Bcl-2 protein level in blood of patients with acute myeloid leukaemia

Bruno Baudin [1,3], Françoise Isnard [2], Agnès Mailloux [1], Patricia Zunic [2], Abdelkrim Tahraoui [1,4], Bénédicte Bénéteau-Burnat [1], Laurent Garderet [2] et Michel Vaubourdolle [1]

[1)] Service de Biochimie, [2)] Service d'Hématologie Clinique, Hôpital Saint-Antoine, AP-HP, 184 rue du Faubourg Saint-Antoine, 75571 Paris Cedex 12, France.
[3)] JE-2493, Faculté de Pharmacie - Châtenay-Malabry, France.
[4)] Laboratoire de Biologie Animale Appliquée, Département de Biologie, Faculté des Sciences, Université Badji Mokhtar, Annaba 23000, Algérie.

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ملخص

بحثنا عن العلاقة بين البروتين bcl-2 في الدم و وجود اللوكيميا النخاعية الحادة و المعبر عنها بالمختصر (LMA) والبروتين bcl-2 يعتقد أن له دور في السرطان. تمت دراسة عينات من الدم المأخوذ من 28 مريضا مصابون بـLMA ناتجة عن حالات مختلفة وقورنت ب 25 عينة غير مصابة و ليس لها نسب بالمرضى. في بعض العينات قسنا البروتين p53 في العينات الـ 25، bcl-2 كانت (90,6 ± 27,8 U/ml) بينما البروتين p53 لم يتم اكتشافها إلا عند 72 % من العينات (0,15 ± 0,17 ng/ml)؛ النتيجة تظهر أن bcl-2 و p53 تتناسب عكسيا. نسبة bcl-2 كانت مرتفعة أكثر عند المصابين بـ LMA الثانوية. العينات الـ 28 أظهرت أن bcl-2 تتناسب مع اللبنات المزهرجة و مع النمط الوراثي mdr-1. اما p53 فلم يتم العثور عليها إلا عند مصاب واحد بـ LMA. أما المواد النشطة لعمل الأجسام فكانت مرتفعة عند كل العينات المصابة مقارنة بالغير مصابة. تظهر هذه الدراسة ان الاعتماد على هذا البروتين لكشف الإصابة بـLMA غير ناجع، وبقي البروتين bcl-2 مهم لدراسة أمراض أخرى متفاقمة، متدنية او مؤجلة.

الكلمات المفتاحية: بروتين *bcl-2*؛ اللوكيميا النخاعية الحادة؛أعراض مرض الميلوديسبلاستيك؛ بروتين *p53*.

Résumé

Nous avons recherché la relation entre la protéine bcl-2 dans le sang et l'existence d'une leucémie myéloïde aiguë (LMA), bcl-2 étant une protéine anti-apoptotique incriminée dans le cancer. Des échantillons sanguins ont été prélevés chez 28 patients présentant une LMA, soit *de novo* soit à la suite d'un syndrome myélodysplasique, et chez 25 contrôles sains non reliés. Dans quelques échantillons, la protéine p53 pro-apoptotique et les oligonucléosomes ont aussi été mesurés. Chez les contrôles, bcl-2 était de 90,6 ± 27,8 U/ml, alors que p53 n'a été détectée que dans 72 % des échantillons (0,15 ± 0,17 ng/ml); bcl-2 et p53 étaient inversement corrélées. Les oligonucléosomes ont été détectés dans tous les échantillons contrôles mais à un niveau faible et sans corrélation à bcl-2 ni p53. Le niveau de bcl-2 était plus élevé chez les patients avec LAM que chez les contrôles, et même plus élevé chez les patients avec une LAM *de novo* que chez ceux avec une LMA secondaire. Chez l'ensemble des patients, bcl-2 était corrélée à la lactate-déshydrogénase et aux leucocytes sanguins, mais plus faiblement aux blastes, et pas du tout au caryotype, à l'antigène cd-40 et au génotype mdr-1. p53 n'a été détectée que chez un patient avec LAM et les oligonucléosomes étaient plus élevés chez les patients que chez les contrôles. Etudiée chez 12 patients sans mauvais pronostic cytogénétique, le niveau de bcl-2 était corrélé à un mauvais dénouement. Ces résultats suggèrent que les blastes qui sur-expriment bcl-2 sont devenus résistants à l'apoptose, qualifiant cette protéine de facteur de mauvais pronostic dans la LAM. De plus, la détermination des valeurs normales de la protéine bcl-2 pourrait être utile aux études portant sur diverses maladies où l'apoptose est soit augmentée, soit diminuée ou encore différée.

Mots clés : *proteine bcl-2; leucémie myéloïde aiguë; apoptose; syndrome de myelodysplastique;proteine p53.*

Abstract

We investigated the association between blood bcl-2 protein and the occurrence of acute myeloid leukaemia (AML), bcl-2 being an anti-apoptotic protein incriminated in cancer. Blood specimens were collected from 28 patients with AML, either *de novo* or following myelodysplastic syndrome, and from 25 healthy unrelated

Auteur correspondant: bruno.baudin@sat.aphp.fr (Bruno Baudin)

controls. In some specimens, the pro-apoptotic p53 protein and oligonucleosomes were also determined. In controls, bcl-2 was at 90.6 ± 27.8 U/ml, whereas p53 was detected in only 72 % of the specimens (0.15 ± 0.17 ng/ml); bcl-2 and p53 levels were inversely correlated. Oligonucleosomes were detected in all the controls but at low level and with no correlation to bcl-2 or p53. Bcl-2 level was higher in AML patients than in controls and some more increased in patients with *de novo* AML than in patients with secondary AML. In overall patients, bcl-2 correlated to lactico-dehydrogenase and to blood leukocytes, but weakly to blasts, and not to caryotype, cd-34 antigen and mdr-1 genotype. p53 was detected in only one patient with AML and oligonucleosomes were higher in patients than in controls. Studied on 12 patients without cytogenetic poor prognosis, bcl-2 level correlated to bad outcome. These data suggest that blasts that over express bcl-2 are resistant to apoptosis, defining this protein as a factor of bad prognosis in AML. Moreover, the determination of normal ranges for bcl-2 protein may be instrumental in the study of various diseases where apoptosis is enhanced, disrupted or delayed.

Key words: *bcl-2; acute myeloid leukaemia; apoptosis; myelodysplastic syndrome; p53.*

1. INTRODUCTION

Programmed cell death, or apoptosis, is an important physiological process in most, if not all, multicellular organisms, and in particular during development and for homeostasis in adult organisms. For example, in the immune system, B and T lymphocytes are removed when they fail to see foreign antigens; animals have evolved mechanisms to protect against viral infections by targeting infected cells for death; apoptosis may also be used to minimize the risk from cells frequently subjected to mutagenic chemicals or radiation [1]. With cell death being a normal part of so many different systems, the machinery that carries it out must be properly regulated to maximize benefit to the individual; when this regulation is disturbed, disease can result. Both inhibition of apoptosis and inappropriate cell death may be deleterious. For example, degenerative neurological diseases, such as Alzheimer disease and Parkinson disease, are associated with the premature death of particular subsets of neurons [1,2] ; the death of T cells in AIDS resembles physiological apoptosis [3] ; and the rejection of transplants may, in part, be from apoptotic mechanisms [4]. Conversely, inhibition of apoptosis may contribute to promote autoimmune disease by allowing persistence of self-reactive B and T cells [5,6] ; most importantly, cancer may result when cells that fail to die undergo further mutations, leading to a transformed state. Otherwise, in the treatment of cancers and leukaemia, most of the chemotherapeutic agents exert their cytotoxic effect by induction of apoptosis [7,8]; therefore, poor response to induction therapy or persistence of residual disease responsible for following relapses may be caused by resistance of tumour cells or leukaemia blasts to induction of apoptosis. Two major endogenous regulators of apoptosis were identified, i.e. p53 and bcl-2 proteins. The wild-type p53 protein functions as an inductor of cell death, especially in response to DNA damaging events [9] ; corollary, aberrations of the *p53* gene are the most commonly detected abnormalities in human cancer and loss of p53-dependent apoptosis correlates with tumour aggressiveness [7]. *Bcl-2* (B-cell lymphoma 2) is a proto-oncogene encoding of a protein with anti-apoptotic functions; in particular, from its location in the outer membrane of mitochondria, bcl-2 protein suppresses signals that induce apoptotic cell death and, in many cancers, bcl-2 expression is correlated to tumoural or blastic proliferative capacity. In particular blasts of acute myeloid leukaemia (AML), with autonomous proliferation in culture, express high levels of bcl-2 protein and have increased resistance to chemotherapy [10,13]. Higher expression, in bone marrow mononuclear cells, of pro-apoptotic bcl-2

family proteins (such as bak, bad and bcl-x_S) was associated with longer survival whereas expression of anti-apoptotic proteins (such as bcl-2 and bcl-x_L) was associated with decreased survival in patients with myelodysplastic syndromes [14]. Otherwise, high expression of bcl-2 protein or *bcl-2* mRNA in bone marrow appeared as determinant of poor prognosis in AML [15-17]. But high expression of bcl-2 protein in lymphoblasts from patients, adults or children, with acute lymphoblastic leukaemia was not correlated with a bad prognosis or a poorer response to therapy [18,19].

In this study, we investigated the association between blood bcl-2 protein and the occurrence of AML. Bcl-2 levels were compared to those of p53 protein and to the appearance of oligonucleosomes in blood. Bcl-2 data were also correlated to usual parameters of prognosis in AML such as cd-34 antigen and caryotype.

2. MATERIALS AND METHODS

2.1 Sample collection

Blood samples were collected from 25 apparently healthy adults (17 women and 8 men, mean age 33.4 ± 9.7 years, range 23-50 years), in particular without cardiac, renal or neurological disorders, and no known neoplasm or recent infectious disease (control group). 28 patients (26 - 72 years old) with AML were divided into two groups: group 1 consisted of 17 patients with *de novo* AML (9 women and 8 men, mean age 53.4 ± 14.8 years) and group 2 consisted of 11 patients with AML following myelodysplastic syndrome or chronic myeloid leukaemia, i.e. secondary AML (5 women and 6 men, mean age 55.5 ± 11.8 years). The slight difference between control and patient groups as regards elderly individuals might not influence the results; nevertheless the practical use of the proposed biomarkers must be restricted to patients under 60 years before confirmation of these data on larger cohorts. Blood samples were collected by vein-puncture into EDTA-containing tubes and immediately processed with specific lysis solutions as stated by the manufacturers. Briefly, (i) for bcl-2, 500 µL of blood were mixed with 100 µL of lysis solution from Endogen, homogenized then frozen at -80°C until determination; (ii) for p53, 200 µL of blood were mixed with 20 µL of lysis solution from Calbiochem, homogenized, then at rest on ice for 30 min, further centrifuged (12.000 g x 3 min.) and supernatant was frozen at -80°C; (iii) for oligonucleosomes, 200 µL of blood were mixed with 400 µL of lysis solution from Boehringer, homogenized, then at rest for 30 min at room temperature, centrifuged and treated as for p53.

2.2 Methods for bcl-2, p53 and oligonucleosomes quantification

Bcl-2 and p53 proteins were quantified by specific ELISAs using monoclonal antibodies recognizing the human proteins, in sandwich assays and with peroxydase activity revelation at 450 nm; i.e. Cytoplus bcl-2 ELISA kit (DK1210) from Endogen, Bio-Advance (Emerainville, France) and Pantropic p53 rapid format ELISA kit (QIA26) from Calbiochem (La Jolla, CA). Bcl-2 quantification was linear between 5 and 200 U/ml and the limit of detection was 0.01 ng/mL for p53 quantification. DNA fragmentation was detected by an ELISA specific for histone-associated DNA fragments or oligonucleosomes (Cell Death Detection kit from Boehringer-Mannheim, Germany). Oligonucleosomes were expressed as the variation of absorbance between 405 and 490 nm ($U = A_{405} - A_{490}$) and with a detection limit at 0.1 U; a positive control was at 2.1 ± 0.2 U [20]. Oligonucleosomes and p53 were measured in all the controls but in only 11 patients with AML.

2.3. Other determinations

Plasma lactico-dehydrogenase activity (LDH) and blood leukocytes were determined using standard methods. Medullar cells were collected from patients by biopsy for AML diagnosis and prognosis with (i) caryotype determination using the banding method and after 24 hours cell culture (in particular, in group 1, when 5 patients had a abnormal caryotype related to poor prognosis, 12 patients had a normal caryotype or minor modifications recognized as of better prognosis), (ii) cd-34 antigen determination as analysed by immunofluorocytometry using a specific monoclonal antibody (the expression was considered positive when more than 20 % of the medullar mononuclear cells bound the antigen), and (iii) genotyping of the *Multidrug Resistance* gene 1 (mdr-1) as performed by Reverse Transcriptase Polymerase Chain Reaction after RNA extraction from the medullar mononuclear cells (results were semi-quantitative from 0 to 5 +).

2.4. Statistics

Quantitative data are expressed as mean plus or minor standard deviation (m ± SD) and Mann-Whitney (non parametric) U-test was used for statistical analysis.

3. RESULTS

The bcl-2 protein was detected in blood of all the individuals of the control group and with the mean concentration of 90.6 ± 27.8 U/ml (range from 40 to 154 U/ml); no statistical significant difference was shown between males and females (93.0 ± 23.7 versus 89.5 ± 30.3 U/ml, respectively) (fig. 1a), nor with age. The p53 protein was detected in 18 healthy subjects (72 %) and, when positive, only weakly expressed and with large inter-individual differences (0.15 ± 0.17 ng/ml, range from < 0.01 to 0.56 ng/ml); p53 levels were higher in men (0.27 ± 0.20 ng/ml) than in women (0.105 ± 0.125 mg/ml, $p<0.05$) (fig. 1b). Bcl-2 and p53 levels were inversely correlated ($r = -0.403$, $p<0.05$) (fig. 2), but they were not correlated to blood leukocytes. Oligonucleosomes could be detected in all the individuals but at low level (0.212 ± 0.136 U) and without difference in function of sex (0.200 ± 0.116 U for women, 0.239 ± 0.192 U for men, NS) (fig. 1c), or age. Oligonucleosomes were not correlated to bcl-2 or p53 level.

Bcl-2 was detected in all the patients with AML at diagnosis (groups 1 and 2) and was higher in patients than in controls, whereas p53 was only detected in one patient among 11, and oligonucleosomes were significantly increased in patients in comparison to controls (Table 1).

Table 1. *Bcl-2, p53 and oligo-nucleosomes levels, as determined by ELISAs, in blood of patients with AML versus controls.*

	Controls (n=25)	AML
Bcl-2 (U/ml)	90.6 ± 27.8	483.5 ± 1 025.6 (n = 28)
p53 (ng/ml)	0.15 ± 0.17	ND‸ (n = 11)
Oligonucleosomes (U)	0.21 ± 0.14	1.16 ± 1.07***** (n=11)

‸ND: not detectable in the 11 patients studied but one at 0.07 ng/ml; *****$p < 0.02$

Bcl-2 more drastically increased in group 1 (*de novo* AML) than in group 2 (secondary AML) but without reaching statistical significance, whereas oligo-nucleosomes were significantly more elevated in blood of patients from group 1 than in patients from group 2 (fig. 3). No difference for p53 was shown between groups. In AML patients, bcl-2 level correlated to plasma LDH activity ($r = 0.45$, $p<0.05$) and to blood leukocytes in both groups (fig. 4). Moreover bcl-2

weakly correlated to blasts in group 1, but not in group 2 (fig. 5), and did not correlate to caryotype neither to the expression of cd-34 antigen on blasts or to mdr-1 genotype (not shown).

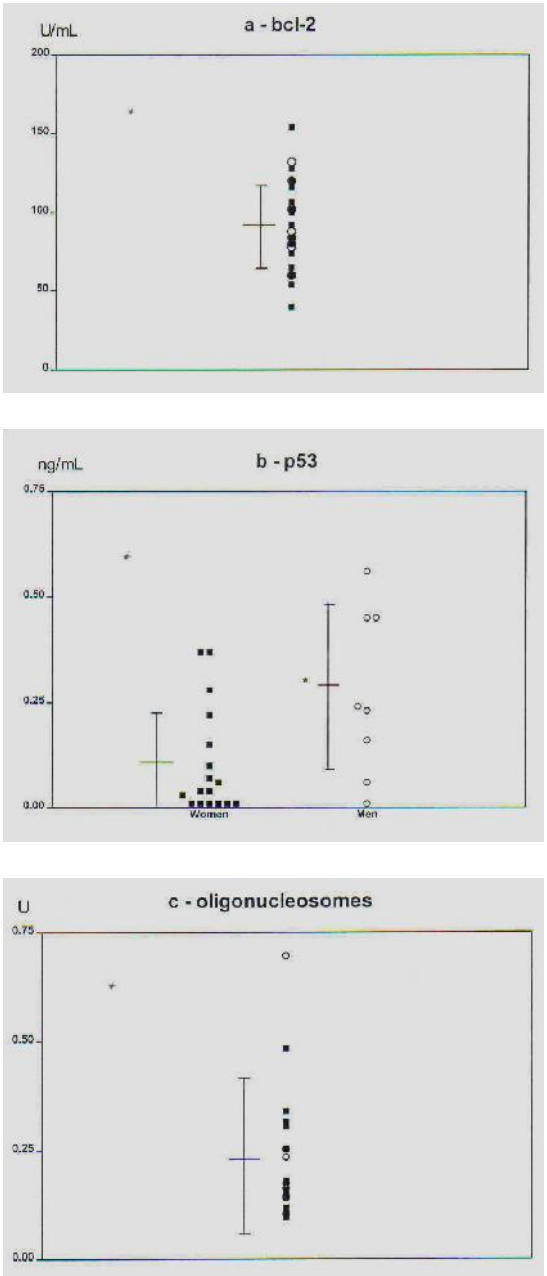

Figure 1. *Distribution of bcl-2 (a), p53 (b) and oligonucleosomes (c) levels in blood of healthy individuals : ■ women (n= 17), ○ men (n= 8),* $^{*}p < 0.05$.

Bcl-2 level did not globally correlate to the response to chemotherapy or to the duration of the remission, but studied on the 12 patients with intermediary caryotype, i.e. without cytogenetic poor prognosis, bcl-2 correlated to bad outcome, in particular to a duration of remission below 18 months and to a fatal outcome below 24 months (p= 0.05 for each).

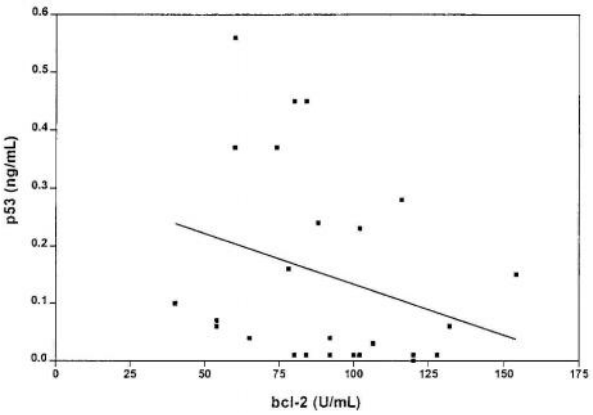

Figure 2. *Inverse correlation between bcl-2 (x) and p53 (y) levels in blood of healthy individuals (n = 25):* $y = -1.77.10^{-3} x + 0.31$.

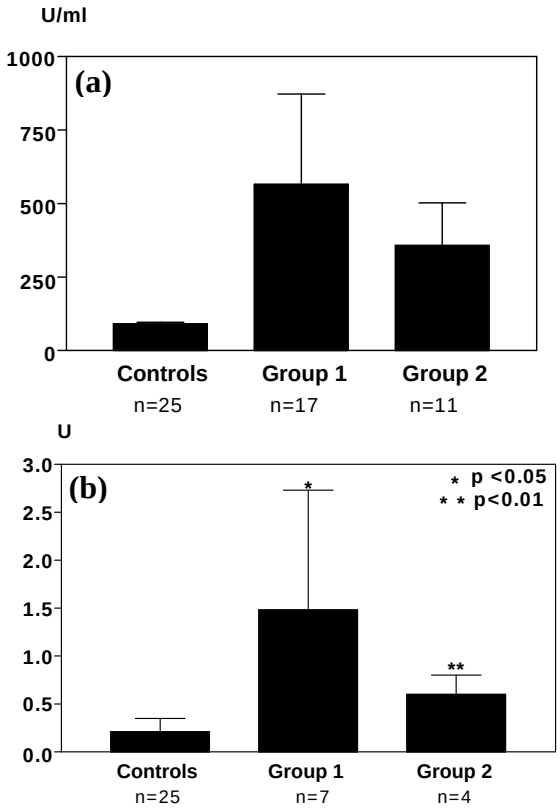

Figure 3. *Bcl-2 (a) and oligonucleosomes (b) in blood of patients with acute myeloid leukaemia (AML) (group 1: de novo AML, group 2: secondary AML) versus controls.*

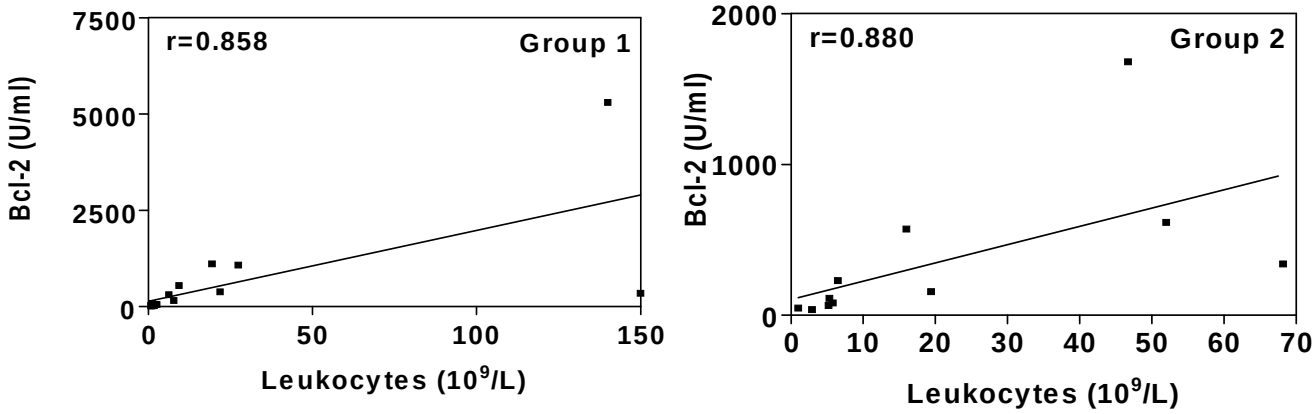

Figure 4. *Correlations between bcl-2 and blood leukocytes in patients with de novo AML (group1) or secondary AML (group2).*

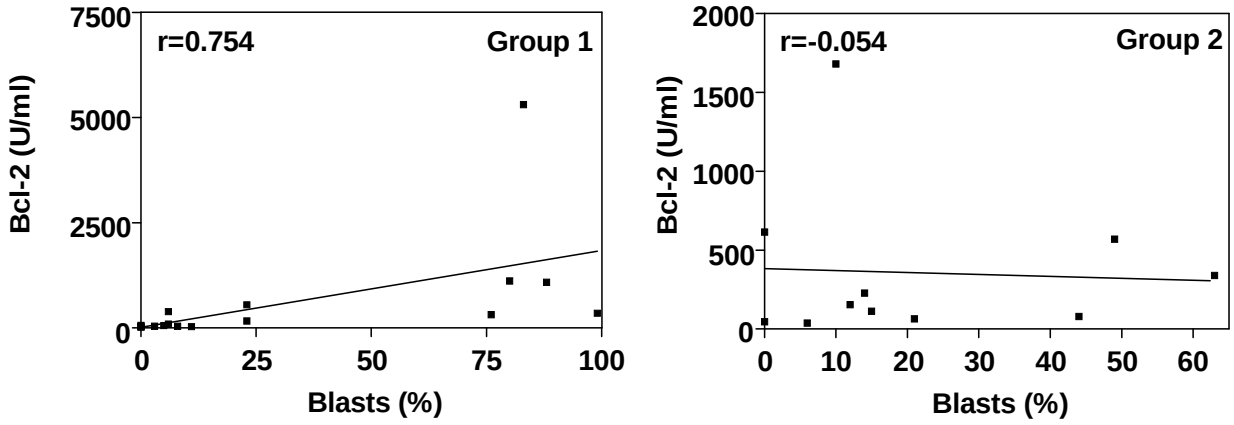

Figure 5. *Correlations between bcl-2 and blasts (percentage of blood leukocytes) in patients with de novo AML (group1) or secondary AML (group2).*

4. DISCUSSION

We have determined bcl-2 protein level in blood of healthy individuals and of patients with newly diagnosed acute myeloid leukaemia; bcl-2 level was compared to that of p53 and to DNA fragmentation, all being putative markers of apoptosis. The first remarkable result is that DNA fragmentation in blood of healthy subjects is weak but not null, that could represent the spontaneous death of leukocytes. This low level of physiological DNA fragmentation can be compared to p53 level that is also low and irregularly found in blood of healthy subjects. The over-expression of p53 protein in blood cells should be easily quantified, in particular in relation to pathological enhanced apoptosis. Conversely, it will be difficult to search a defect in p53 synthesis in human blood, a fail that could be related to pathological states where apoptosis is disrupted or delayed. Levesque et al. [21] also reported by ELISA p53 positivity in non cancerous patients but by analysing sera and with reference interval between 0.04 and 0.35 ng/ml, thus not far from our interval as determined on whole blood. Maybe the most interesting result on the group of healthy subjects is the homogeneous level of bcl-2 protein, and interestingly, bcl-2 and p53 levels inversely correlated

showing a physiological equilibrium between pro- and anti-apoptotic factors. Other apoptotic controlling factors could be investigated, e.g. members of the bcl-2 family such as bax and bcl-x; but so far only few ELISAs are available or tested for human samples. On the other hand the discrepancies found in function of sex should be further studied, especially in relation to hormonal and haematological parameters. The establishment of the normal ranges for bcl-2 and p53 proteins could be useful for clinical studies on human diseases involving apoptosis, such as Alzheimer and Parkinson diseases, AIDS, autoimmune diseases, graft rejection, cancer and their therapies.

The prognostic value of caryotype for *de novo* AML treatment is well recognized [22]; for example, the translocations t(15;17), t(8;21) and inu(16) are of good prognosis with a patient survival between 60 and 70 % at 5 years, whereas abnormalities on chromosome 5 or 7 are of bad prognosis with less than 20 % of survivors at 5 years. The high expression of cd-34 is also a factor of bad prognosis and the genotype of mdr-1 correlates to the response to chemotherapy [23,24] ; in particular, cd-34+ blast cells seem to express significantly higher bcl-2 levels compared with cd-34- cells [25]. We determined bcl-2 and p53 protein levels in blood of patients with newly diagnosed AML in the goal to evaluate these apoptosis regulators as blood determinants of prognosis and of clinical response to chemotherapy. The bcl-2 protein was always detected and was higher in patients, in particular from the *de novo* AML group, than in controls, whereas the p53 protein was not detected but in one patient. Because bcl-2 correlates to white blood leukocytes and is expressed at high level in blasts isolated from patients with *de novo* AML [26], we can argue that cells resistant to apoptosis may circulate in the blood stream. Curiously, an intense apoptosis simultaneously occurred since DNA fragmentation was increased in comparison to controls and p53 level was decreased. Bcl-2 also correlated with plasma LDH, a marker of cytolysis, suggesting that resistance to apoptosis may simultaneously occur with cell death by necrosis. These data suggest that blasts that over express bcl-2 could be resistant to apoptosis, thereby being of poor prognosis, whereas other lymphoid cells would die either by necrosis or apoptosis. Nevertheless, bcl-2 did not correlate to blasts, or poorly, in patients with *de novo* AML, nor to the expression of cd-34 as well as the caryotype and the mdr-1 genotype, i.e. recognized factors for prognosis. Interestingly, in patients without cytogenetic poor prognosis, high bcl-2 level was related to bad outcome. Moreover, kinetically studied on seven patients, the decrease in bcl-2 level (mean decrease of about 90 % in two months) seemed to be correlated to the benefit of the chemotherapy. On the other hand, Karakas et al. [17] showed that *bcl-2* mRNA was of poor prognosis in AML. Close to our data, Maung et al. [27] showed an over-expression of bcl-2 in blasts obtained from the bone marrow of patients with *de novo* AML but not secondary AML; moreover, Campos et al. [16] found that among three markers studied by flow cytometry, i.e. bcl-2, cd-34 and P170-glycoprotein, high expression of bcl-2 was an independent factor to influence survival after chemotherapy in patients with AML. Robertson et al. [28] also suggested that quantification of bcl-2 protein in lymphocytes of patients with chronic lymphoid leukaemia may provide useful prognosis information. Further data must be obtained in order to better characterize bcl-2, protein and mRNA, as determinants of poor prognosis and of bad clinical response to chemotherapy in leukaemia.

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