanisms and interactions of the resulting anti- and pro-apoptotic gene products (28). We found that the level of short variant of MCL1 mRNA was significantly depressed in AML and ALL cells. However, the relatively small difference between normal and leukaemic cells does not favour the idea that a decreased level of MCL1s mRNA is associated with apoptosis dysregulation. In order to explain possible cause of mitochondrial apoptosis dysregulation in AML cells, we have shown that acute leukaemia is not associated with the increased transcription of the *hsp70.1* gene, which product is considered to be a potent inhibitor of mitochondrial apoptosis (29,30). Notably, transcription of *gapdh* was significantly increased in leukaemic cells isolated from AML patients. Induction of glycolysis represents another hallmark of cancer cells (39). In addition, recent studies have reported that increased glycolysis observed in malignant diseases is often associated with defects of mitochondria (16). But, whether changes in the metabolic profile of cancer cells are associated with dysregulation of mitochondrial apoptosis is unclear. In our experiments we discovered significant diversity of Bcl-xL and gapdh mRNA levels between individual cases of AML. However, we did not find any relationship between mRNA levels of Bcl-xL and gapdh. Thus, the association between transcription of *gapdh* and Bcl-xL mRNA levels seems to be unlikely in the case of AML.

In conclusion, we have demonstrated that acute leukaemia, AML and ALL, are associated with changes in the transcription of some genes whose products are involved in p53-dependent apoptosis. Significantly elevated levels of Bax mRNA in MNCs isolated from AML and ALL patients open the possibility that p53-dependent apoptosis in the case of acute leukaemia is initiated at least at the level of Bax transcription but is not executed. Regarding ALL, significantly elevated Bcl-xL mRNA may explain the relative resistance of ALL cells to p53-dependent apoptosis. It seems that molecular mechanisms involved in dysregulation of p53-dependent apoptosis in the case of AML are more complex and other possibilities such as activation of phosphoinositide 3-kinase/ Akt signalling pathway (46) should be considered.

Acknowledgements

This work was supported by the Ministry of Education of Slovak Republic (grant no. aAV/1106/2004).

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